AVIAN INFLUENZA AT THE WILD BIRD-POULTRY INTERFACE

S.A. BERGERVOET

Avian Influenza at the Wild Bird-Poultry Interface

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CHAPTER 1

1

General introduction

Saskia A. Bergervoet

General introduction

Avian influenza (AI), also known as avian flu or bird flu, is an infectious viral disease of birds. AI viruses predominantly circulate in wild aquatic birds, but can occasionally be transmitted to other animals, including poultry. Frequent mutations and the exchange of genomic material between viruses have led to a high genetic diversity and drives the constant emergence of novel virus strains. AI viruses are divided into numerous subtypes based on the hemagglutinin (HA) and neuraminidase (NA) surface proteins. Most AI viruses are low pathogenic avian influenza (LPAI) viruses that circulate in birds without causing clinical disease. However, LPAI viruses of two subtypes, H5 and H7, can mutate into highly pathogenic avian influenza (HPAI) viruses, causing severe disease and sudden death. Therefore, outbreaks of AI viruses can have a major impact on animal health and the economies of poultry industries. In addition, some AI viruses have shown to infect humans, and thus pose a substantial threat to public health. Recent outbreaks have highlighted the importance of the early detection, control and prevention of AI introductions into poultry. Globally, many countries have implemented surveillance programs to monitor AI viruses in wild birds and detect virus introductions into poultry. In the Netherlands, intensive surveillance has been performed for more than a decade now. Comprehensive analysis of surveillance data provides more insight into the circulation of AI viruses in the wild bird and poultry population. Detailed analysis of the viral genome has proved to be a valuable tool to investigate the origin and transmission patterns of viruses, in particular when combined with spatiotemporal information or phenotypic traits, such as the capacity of viruses to cause disease or infect new hosts. A better understanding of evolution and transmission patterns of AI viruses at the wild bird-poultry interface is important for more efficient monitoring and to prevent introductions into poultry.

VIRUS GENOME AND STRUCTURE

AI viruses are influenza A viruses that belong to the family of *Orthomyxoviridae* [1]. The AI virus particle consists of 8 negative-sense single-stranded RNA gene segments enclosed within a lipoprotein envelope (Figure 1). Two of the gene segments encode for the HA and NA glycoproteins expressed on the surface of the virus, which interact with sialic acid receptors on host cells and mediate viral entry and release, respectively [1]. The genetic and antigenic properties the HA and NA proteins are used for the classification of AI viruses into subtypes. At present, 16 HA (H1-H16) and 9 NA (N1-N9) subtypes have been identified in birds, which are found in a wide variety of combinations [1-3].

The other six gene segments code for the essential internal proteins polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), nucleoprotein (NP), matrix protein 1 (M1), matrix protein 2 (M2), nonstructural protein 1 (NS1) and nonstructural protein 2 (NS2, also known as the nuclear export protein (NEP)), and several nonessential accessory proteins [1,4]. The polymerase proteins PB1, PB2 and PA form a RNA-dependent RNA polymerase complex, which drives transcription and replication of the viral genome in the nuclei of infected cells. The polymerase complexes bind to the viral RNA gene segments that are wrapped around NP proteins, forming ribonucleoprotein (RNP) complexes. The M1 protein encloses the core of the virus particle and supports the viral

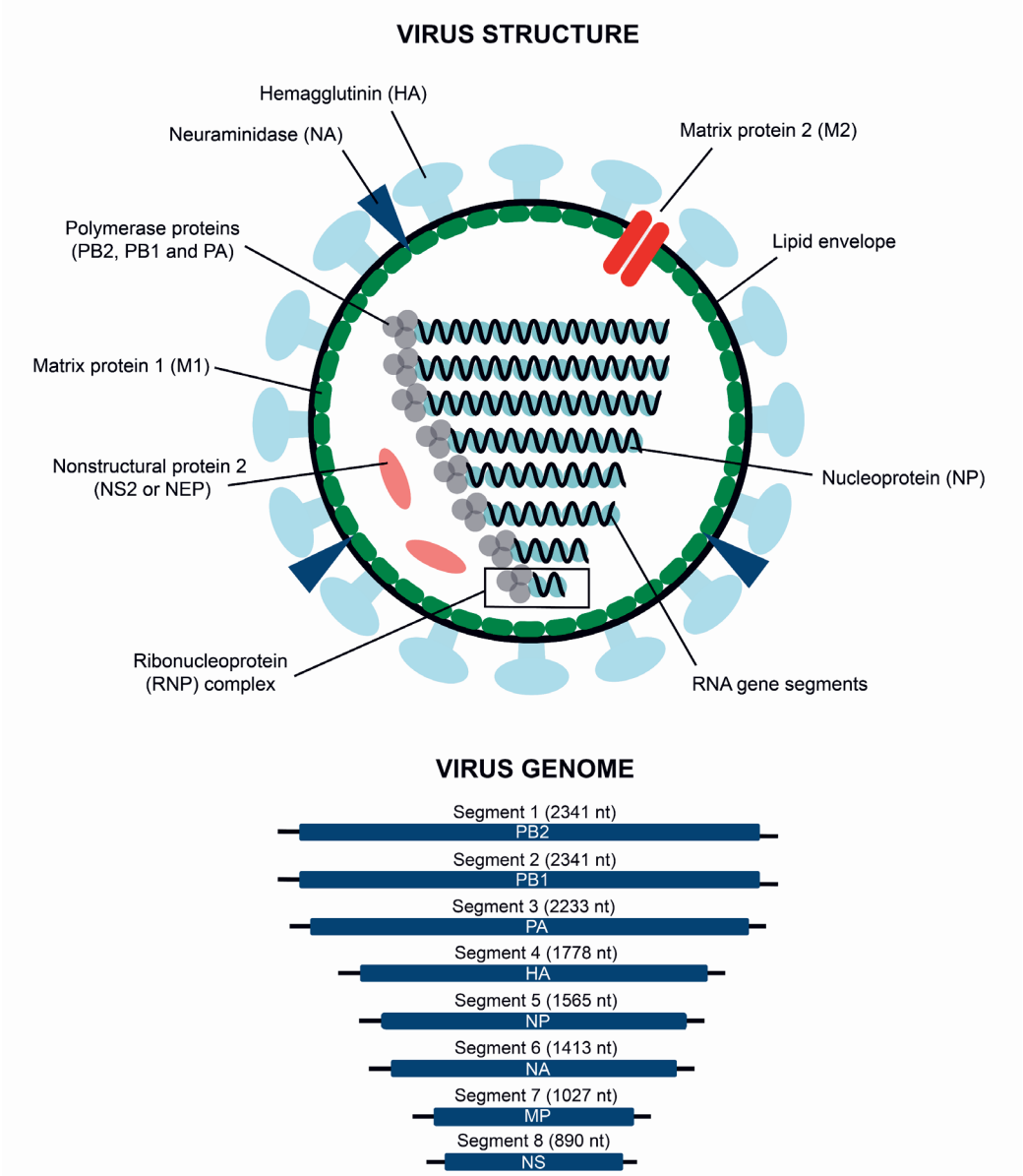

FIGURE 1. Influenza A virus structure and genome.

envelope, whereas the M2 protein is present in the viral envelope as an ion channel needed for viral fusion and the transfer of the RNP complexes into the cytoplasm. The NS1 protein modulates virus replication and interacts with the host innate immune response, while the NS2 protein is responsible for the export of RNP complexes from the nucleus. The NS

proteins circulate in two variants (NS allele A and B) that differ by around 30% of their amino acids [5]. The PB2, PB1, PA, HA, NP and NA proteins are encoded by gene segments 1-6, whereas the M1/M2 and NS1/NS2 proteins are produced upon alternative splicing of the mRNA transcripts from gene segments 7 and 8, respectively.

The genome of AI viruses mutates constantly due to the lack of proofreading activity of the polymerase during virus replication. This generates genetically heterogeneous virus populations, also referred to as quasi-species or minority variant subpopulations. In addition, the segmented genome of influenza A viruses enables the exchange of genomic material between two or more AI viruses, also known as genetic reassortment . The rapid evolution of AI viruses results in the emergence of strains with novel gene constellations and subtype combinations [6-9]. It also leads to the production of strains with novel virus characteristics, such as increased virulence or a broader host range, and allows viruses to rapidly adapt to new environments and to evade host immune responses.

HOST RANGE AND TRANSMISSION

AI viruses can infect a broad range of hosts that includes birds and mammals. Wild birds are the natural hosts of AI viruses, in particular waterfowl of the orders *Anseriformes* (mainly ducks, geese, and swans) and *Charadriiformes* (mainly gulls, terns, and waders) [10]. Some of these wild bird species migrate over long distances, contributing to the dispersal of AI viruses to susceptible wild bird populations worldwide [3]. AI viruses can also be transmitted to domesticated birds when wild birds travel to areas where poultry is kept. Among poultry, AI viruses are mainly found in outdoor layer chickens, domestic ducks and turkeys, which is likely due to their close contact to wild birds, the lack of species barrier, and higher susceptibility to infection, respectively [11-13].

Besides their frequent detection in birds, certain AI virus strains are able to infect mammals, such as pigs, horses, dogs, cats, seals [14], and even humans [15]. The host range is mainly determined by the receptor specificity of the HA protein. The HA protein of AI viruses binds to sialylated-glycan receptors expressed on the host cell membrane, but the specificity for certain sialic acid structures varies between virus strains of different origin. In addition, the expression of specific sialylated-glycan receptors differs between cell types and host species. For example, the HA protein of avian-origin AI viruses preferentially bind alpha 2,3-linked sialic acids that are common in the avian intestinal epithelium, whereas human-origin AI viruses preferentially bind alpha 2,6-linked sialic acids that are expressed in the upper respiratory tract of humans [16-18]. However, other viral factors, such as variations in the internal genes that determine the virus replication efficiency or the capacity of the virus to evade host immune responses, may also influence the host-range [19].

Transmission of AI viruses between wild birds typically occurs via the faecal–oral route, when wild birds reside (eat and drink) at faeces-contaminated surface water [1,20]. Infected birds can shed infectious virus particles via their faeces for several days or weeks [20]. AI viruses can persist for extended periods of time in the environment, such as water, soil and surfaces, especially at low temperatures [21,22]. Infection of poultry may occur by direct contact with wild birds or when wild birds drop their faeces in outdoor poultry facilities. Indirectly, transmission to poultry may occur via vectors or transport of faeces-contaminated materials into farms [23]. Some studies have also suggested virus transmission via air or dust particles [24,25]. The sporadic zoonotic transmission to humans often involves close contact

with infected poultry at farms or bird markets [26,27]. In rare cases, limited human-to-human transmission has been reported for avian-origin influenza viruses [28]. In the last century, sustained transmission between humans has only been observed for influenza A viruses of subtypes H1N1 (the Spanish flu in 1918 and the Mexican flu in 2009), H2N2 (the Asian flu in 1957) and H3N2 (the Hong Kong flu in 1968) [29-31].

PATHOGENICITY AND CLINICAL DISEASE

AI viruses can be classified based on their HA and NA antigens as well as on their capacity to cause disease in chickens, called pathogenicity. Most AI viruses are LPAI viruses that exhibit low pathogenicity for chickens. These viruses usually circulate in birds without clinical signs of disease, but sometimes cause mild clinical symptoms in poultry, such as mild respiratory disease, a reduction in egg production or low mortality [32]. LPAI viruses of subtypes H5 and H7 pose the most serious health risk, as they can mutate into highly pathogenic forms [33]. HPAI virus infections in chickens are characterized by severe clinical symptoms and a sudden onset of death, up to 100% mortality over the course of a few days [34]. Fatal infections have also been observed in wild birds, but to a much lesser extent as compared to poultry.

The pathogenicity of AI viruses is mainly determined by the HA protein, which is cleaved post-translationally into HA1 and HA2 subunits by host proteases to enable viral entry into host cells. LPAI viruses have a mono-basic cleavage site that can be cleaved by proteases such as trypsin-like enzymes present in the respiratory and intestinal tract [35]. Therefore, replication of LPAI viruses is generally restricted to these organs. Currently, H5 and H7 are the only known subtypes that have the potential to mutate into HPAI forms under natural conditions. The transition from LPAI to HPAI occurs when basic amino acids are inserted at the cleavage site of the HA protein [33]. This commonly occurs after the virus has been introduced to a high density population, such as a poultry flock. The multi-basic cleavage site, containing consecutive arginine and lysine residues, can be cleaved by ubiquitous furin-like proteases present in all organs, resulting in systemic virus replication. Virus pathogenicity is also influenced by other changes in the viral genome, such as alterations in the genes encoding the polymerase proteins [36,37], or a deletion in the stalk region of the NA gene, which is known as an important adaptation marker for poultry of *Galliformes* species (including chickens and turkeys) [38,39].

DIAGNOSTICS AND VIRUS CHARACTERIZATION

AI virus infections can be diagnosed using both serological and virological methods. Serological tests are used for routine screening for virus-specific antibodies in blood samples, which are generated in response to infection and detectable for several weeks or months after the virus is cleared. The diagnosis based on serology is generally done by performing an enzyme-linked immunosorbent assay (ELISA) for the detection of influenza A virus-specific antibodies, followed by antibody subtyping using subtype-specific hemagglutinin inhibition (HI) tests, neuraminidase inhibition (NI) tests or multiplex serological assays [40,41].

Virological tests are used to detect current infections. The traditional method for the detection and subtyping of AI viruses consists of virus isolation in embryonated chicken

eggs followed by antigenic characterization using HI and NI tests [40]. Nowadays, molecular techniques based on reverse transcription polymerase chain reaction (RT-PCR) are often used to detect and characterize AI viruses in clinical samples. As recommended by the World Organization of Animal Health (OIE), this is done by performing a RT-PCR targeting the universal matrix gene (M-PCR) [42], followed by subtype-specific RT-PCR [43,44]. These virological techniques enable high-throughput screening, especially when samples are pooled prior to diagnostic testing [45,46]. Further virus characterization is done by sequencing fragments of the HA and NA gene segments, including the HA proteolytic cleavage site to infer pathogenicity [47,48]. The pathogenicity of the virus in live birds is assessed using the intravenous pathogenicity index (IVPI) test, in which a high viral dose is inoculated into the blood stream of ten six-week-old chickens and mortality is measured [40].

In recent years, next-generation sequencing (NGS) has proved to be a valuable tool to investigate the genetic diversity and evolution of AI viruses. In particular, phylogenetic analysis using complete genome sequences is nowadays a widely adopted approach to study the origin of newly emerging viruses and their genetic relationship with other circulating AI viruses. To improve these analyses, researchers are encouraged to share genome sequencing data on online platforms [49]. NGS also allows the detection of so-called minority variants that arise from biological variation in the virus population.

SURVEILLANCE PROGRAMS

Globally, many surveillance studies have been implemented for the early detection and control of AI viruses in wild birds and poultry. The surveillance programs are mainly focused on the detection of H5 and H7 subtyped viruses, which are classified as notifiable diseases by the OIE because of their ability to mutate into highly pathogenic forms [50,51]. This means that it is compulsory to report, control and eradicate AI virus infections of subtypes H5 and H7 in poultry. Measures to control outbreaks include eradication of infected flocks, movements bans and additional testing of neighbouring farms. The surveillance programs vary between countries, but often consist of a combination of active and passive monitoring methods.

In the Netherlands, active surveillance for AI viruses in the wild bird population has been performed since 1998 [52,53]. This surveillance program mainly focuses on the detection of LPAI viruses, but has been adapted during outbreaks to detect HPAI viruses as well. For active monitoring for AI viruses, approximately 15,000 samples are collected from live wild birds of various species at breeding, staging or wintering sites in the Netherlands each year. Most of these samples are collected from mallards and gulls, which are common wild bird species in the Netherlands, relatively easily accessible for sampling, and considered important reservoirs of AI viruses. Passive surveillance in wild birds is performed for the early detection of HPAI viruses, and consists of sampling and testing of sick or dead wild birds. Both surveillance programs are mainly based on virological methods, which consist of the collection of swabs or faeces for the detection of viral genomic material [53].

Surveillance in poultry has been implemented in the Netherlands since the early 2000s, and also consists of both active and passive monitoring programs [54]. Active monitoring is performed by routine screening for the presence of influenza A virus-specific antibodies in blood samples. Each commercial poultry farm is tested at least once a year, but some poultry species are sampled more often, dependent on poultry type, housing system

and estimated risk for virus introduction [54]. Indoor layer chickens, broiler chickens or ducks are tested once a year, while outdoor layer chicken and turkey farms are tested four times a year and each production cycle, respectively. The serology-based screening method enables the detection of LPAI viruses, which often remain unnoticed due to the lack of obvious clinical signs. This method not only enables the detection of the notifiable LPAI H5 and H7 viruses, but also the detection of LPAI viruses of other (non-notifiable) subtypes. Passive surveillance consists of virological testing of poultry upon notification of suspected AI virus infection based on clinical signs [55], or to confirm positive serology. This program serves as an early warning system for HPAI virus infections.

RECENT OUTBREAKS

Outbreaks of HPAI virus infections have been reported frequently in poultry since the early 1990s. Historically, outbreaks occurred when LPAI viruses of subtypes H5 or H7 mutated into HPAI viruses after introduction into poultry. These outbreaks were generally rapidly controlled by preventive measures, such as culling of infected poultry and movement bans. In some cases, virus transmission between poultry farms has led to large outbreaks. In the Netherlands, a large outbreak of HPAI H7N7 virus occurred in 2003, affecting both poultry and humans [56-58]. In recent years, HPAI H5 viruses have been circulating in the wild birds, acting as a direct source for HPAI virus infection of poultry.

The HA gene of the recent HPAI viruses descend from the H5N1 A/Goose/Guangdong/1/96 (GsGd) lineage virus, which was first detected in China in 1996 [59]. Since 1997, descendants of the HPAI H5N1 GsGd lineage virus circulate enzootically in poultry in Asia [60,61]. In 2005, the H5N1 virus caused massive die-offs among migratory wild birds in the Qinghai Lake region of China [62-64], followed by infections of poultry and wild birds in Russia and Kazakhstan [65]. The virus has subsequently spread intercontinentally from Asia to Europe, the Middle East and Africa [66-68], causing numerous outbreaks of severe disease and high mortality among wild birds and poultry. In addition, transmissions to humans have been reported [69-71]. Due to the global expansion and rapid evolution, GsGd lineage viruses have developed into numerous lineages and reassortant viruses of different NA subtypes, including H5N2, H5N3, H5N5, H5N6 and H5N8. The HA gene of GsGd lineage viruses has diversified into numerous genetic subgroups, called clades [72].

From 2014 onwards, multiple reassortant variants of HPAI H5 clade 2.3.4.4 viruses have been detected in Europe, including the Netherlands. The viruses descend from H5N8 clade 2.3.4.4 viruses of two phylogenetic groups, referred to as group A and B, that were first detected in China and South Korea in 2013-2014 [73,74]. HPAI H5N8 viruses belonging to clade 2.3.4.4 group A were first identified in Europe by the end of 2014 [42,75], resulting in outbreaks in poultry in several European countries. In the Netherlands, five commercial poultry farms were infected [42]. Influenza virus and virus-specific antibodies were found in few live wild birds in Russia [76] and several European countries, including the Netherlands [77,78], but lethal infections were rarely reported in wild birds during this outbreak. In the same period, H5N8 clade 2.3.4.4 group A virus was also identified in North America, where it reassorted with co-circulating LPAI viruses to generate H5N1 and H5N2 reassortant viruses, resulting in a large epizootic in commercial turkeys in 2014-2015 [79].

In early 2016, HPAI H5N8 clade 2.3.4.4 viruses belonging to group B re-emerged in wild birds in China and at the Russian-Mongolian border [80-82]. Related viruses were

introduced into Europe by late 2016 [83]. The virus also spread to other continents, including the Middle East and Africa [84-86]. In contrast to the H5N8 outbreak in 2014-2015, this H5N8 virus caused massive deaths among wild birds [87]. The virus was also detected in apparently healthy birds, including mallards [88]. In the winter of 2016-2017, more than 2000 outbreaks of severe disease and high mortality have been reported in wild birds and poultry, affecting most European countries [89]. During this epizootic, multiple reassortant viruses have been detected, including viruses of subtype H5N5 in several European countries [83,90-95] and a single detection of H5N6 in Greece [96].

In the winter of 2017-2018, outbreaks of a novel reassortant HPAI H5N6 clade 2.3.4.4 group B virus were reported in wild birds and poultry in several European countries [97,98]. This H5N6 virus emerged from H5N8 clade 2.3.4.4 group B viruses in Asia [99], but obtained novel PB2 and NA genes. H5N6 virus infections have been associated with acute disease and mortality in both poultry and wild birds, but the number of outbreaks was limited compared to the H5N8 epizootic in 2016–2017 [96]. In the Netherlands, the H5N6 virus caused infections in three commercial poultry farms, two hobby holdings and several wild birds found dead [98].

THESIS SCOPE AND OUTLINE

AI viruses circulating in the wild bird population pose a continuous risk for infection of poultry. A better understanding of how AI viruses are transmitted from wild birds to poultry is important to prevent introductions. In addition, it will contribute to more efficient surveillance for AI viruses, which is essential to detect and control potentially dangerous strains at an early stage. This thesis aims at improving our knowledge on the spread of AI viruses at the wild bird-poultry interface. In **Chapter 2**, routinely collected surveillance data was used to explore potential links between LPAI viruses circulating in wild birds and poultry in the Netherlands between 2006-2016. The objective of the study described in **Chapter 3** was to determine the susceptibility of chickens to experimental infection with LPAI viruses of various subtypes and genotypes. **Chapter 4** describes a study in which the contribution of between-farm transmission to the overall incidence of LPAI virus introductions in poultry was assessed. In **Chapter 5**, we determined differences in the timing of reassortment and replication kinetics between three HPAI H5N5 genotypes that were detected in the Netherlands and other European countries in 2016-2017. In **Chapter 6**, we investigated histopathology and tissue distribution of HPAI H5N6 virus in chickens and Pekin ducks to elucidate differences in infection between poultry species during the 2017-2018 epizootic. Finally, the main results of the studies described in this thesis were summarized and discussed in a broader perspective in **Chapter 7**. This thesis contributes to a better understanding of AI virus spread at the wild bird-poultry interface, thereby improving the knowledge base for more efficient monitoring and prevention of introduction and spread of AI viruses in poultry.

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CHAPTER 2

2

Circulation of low pathogenic avian influenza (LPAI) viruses in wild birds and poultry in the Netherlands, 2006-2016

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Abstract

In this study, we explore the circulation of low pathogenic avian influenza (LPAI) viruses in wild birds and poultry in the Netherlands. Surveillance data collected between 2006 and 2016 was used to evaluate subtype diversity, spatiotemporal distribution and genetic relationships between wild bird and poultry viruses. We observed close species-dependent associations among hemagglutinin and neuraminidase subtypes. Not all subtypes detected in wild birds were found in poultry, suggesting transmission to poultry is selective and likely depends on viral factors that determine host range restriction. Subtypes commonly detected in poultry were in wild birds most frequently detected in mallards and geese. Different temporal patterns in virus prevalence were observed between wild bird species. Virus detections in domestic ducks coincided with the prevalence peak in wild ducks, whereas virus detections in other poultry types were made throughout the year. Genetic analysis of the surface genes demonstrated that most poultry viruses were related to locally circulating wild bird viruses, but no direct spatiotemporal link was observed. Results indicate prolonged undetected virus circulation and frequent reassortment events with local and newly introduced viruses within the wild bird population. Increased knowledge on LPAI virus circulation can be used to improve surveillance strategies.

Keywords: avian influenza virus; low pathogenic avian influenza; subtypes; wild birds; poultry; genetic analysis

Introduction

Avian influenza (AI) is an infectious disease of birds caused by influenza A viruses. Wild aquatic birds of the orders *Anseriformes* (ducks, geese and swans) and *Charadriiformes* (gulls and waders) are the natural reservoirs of AI viruses [1]. The prevalence of AI viruses in wild birds varies by species, age, season and geographical location [1]. During wild bird migration, AI viruses can be carried over large geographical distances, enabling virus transmission to susceptible host populations across the globe [2]. AI viruses can be transmitted from wild birds to poultry when breeding, stopover and wintering regions overlap with areas of commercial poultry production.

AI viruses are classified into subtypes based on the antigenic structures present on the surface of the virus [3]. Currently, 16 hemagglutinin (HA) and 9 neuraminidase (NA) antigenic subtypes have been identified in birds, which can be found in numerous combinations [2,4]. Most AI viruses are low pathogenic avian influenza (LPAI) viruses that remain subclinical or cause mild infection of the intestinal or respiratory tract [5]. LPAI viruses of subtypes H5 and H7 can evolve into highly pathogenic avian influenza (HPAI) virus variants that are associated with multi-organ systemic infection, which can cause severe disease and high mortality in birds [5].

Outbreaks of AI virus infections can have serious consequences for animal health and may result in major economic losses for the poultry industry. In addition, human cases of AI virus infections have been reported upon direct or indirect exposure to infected poultry [6]. The rapid and unpredictable evolution of AI viruses leads to the emergence of new influenza virus strains and subtype combinations [7-9]. Alterations in the genetic material of a virus can lead to changes in the virus characteristics, such as increased virulence or expanded host range, and may give rise to virus variants that are more prone to infect poultry. The recurrence of AI outbreaks in poultry highlights the importance of global surveillance efforts for early detection and rapid response.

In the Netherlands, the circulation of AI viruses in wild birds and poultry has been monitored for more than a decade [10,11]. The collection of wild bird swab specimens enables virological detection of AI viruses within the wild bird population. AI virus detection and monitoring in commercial poultry includes both active and passive surveillance methods. Active surveillance is performed by serological screening for AI viruses. The sampling frequency depends on poultry type, housing system and estimated risk for virus introduction [11,12]. Farms holding indoor layer chickens, broiler chickens or ducks are tested once a year for the presence of influenza virus-specific antibodies, while outdoor layer chicken and turkey farms are tested four times a year and each production cycle, respectively. Passive surveillance consists of virological testing of poultry upon notification of AI suspicions based on clinical signs or to confirm positive serology. AI virus surveillance in poultry focuses mainly on the early detection of viruses of subtypes H5 and H7, because of their potential to become highly pathogenic. However, samples collected in these programs are also used to monitor introductions of LPAI viruses of other subtypes.

Although a close relationship between AI viruses originating from wild birds and poultry has been described [13-16], wild bird species that act as source of infection for poultry and the actual virus transmission route has not yet been identified. In this study, surveillance data collected in the Netherlands between 2006 and 2016 was analysed to obtain more insight in the circulation of LPAI viruses in wild birds and poultry. We analysed the subtype diversity

among LPAI viruses from wild birds and poultry to identify potential hosts for viruses that infect poultry. In addition, spatiotemporal patterns of LPAI virus detections in wild birds and poultry were inferred to identify potential geographical locations or periods in a calendar year associated with infection of poultry. Finally, the genetic relationship between LPAI viruses isolated from wild birds and poultry was determined by phylogenetic analysis of the HA and NA sequences. Expanded knowledge on the circulation of LPAI viruses in wild birds and poultry can be used to improve surveillance strategies and control virus spread in the Netherlands.

Methods

ETHICAL STATEMENT

The capture of live wild birds was approved by the Dutch Ministry of Economic Affairs (Flora and Fauna permit FF/75A/2009/067). Wild bird handling and sampling methods were approved by the Animal Experiment Committee of the Erasmus MC (permit numbers 122-07-09, 122-08-12, 122-09-20, 122-10-20 and 122-11-31). Sampling of poultry was carried out in accordance with the European Union Council Directive 2005/94/EC [17].

COLLECTION OF WILD BIRD AND POULTRY SAMPLES

Active virological surveillance of AI virus infections in live wild birds was conducted by Erasmus MC. Individual faecal, cloacal, oropharyngeal or tracheal swabs from wild birds were collected, transported and stored as described previously [18]. Samples collected from wild birds found dead were not included in this study. Serological monitoring of AI virus infections in commercial poultry was conducted by the Dutch Animal Health Service (GD). Blood samples were collected from all poultry farms one or more times a year, depending on the type of farm. Seropositive samples were forwarded to the national reference laboratory Wageningen Bioveterinary Research (WBVR) for confirmatory testing and stored at -20°C. Virological surveillance of AI virus infections in commercial poultry was conducted when clinical signs were notified or antibodies against virus subtypes H5 or H7 were detected. Individual cloacal, oropharyngeal or tracheal swabs from poultry were collected by a specialist team of the Netherlands Food and Consumer Product Safety Authority (NVWA). Swabs were tested for the presence of influenza virus at WBVR and stored at -80°C. Information on species, location and date was provided for all samples collected.

ANTIBODY DETECTION

Antibody detection in poultry serum samples was performed using the FlockChek AI MultiS-Screen Ab Test Kit (IDEXX) according to the manufacturer's protocol. Serum samples identified as influenza virus-positive were subsequently tested in a H5 and H7 subtype-specific hemagglutination inhibition (HI) test according to the OIE Manual of Standards for Diagnostic Tests and Vaccines [19]. Further antibody characterization was done using a multiplex serological assay based on HA and NA antigens [20]. The results were confirmed using HI tests, neuraminidase inhibition (NI) tests and NA-specific ELISAs [19].

VIRUS DETECTION AND ISOLATION

Wild bird virus detection and isolation were performed as described previously [18]. To detect poultry viruses, RNA was extracted from swab specimens or allantoic fluids using the MagNA Pure 96 instrument (Roche) with the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche). Influenza virus was detected by the real-time reverse transcription polymerase chain reaction method targeting the matrix gene (M-PCR) [21]. M-PCR positive poultry samples were subsequently tested for the presence of virus subtypes H5 and H7 by the subtype-specific PCRs as recommended by the European Union reference laboratory [22,23]. The pathogenicity of the virus was determined by amplification of a gene fragment spanning the HA proteolytic cleavage site [24]. Subtyping was done by using universal primer sets for amplification of HA and NA gene fragments of all influenza A viruses, as previously described [24,25]. PCR fragments were sequenced by standard Sanger sequencing and compared to publicly available sequences using the BLAST algorithm for subtype identification. To isolate viruses, M-PCR positive samples were inoculated into the allantoic cavity of specific-pathogen-free (SPF) embryonated chicken eggs (ECEs) [19]. Allantoic fluid was collected and tested for hemagglutination activity by standard procedures [19]. Virus isolates were characterized in a HI test using in-house prepared antisera. A second passage in eggs was performed in case no virus was detected in the first passage.

SEQUENCING

The HA and NA sequences of LPAI viruses were generated by next-generation sequencing (NGS). Wild bird viruses were selected for NGS based on surveillance data. We selected 129 wild bird viruses of subtypes that were also detected in poultry and 33 wild bird viruses of subtypes that were not detected in poultry to a maximum of two viruses per subtype, species, year and geographical region. Consensus sequences of wild bird viruses were generated as described previously [26]. For sequencing of poultry viruses, 42 LPAI viruses obtained from 58 virus-positive field samples were included. RNA was isolated from swab specimens or allantoic fluid using the High Pure Viral RNA Kit (Roche). The SuperScript III One-Step RT-PCR System with the Platinum Taq DNA Polymerase kit (Invitrogen) and purified universal primers were used for multi-segment amplification of influenza viruses [27]. The PCR products were visualized on agarose gel and purified using the High Pure PCR Product Purification Kit (Roche). Purified amplicons were prepared for sequencing using the Illumina Nextera DNA Sample Preparation kit. Sequencing was performed with a minimum sequence coverage of 1,000x using the paired-end 200 Illumina MiSeq platform. To determine the consensus sequence for each HA and NA gene segment, reads were mapped using the ViralProfiler-Workflow, an extension of the CLC Genomics Workbench (Qiagen, Germany), as described previously [28]. Consensus sequences were generated by a reference-based method using a set of Eurasian AI virus submitted to Genbank (https://www.ncbi.nlm.nih.gov) (Supplementary Table S1) and GISAID's EpiFlu Database [29] (http://www.gisaid.org) (Supplementary Table S2), respectively.

PHYLOGENETIC ANALYSIS

To construct phylogenetic trees of HA and NA gene segments, cluster representatives for each virus subtype were selected from around 21,000 HA and 17,000 NA sequences of AI viruses available in GISAID's EpiFlu Database [29] as of July 2016. Sequences outside the 75-125% range of the cluster median sequence length, containing sequencing errors or gaps were excluded for analysis. Remaining sequences were clustered at 90% sequence identity using CD-HIT version 4.6.6 per gene segment [30]. Each cluster was represented by one sequence, known as the centroid sequence or cluster representative. The BLAST algorithm was used to select the top 50 sequence matches from publicly available HA and NA sequences for each poultry virus. Nucleotide sequences of cluster representatives, poultry viruses and BLAST hits were aligned using CLC Genomics Workbench version 8.5. Alignments were edited manually for frameshifts, sequence duplicates and length. A phylogenetic tree was constructed for each HA and NA gene segment using the Neighbour-Joining method [31] within the MEGA7 software package [32] using the Tamura-Nei substitution model with a gamma distribution (shape parameter = 1) for rate variation. Bootstrap support values (1,000 replicates) of more than 70 are shown at the branches.

DATA ANALYSIS

Cases were defined as subtyped if the HA or NA subtype of the virus or the subtype-specificity of the influenza virus-specific antibodies was determined. The number of virus detections mentioned in this study may differ from previous studies that have also included non-subtyped M-PCR positive samples [12,16,33]. The association between bird species and virus subtype was assessed performing corresponding analysis where host-virus subtype dependencies where graphically explored in a two dimensional plot. To estimate the temporal prevalence of LPAI viruses circulating in the wild bird population, cases were treated as epidemiological units defined as sampling clusters (groups of birds of same species sampled at one time and one place) where subtyped viruses were detected. Cluster prevalence was quantified at a monthly level for each year of the study for each wild bird species monitored. Data analysis was done using the statistical software package R version 3.4.0 [34]. The geographical distribution of LPAI viruses in wild birds and poultry was explored by mapping the sampling efforts (total number of wild birds or poultry farms sampled) and the number of subtyped cases during the study period. Geographical maps were plotted using the QGIS desktop application version 2.18.2.

Results

COLLECTION AND SUBTYPING OF WILD BIRD AND POULTRY SAMPLES

During the surveillance period, in total 111,114 wild birds (9,281 sampling clusters) belonging to 148 species of 17 orders were sampled for virological testing (Supplementary Table S3). Most birds belonged to species of the order *Anseriformes* (77%), of which the majority were mallards (55%), followed by geese (26%), other wild duck species (16%), and swans (3%) (Fig 1A). Fewer birds belonged to species of the order *Charadriiformes* (19%), of which 86% were

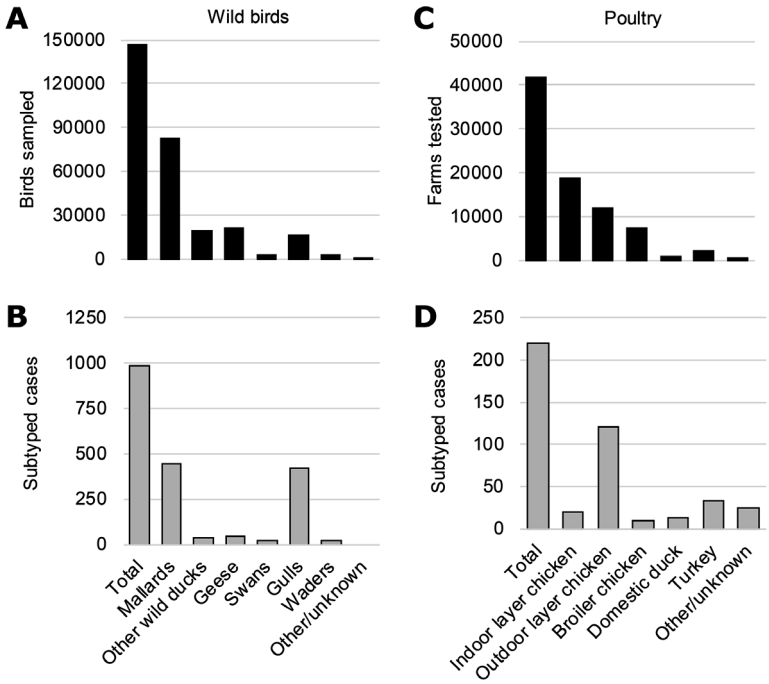

FIGURE 1. COLLECTION AND SUBTYPING OF WILD BIRD AND POULTRY SAMPLES.
(A) Number of wild birds sampled and (B) number of subtyped cases of low pathogenic avian influenza (LPAI) virus detections in wild birds per wild bird species. (C) Number of poultry farms tested and (D) number of subtyped cases of LPAI virus detections in poultry farms per poultry type. All samples were collected as part of the national avian influenza (AI) surveillance program in the Netherlands, January 2006-September 2016. A case is considered subtyped if the hemagglutinin (HA) or neuraminidase (NA) subtype of the virus or the subtype-specificity of the influenza virus-specific antibodies is determined.

gulls, 12% waders and 2% other *Charadriiformes* species. The HA or NA subtype was characterized for 981 swab samples collected from 21 wild bird species. Most subtyped samples were obtained from mallards (45%) and gulls (43%) (Fig 1B).

In contrast to the wild bird monitoring program, surveillance in poultry was performed by both serological and virological testing. As part of serological monitoring in poultry, in total 41,769 farms were tested, including farms holding indoor layer chickens (45%), outdoor layer chickens (28%), broiler chickens (17%), turkeys (6%) and ducks (2%) (Fig 1C; Supplementary Table S4). For virological monitoring in poultry, swab samples from 980 farms were tested to confirm positive serology or suspicions raised by clinical surveillance. The HA or NA subtype was characterized for 220 LPAI virus detections in 152 poultry farms. Subtyped cases were most often detected in chicken farms (76%), in particular layer farms with a free-ranging facility, followed by turkey farms (15%) and duck farms (6%) (Fig 1D). Most infections in poultry were detected through antibody detection (162 subtyped cases), whereas a quarter of the cases were subtyped based on virology (58 subtyped cases).

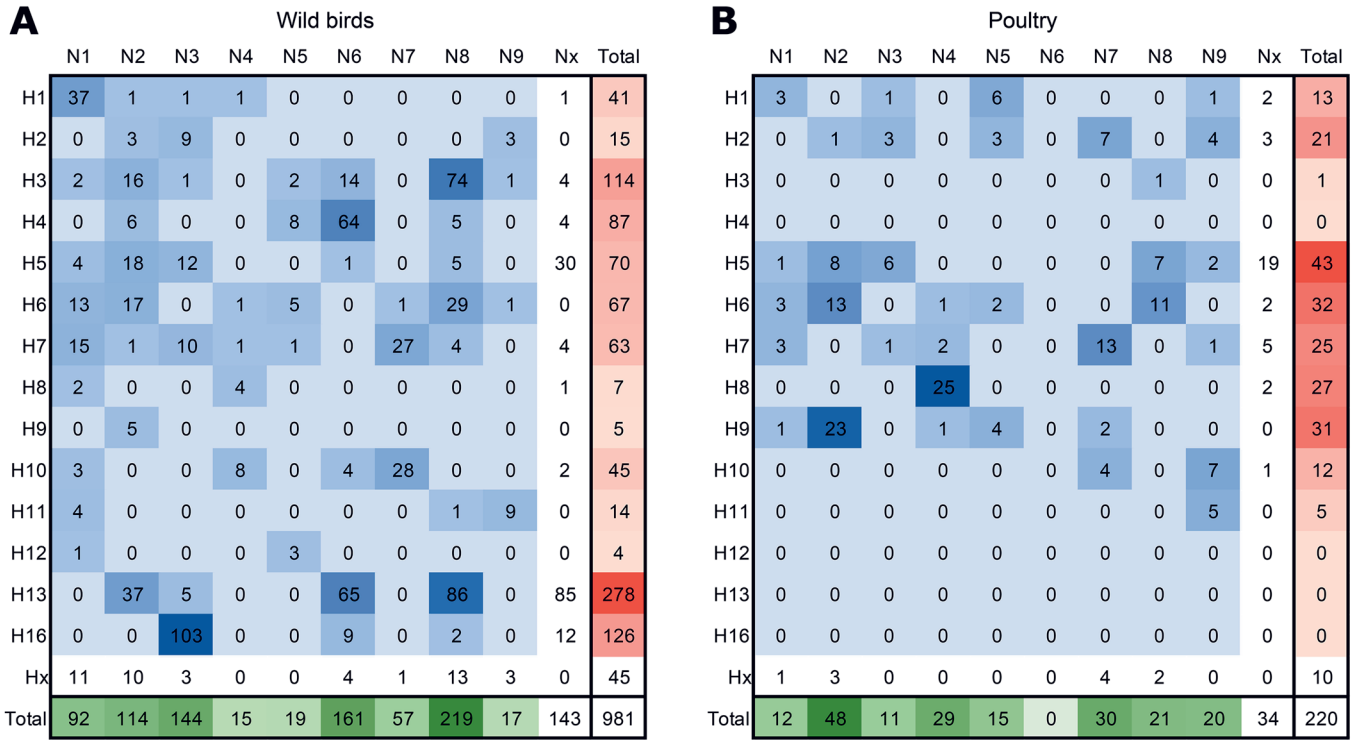

FIGURE 2. VIRUS SUBTYPES AND SUBTYPE COMBINATIONS DETECTED IN WILD BIRDS AND POULTRY.
Number of hemagglutinin (HA) subtypes, neuraminidase (NA) subtypes, and HA/NA subtype combinations of low pathogenic avian influenza (LPAI) viruses detected in (A) wild birds and (B) poultry, as part of virological and serological surveillance for avian influenza (AI) virus infections in the Netherlands, January 2006-September 2016. HA subtypes (red), NA subtypes (green), HA/NA subtype combinations (blue) were coloured according to the frequencies of detection.

ANALYSIS OF LPAI VIRUS SUBTYPES CIRCULATING IN WILD BIRDS AND POULTRY

To obtain more insight into the circulation of LPAI virus subtypes in the Netherlands, we analysed the HA and NA subtypes and subtype combinations that were detected in wild birds (Fig 2A) and poultry (Fig 2B). The HA subtype was identified for 937 wild bird viruses and 211 virus detections in poultry. All 16 HA subtypes except H14 and H15 were detected during surveillance in live wild birds. Of the most frequently identified HA subtypes in wild birds, H13 (30%) and H16 (13%) were exclusively detected in gulls, whereas H3 (12%) and H4 (9%) were primarily detected in wild ducks (Fig 3A). H8, H9 and H12 were detected in wild birds only sporadically (frequency of <1%). In poultry, the most frequently detected HA subtypes were H5 (20%), H6 (15%), H9 (14%), H8 (12%) and H7 (11%). HA subtypes H4 and H12-H16 were

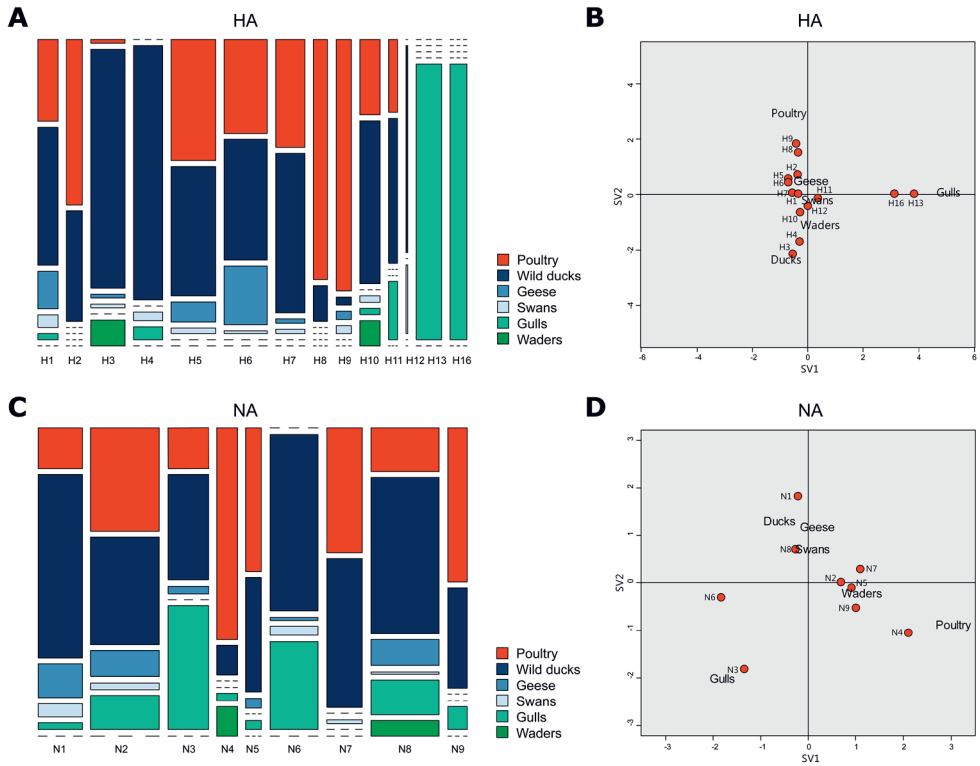

FIGURE 3. VIRUS SUBTYPE DISTRIBUTION AMONG WILD BIRD SPECIES AND POULTRY.
(A) Relative hemagglutinin (HA) subtype distribution among wild bird species and poultry. The bar width represents the number of cases within each HA subtype. (B) Correspondence plot showing the association between bird species and HA subtype in two dimensions (singular value (SV) 1 and SV2). (C) Relative neuraminidase (NA) subtype distribution among wild bird species and poultry. The bar width represents the number of cases within each NA subtype. (D) Correspondence plot showing the association between bird species and NA subtype in two dimensions (SV1 and SV2). All subtyped cases were detected as part of the national avian influenza (AI) surveillance program in the Netherlands, January 2006-September 2016.

not detected in poultry, and H3 was detected only once in domestic ducks. A two dimensional correspondence analysis plot combining HA subtypes and bird species shows that H13 and H16 viruses are closely associated with gulls, and indicates an association of H3 and H4 viruses with wild ducks, and H8 and H9 viruses with poultry (Fig 3B). Other HA subtypes fell around the centre of the correspondence plot, indicating their occurrence is host-independent.

The NA subtype was identified for 838 wild bird viruses and 186 virus detections in poultry. The most frequently detected NA subtypes in wild birds were N8 (26%), N6 (19%), and N3 (17%). In poultry, the most frequently identified NA subtypes were N2 (26%), N7 (16%) and N4 (16%). These NA subtypes were found in all wild bird species except waders (Fig 3C). Correspondence analysis of NA subtypes and bird species combined indicates an association of N3 viruses with gulls and N4 viruses with poultry (Fig 3D). Due to the absence of detection, N1 was negatively associated with gulls and N6 was negatively associated with poultry. For other NA subtypes, no clear association between bird species and virus subtype was observed.

We identified 55 HA/NA subtype combinations for 796 wild bird viruses and 35 HA/NA subtype combinations for 177 virus detections in poultry. The most frequently detected HA/NA subtype combinations in wild birds were H16N3 (13%), H13N8 (11%), H13N6 (8%) in gulls, and H3N8 (9%) and H4N6 (8%) in other wild bird species. The most frequently detected HA/NA subtype combinations in poultry were H8N4 (14%) and H9N2 (13%), followed by H7N7 (6%), H6N2 (6%) and H6N8 (5%). Of these subtype combinations, H8N4 and H9N2 were rarely detected in wild birds (frequency of <1%). In contrast, H6N2, H6N8 and H7N7 were frequently isolated from wild birds, in particular from mallards and geese.

SPATIOTEMPORAL ANALYSIS OF LPAI VIRUSES IN THE NETHERLANDS

Wild bird samples were mainly collected in water-rich areas along the coastline of the Netherlands, in the provinces Zuid Holland (51%), Noord Holland (15%) and Friesland (9%) (Fig 4A), while most tested farms were located in poultry dense areas in the central and south-eastern part of the Netherlands, in the provinces Gelderland (24%) and Noord Brabant (22%) (Fig 4B). Wild bird viruses were relatively more frequently detected in the provinces Groningen and Friesland, whereas the distribution of virus detections in poultry was proportional to the distribution of the farms.

To analyse temporal patterns in the detection of LPAI viruses, we estimated the monthly cluster prevalence for each wild bird species for each year of the study period. A significant increase in prevalence of virus detections in wild birds was found in August, September, October and December compared to January ($p<0.05$). LPAI viruses in gulls were most frequently detected in summer season (June-September) (Fig 5A). Although belonging to the same species order, LPAI viruses in waders were more often detected during autumn (September-November). LPAI viruses in mallards and other wild ducks were primarily observed between late summer and early winter (August-December). LPAI viruses in geese and swans were most often detected in winter and spring season (November-April). In chickens and turkeys, LPAI virus detections were made throughout the year, with an increase in incidence in chickens in March based on both serological and virological surveillance data (Fig 5B). LPAI virus detections in domestic ducks were solely observed during summer and autumn (July-November).

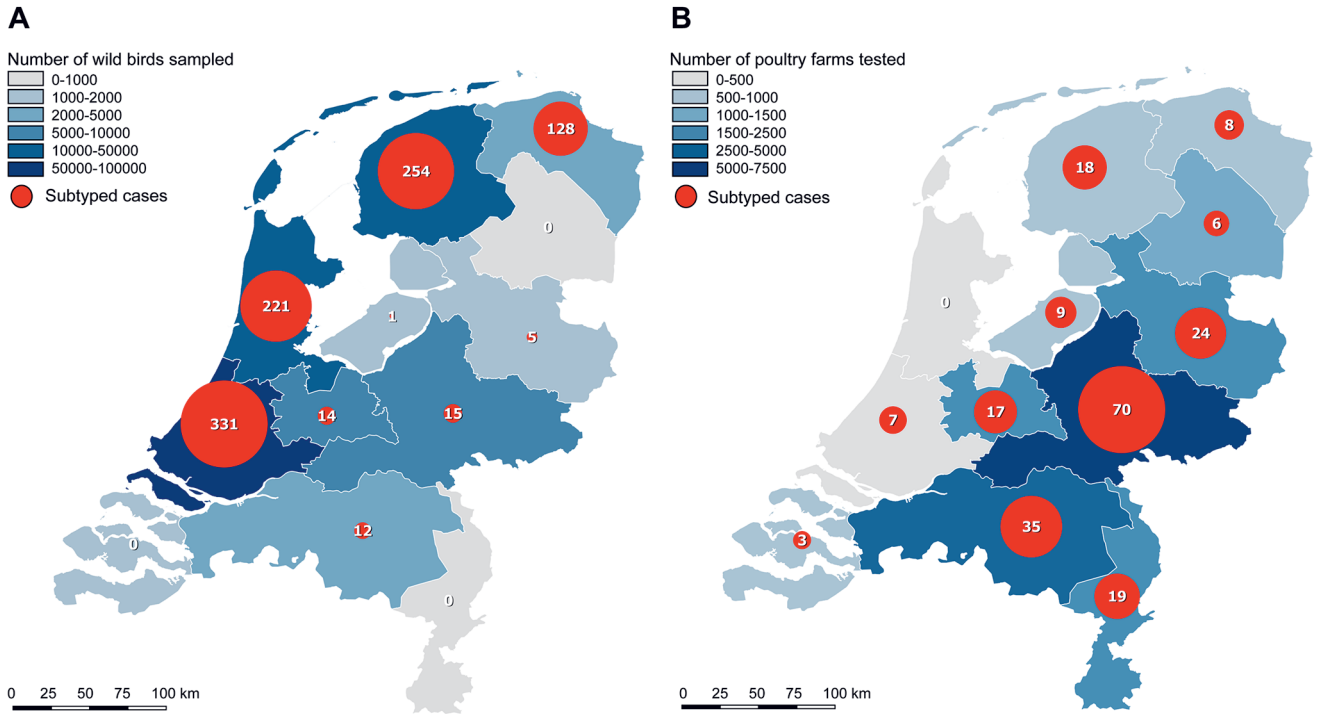

FIGURE 4. GEOGRAPHICAL DISTRIBUTION OF SAMPLING EFFORTS AND SUBTYPED CASES IN WILD BIRDS AND POULTRY.
Geographical distribution of (A) wild bird samples collected (blue) and the number of subtyped cases of low pathogenic avian influenza (LPAI) virus detections in wild birds (red), and (B) poultry farms tested (blue) and the number of subtyped cases of LPAI virus detections in poultry farms (red), by province, as part of the national avian influenza (AI) surveillance program in the Netherlands, January 2006-September 2016. A case is considered subtyped if the hemagglutinin (HA) or neuraminidase (NA) subtype of the virus or the subtype-specificity of the influenza virus-specific antibodies is determined.

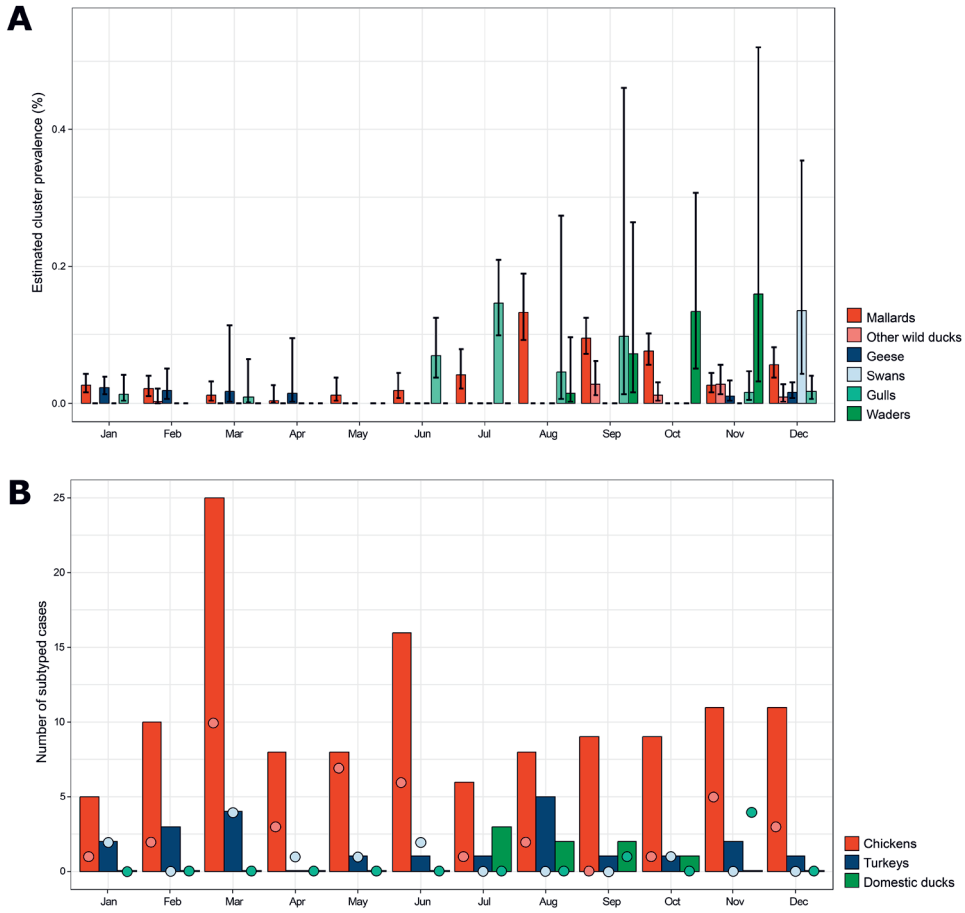

FIGURE 5. TEMPORAL PATTERNS OF VIRUS DETECTIONS IN WILD BIRDS AND POULTRY.
(A) Estimated cluster prevalence of low pathogenic avian influenza (LPAI) viruses in wild birds per month of the year. For this analysis, subtyped cases in wild birds were clustered based on identical host species, subtype combination, sampling location and collection date. The error bars show the standard deviation between different years. (B) Number of subtyped cases in poultry per month of the year based on serology (bars) and virology (dots). The black line represents the average number of poultry farms tested per month of the year. The error bars show the standard deviation between different years. Data was collected as part of the national avian influenza (AI) surveillance program in the Netherlands, January 2006-September 2016. A case is considered subtyped if the hemagglutinin (HA) or neuraminidase (NA) subtype of the virus or the subtype-specificity of the influenza virus-specific antibodies is determined.

GENETIC ANALYSIS OF HA AND NA GENE SEGMENTS

To investigate the genetic relationship between LPAI viruses from wild birds and poultry, the sequences of the HA and NA gene segments were determined for all 42 poultry viruses and a selection of 162 wild bird viruses. The sequences of the poultry viruses were subsequently

compared to the sequences of wild bird viruses determined in this study, and publicly available sequences. Phylogenetic analysis was performed for all HA subtypes (H1-H3 and H5-H10) (Supplementary Fig. S1) and NA subtypes (N1-N5 and N7-N9) (Supplementary Fig. S2) detected in poultry. Most LPAI viruses isolated from poultry clustered phylogenetically with European virus strains. The HA gene of 21 poultry viruses and the NA gene of 25 poultry viruses clustered phylogenetically with viruses collected in the Netherlands. One poultry virus was genetically most closely related to viruses outside Europe: the HA and NA gene of A/Chicken/Netherlands/14003323/2014 (H5N2) clustered phylogenetically with Asian strains.

To detect potential precursor viruses in wild birds, we determined the most identical wild bird viruses for each poultry virus HA and NA gene by BLAST (Supplementary Table S5). Related wild bird viruses were of different HA/NA subtype combination in approximately half of the cases. In 33 cases, the poultry virus shared the highest sequence identity with the HA and NA gene of two different wild bird viruses. For nine poultry viruses, a single wild bird virus was identified as most identical for both gene segments (Table 1). These poultry viruses showed nucleotide sequence identities of 97.8-99.8% (HA) and 98.2-99.9% (NA) to the most identical wild bird virus. The distance between the sampling sites of these poultry and wild bird viruses varied from 27 to 216 km. Two of the wild bird viruses were collected within a three-months period prior to detection in poultry.

Most identical wild bird viruses were often isolated from mallards (75%), whereas a smaller subset was isolated from other duck species, swans, geese, and gulls (25%). Genetic analysis also revealed a close relationship between viruses derived from different poultry farms. Poultry viruses that were related based on both the HA and NA gene segment were collected within the same year: H1N5 (2007), H10N7 (2009), H6N1 (2010), H7N7 (2011), H10N9 (2012), H5N3 (2013), and H6N2 (2014). These poultry viruses showed nucleotide sequence identities of 99.5-100.0% (HA) and 99.7-100.0% (NA). In seven cases, poultry viruses clustered together in the phylogenetic tree based on only one of the two gene segments. These poultry viruses were of different HA/NA subtype combination or collected in separate years. An exception is A/Chicken/Netherlands/13003601/2013 (H7N7) that clustered phylogenetically together with A/Chicken/Netherlands/13003983/2013 (H7N7) based on HA, but not NA.

Discussion

Analysis of surveillance data obtained in the Netherlands between January 2006 and September 2016 demonstrated that wild birds were frequently infected with LPAI viruses and infection of poultry was not uncommon. Most wild bird LPAI viruses were detected in mallards, which were the most sampled species among the waterfowl breeding population in the Netherlands. Mallards belong to the group of dabbling ducks, which are considered the main reservoir hosts of LPAI viruses [35]. As the most abundant dabbling duck species, mallards have been the focus of many influenza monitoring programs [1,2,35]. In poultry, most LPAI viruses were detected in chickens, which represent 98% of the commercial poultry population in the Netherlands [36]. Detections were relatively more frequently made in outdoor layer chickens, domestic ducks and turkeys compared to indoor layers and broiler chickens, as reported previously [12,37,38]. Outdoor-ranged poultry is considered to have an increased risk for AI virus

TABLE 1. Poultry viruses and their most identical wild bird virus.

Poultry virus	Collection date poultry virus	Collection location poultry virus (country – province)	Most identical wild bird virus	Collection date wild bird virus	Collection location wild bird virus (country – province)	Time interval (days)	Distance (km)	Identity HA (%)	Identity NA (%)
A/Duck/Netherlands /06027358/2006 (H3N8)	2006-09-27	NL - Gelderland	A/Mallard Duck/Netherlands /60/2006 (H3N8)	2006-09-18	NL - Noord Holland	9	135	99.80	99.80
A/Chicken/Netherlands /10008427/2010 (H10N7)	2010-05-20	NL - Friesland	A/Mallard/Netherlands /67/2008 (H10N7)	2008-12-13	NL - Zuid Holland	523	172	99.00	99.00
A/Chicken/Netherlands /11004004/2011 (H8N4)	2011-03-09	NL - Utrecht	A/Common Teal/Netherlands /12002960/2012 (H8N4)	2012-03-02	NL - Noord Holland	-359	27	99.20	99.50
A/Chicken/Netherlands /11008327/2011 (H7N7)	2011-05-12	NL - Gelderland	A/Mallard Duck/Netherlands /1/2011 (H7N7)	2011-02-23	NL - Noord Holland	78	96	99.40	98.80
A/Chicken/Netherlands /11009919/2011 (H1N1)	2011-05-30	NL – Zuid Holland	A/Greater white-fronted goose /Netherlands/4/2011 (H1N1)	2011-01-17	NL - Noord Brabant	133	50	98.80	99.90
A/Chicken/Netherlands /11011392/2011 (H7N7)[a]	2011-06-22	NL - Flevoland	A/Mallard Duck/Netherlands /1/2011 (H7N7)	2011-02-23	NL - Noord Holland	119	51	99.30	98.80
A/Turkey/Netherlands /11011530/2011 (H7N7)	2011-06-25	NL - Flevoland	A/Mallard Duck/Netherlands /1/2011 (H7N7)	2011-02-23	NL - Noord Holland	122	51	99.30	98.70
A/Chicken/Netherlands /13003601/2013 (H7N7)	2013-03-12	NL - Gelderland	A/Anas platyrhynchos/Belgium /23852cls33/2012 (H7N7)	2012-09-12	BE - Namur	181	216	99.20	99.20
A/Duck/Netherlands /14016168/2014 (H6N8)	2014-11-25	NL - Gelderland	A/Mallard Duck/Netherlands /15/2011 (H6N8)	2011-09-14	NL - Noord Holland	1168	84	97.80	98.20

Note: Low pathogenic avian influenza (LPAI) poultry viruses, detected as part of the national avian influenza (AI) surveillance program in the Netherlands, January 2006-September 2016, for which the same most identical wild bird virus was identified for the hemagglutinin (HA) and neuraminidase (NA) gene segment sequences by BLAST. The time interval between collection dates, the distance between the collection locations and nucleotide sequence identities between the HA and NA gene segments of the poultry and wild bird viruses are shown. We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database [28] on which this research is based. All submitters of data may be contacted directly via the GISAID website (http://www.gisaid.org). Abbreviations: BE, Belgium; NL, The Netherlands.

[a] A/Chicken/Netherlands/11011392/2011 (H7N7); A/Chicken/Netherlands/11011326/2011 (H7N7)

introduction because of its close contact with wild birds [37]. The relatively high rate of introduction in turkey and domestic duck farms is likely due to a higher susceptibility of these poultry species to wild bird LPAI viruses. Experimental studies have demonstrated that turkeys are highly susceptible to influenza viruses of diverse origins [39-41]. Influenza viruses from wild ducks may be more easily transmitted to domestic ducks than other poultry species because of the lack of a species barrier.

During the ten-year surveillance period, a wide range of LPAI virus subtypes was identified. The HA and NA subtypes most frequently found in wild birds and poultry differed, and not all subtypes detected in wild birds were also found in poultry. Differences in the HA and NA subtype distribution between wild birds and poultry suggest that virus transmission is selective, and likely depends on viral factors that determine host range restriction.

Analysis of the HA subtype diversity indicated that H13 and H16 viruses exclusively infect gulls, which is presumably due to a strict host range [4,42,43]. H3 and H4 viruses were primarily isolated from wild ducks and rarely detected in poultry. These observations are consistent with previous surveillance studies conducted in the Netherlands [16], other European countries [2,35,44-47] and North America [48]. In contrast, H3 and H4 virus infections have been repeatedly reported in poultry in Asia, mainly affecting domestic ducks [49-55], and occasionally chickens [49,56-58]. Experimental studies have demonstrated that H3 and H4 viruses are capable of infecting chickens [55,59-61]. However, infection in chickens is often restricted to the upper respiratory tract and replication efficiency differs strongly between virus strains, which may contribute to the observed host bias.

H8 and H9 viruses were frequently detected in poultry, but only sporadically found in wild birds. The low prevalence of H8 and H9 subtypes in wild birds is consistent with previous findings [2,16,35,44,45,62]. Like in previous studies, H8 and H9 subtypes were most commonly found in combination with N4 and N2, respectively [16,63]. H9 viruses have frequently been detected in poultry in Eurasia [64-66], which may be related to increased monitoring of H9 viruses since certain H9N2 strains have caused clinical disease and significant mortality in poultry [67]. Moreover, transmission of H9N2 viruses from poultry to humans have been reported [68]. According to published sequence data, H8 viruses have rarely been isolated from poultry outside the Netherlands. Like H9 viruses, H8 viruses may also predominantly infect poultry but remain undetected during most monitoring studies because no clinical signs are present. Although high incidence in poultry was observed, H8 and H9 virus infections have also been described in wild birds [69]. Therefore, host range tends to be less stringent for these subtypes. The low number H8 and H9 virus detections in wild birds may represent a limitation of sampling.

Associations between bird species and NA subtypes were often linked to HA subtype, e.g. N3 combined with H16 in gulls (H16N3) and N4 combined with H8 in poultry (H8N4). Additionally, N6 was predominantly found in combination with two HA subtypes that were not detected in poultry, H4 and H13, causing a negative association of N6 with poultry. HA and NA subtypes that were detected in various bird species were located around the centre of the correspondence plot, confirming their species independence. These subtypes tend to have a rather broad host range or may rapidly adapt to a new host.

HA/NA combinations H6N2, H6N8 and H7N7, which were commonly detected in poultry, were in wild birds most frequently detected in mallards and geese. Mallards and geese are recognized reservoirs for influenza viruses [2,3]. Geese mainly feed on pastures and agricultural fields allowing contact with poultry [70]. However, a low prevalence of AI viruses has

been reported in goose species [35], and several studies suggest that the role of geese in virus transmission is limited [71-74]. A large diversity of LPAI virus subtypes was observed in mallards, which are likely exposed to a large variety of influenza viruses during migration. Unlike poultry, mallards preferably reside in water-rich areas and feed in surface water [3,75]. Therefore, geese may act as intermediate hosts, that transfer the virus from wild ducks to poultry. Alternatively, geese may be susceptible or exposed to the same viruses as poultry. It should be mentioned that - due to irregular sampling of only a small proportion of the wild bird population and the absence of serological monitoring - the circulation of certain HA and NA subtypes in wild birds may have remain undetected, influencing the corresponding analysis. In addition, the detection of the same subtype in large sampling clusters may also have contributed to a bias in the host-subtype association.

Spatial analysis revealed limited geographical overlap between sites of LPAI virus detections in wild birds and poultry, confirming previous observations [16]. Most wild bird viruses were detected in water-rich areas along the coastline of the Netherlands, containing breeding, stopover and wintering locations of wild birds. Wild bird sampling activities were considerably biased toward these areas because of the abundance of waterfowl and the presence of duck decoys that are used by ornithologists and hunters for wild bird capturing. In addition, the relative high rate of subtyped cases in the provinces Friesland and Groningen could be explained by intensive sampling of gulls in these areas during fledging season. In contrast, LPAI virus introductions in poultry were predominantly detected in the Central and South-Eastern part of the Netherlands, where most poultry farms are located. The differences between the geographical distribution of wild bird and poultry viruses appears to be a result of different sampling strategies.

The analysis of LPAI virus detections over the calendar year revealed discordant temporal patterns between wild bird species and poultry types. LPAI viruses in gulls were most frequently detected in summer, while LPAI viruses in wild ducks were primarily detected between late summer and early winter. These observations are consistent with previous studies [44,46,62,76,77], and likely related to the fledging period of gull chicks [77] and the migration period of wild ducks [45]. LPAI viruses in geese and swans were detected in winter and spring season. This period coincides with the period of increased LPAI virus observations in chickens, supporting the hypothesis that geese may have a role in transmission of LPAI viruses to poultry. It should be noted that serological surveillance in poultry can cause late diagnosis of virus infection, because antibodies can often be detected for many weeks or months post infection [78], when virus has already been cleared. Information on seronegative test results prior to influenza-specific antibody detection may be used to improve estimations of the time of virus introduction, but is limited by the low frequency of sampling (1-4 times a year). Interestingly, LPAI viruses were solely detected in domestic ducks during the seasonal peak of LPAI virus infections in wild ducks. This observation supports the hypothesis that LPAI viruses may be more easily transmitted from wild to domestic ducks.

Genetic analysis of the HA and NA gene segments showed that many LPAI viruses from poultry shared common ancestors with wild bird viruses in the Netherlands. Some poultry viruses were more closely related to wild bird viruses from other countries in Europe and Asia. In these cases, virus circulation has likely been missed during wild bird surveillance in the Netherlands. The HA and NA gene segments of individual poultry viruses were often related to different wild bird viruses, indicating a lack of sequence data on immediate precursor viruses. Most poultry viruses were subtype reassortants compared to their closest related wild

bird virus, due to the emergence of novel gene constellations during genetic reassortment [7]. For nine poultry viruses, a single virus was identified as most identical wild bird virus for both gene segments, but no direct spatiotemporal link was observed. These results suggest prolonged undetected virus circulation and frequent reassortment events with local and newly introduced viruses within the wild bird population.

Genetically related wild bird viruses were often isolated from mallards. However, since we did not identify wild bird viruses that were linked both genetically and spatiotemporally, it is not known whether the viruses were introduced into poultry by mallards or via another (intermediate) host. Wild bird sampling activities should be performed year-round and intensified in areas of commercial poultry production with focus on farm grounds with turkeys, ducks and outdoor chickens, to allow the detection of genetically related wild bird viruses that can also be linked spatiotemporally to poultry viruses. In addition, samples from wild bird species other than mallards should be collected to identify potential wild bird species of importance for virus transmission to poultry.

Most poultry farms were likely infected by separate virus introductions from wild birds. However, some poultry viruses were genetically highly related based on the HA and NA gene segments, suggesting they were introduced from the same wild bird source or by between-farm transmission. A previous genetic analysis of the internal gene segments confirmed their close genetic relationship [79]. In addition, combined genetic and epidemiological analysis has provided information on the possible routes of introduction for these viruses. Better understanding of factors associated with virus transmission into poultry is important to control virus spread and improve surveillance strategies in the Netherlands.

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AUTHOR CONTRIBUTIONS

Conceptualization: S.B., A.B., R.B., R.F., N.B.; Performing experiments and data collection: S.B., S.P., M.P.; Data curation: S.B., S.P., M.P.; Data analysis: S.B., J.G.; Sequencing: S.B., M.P., J.D., Z.K., D.K., H.B.; Programming: A.B., J.G.; Visualization: S.B., J.G.; Writing the manuscript: S.B., R.F., N.B. All authors reviewed the manuscript.

CONFLICTS OF INTEREST STATEMENT

The authors declare no conflict of interest.

CITATION

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Supplementary information

SUPPLEMENTARY TABLES

S1 Table. Genbank accession numbers of wild bird virus sequences.
S2 Table. GISAID accession numbers of poultry virus sequences.
S3 Table. Collection and subtyping of wild bird samples.
S4 Table. Collection and subtyping of poultry samples.
S5 Table. Poultry viruses and their most identical wild bird virus.

SUPPLEMENTARY FIGURES

S1 Figure. Phylogenetic trees of HA genes.
S2 Figure. Phylogenetic trees of NA genes.

S1 TABLE. GENBANK ACCESSION NUMBERS OF WILD BIRD VIRUS SEQUENCES.

Genbank accession numbers of hemagglutinin (HA) and neuraminidase (NA) gene segment sequences of low pathogenic avian influenza (LPAI) viruses from wild birds detected as part of the national avian influenza (AI) surveillance program in the Netherlands, January 2006-September 2016.

Isolate name	Collection date	Genbank HA segment ID	Genbank NA segment ID
A/Barnacle goose/Netherlands/1/2006 (H1N1)	2006-01-09	KX979589	KX977747
A/Barnacle goose/Netherlands/1/2010 (H6N8)	2010-01-08	KX977787	KX979558
A/Barnacle goose/Netherlands/1/2011 (H6N8)	2011-01-06	KX979107	KX979461
A/Barnacle goose/Netherlands/1/2014 (H6N8)	2014-12-15	KX978284	KX978681
A/Bean goose/Netherlands/1/2007 (H1N1)	2007-01-15	KX978559	KX978842
A/Bean goose/Netherlands/1/2008 (H6N8)	2008-01-22	KX977816	KX978984
A/Bean goose/Netherlands/1/2009 (H7N1)	2009-01-21	KX979368	KX979548
A/Bean goose/Netherlands/2/2009 (H7N1)	2009-01-21	KX978247	KX978908
A/Bewicks swan/Netherlands/2/2007 (H4N6)	2007-12-06	KX978511	KX977631
A/Bewicks swan/Netherlands/3/2006 (H1N1)	2006-11-17	KX978266	KX977791
A/Bewicks swan/Netherlands/3/2007 (H4N6)	2007-12-06	KX978846	KX979193
A/Bewicks swan/Netherlands/4/2006 (H9N2)	2006-11-17	KX977700	KX979422
A/Bewicks swan/Netherlands/5/2008 (H5N2)	2008-12-17	KX979015	KX978975
A/Bewicks swan/Netherlands/6/2006 (H1N1)	2006-11-22	KX978574	KX978892
A/Bewicks swan/Netherlands/7/2008 (H7N1)	2008-12-24	KX978000	KX978951
A/Bewicks swan/Netherlands/8/2009 (H6N8)	2008-01-04	KX977884	KX978281
A/Brent goose/Netherlands/1/2006 (H1N1)	2006-01-10	KX979392	KX979523
A/Dunlin/Netherlands/2/2007 (H3N8)	2007-07-31	KX978538	KX979291
A/Dunlin/Netherlands/3/2007 (H3N8)	2007-07-31	KX979366	KX978457
A/Eurasian teal/Netherlands/1/2008 (H6N1)	2008-09-22	KX978922	KX978742
A/Eurasian teal/Netherlands/1/2011 (H3N8)	2011-10-15	KX979458	KX979211
A/Eurasian teal/Netherlands/3/2008 (H6N1)	2008-09-22	KX978950	KX979001
A/Eurasian wigeon/Netherlands/1/2006 (H6N2)	2007-12-21	KX979500	KX978832
A/Eurasian wigeon/Netherlands/1/2008 (H6N1)	2008-09-22	KX978248	KX978405
A/Eurasian wigeon/Netherlands/1/2009 (H5N2)	2009-09-11	KX979575	KX977926
A/Eurasian wigeon/Netherlands/1/2010 (H5N2)	2010-09-27	KX977950	KX978397
A/Eurasian wigeon/Netherlands/2/2007 (H1N1)	2007-10-20	KX978381	KX977776
A/Eurasian wigeon/Netherlands/2/2008 (H7N4)	2008-12-29	KX978318	KX977640
A/Eurasian wigeon/Netherlands/6/2007 (H1N1)	2007-11-15	KX979129	KX977745
A/Gadwall duck/Netherlands/1/2011 (H6N2)	2011-11-04	KX978389	KX977644
A/Gadwall duck/Netherlands/2/2006 (H9N2)	2006-01-01*	KX978145	KX979196
A/Gadwall duck/Netherlands/3/2006 (H3N8)	2006-09-03	KX978829	KX979428
A/Greater white-fronted goose/Netherlands/1/2006 (H6N2)	2006-01-14	KX979150	KX978480
A/Greater white-fronted goose/Netherlands/1/2007 (H1N1)	2007-01-23	KX977711	KX977666
A/Greater white-fronted goose/Netherlands/1/2009 (H6N8)	2009-01-20	KX978830	KX979241
A/Greater white-fronted goose/Netherlands/1/2010 (H6N1)	2010-01-20	KX977696	KX978928
A/Greater white-fronted goose/Netherlands/1/2011 (H6N8)	2011-01-04	KX977775	KX978983
A/Greater white-fronted goose/Netherlands/1/2012 (H1N1)	2012-11-20	KX978558	KX978251
A/Greater white-fronted goose/Netherlands/11/2009 (H6N1)	2009-12-09	KX977968	KX978797
A/Greater white-fronted goose/Netherlands/2/2007 (H6N8)	2007-12-12	KX979168	KX978521
A/Greater white-fronted goose/Netherlands/2/2008 (H6N8)	2008-01-22	KX979494	KX978669
A/Greater white-fronted goose/Netherlands/2/2009 (H5N3)	2009-12-14	KX977974	KX979382
A/Greater white-fronted goose/Netherlands/2/2010 (H5N2)	2010-01-29	KX978688	KX978932
A/Greater white-fronted goose/Netherlands/2/2011 (H6N2)	2011-01-13	KX977613	KX978753
A/Greater white-fronted goose/Netherlands/3/2006 (H6N8)	2006-01-01*	KX979190	KX979264
A/Greater white-fronted goose/Netherlands/3/2007 (H6N5)	2007-12-18	KX978034	KX978798
A/Greater white-fronted goose/Netherlands/3/2011 (H6N2)	2011-01-11	KX978988	KX978364
A/Greater white-fronted goose/Netherlands/4/2006 (H6N8)	2006-01-01*	KX978498	KX979449
A/Greater white-fronted goose/Netherlands/4/2008 (H6N8)	2008-01-22	KX979397	KX979084
A/Greater white-fronted goose/Netherlands/4/2009 (H5N2)	2009-02-21	KX978287	KX978719
A/Greater white-fronted goose/Netherlands/4/2010 (H6N8)	2010-02-04	KX978377	KX978184

Isolate name	Collection date	Genbank HA segment ID	Genbank NA segment ID
A/Greater white-fronted goose/Netherlands/4/2011 (H1N1)	2011-01-17	KX978591	KX978181
A/Greater white-fronted goose/Netherlands/5/2008 (H5N2)	2008-12-30	KX979073	KX978387
A/Greater white-fronted goose/Netherlands/5/2010 (H5N3)	2010-11-13	KX978851	KX979439
A/Greater white-fronted goose/Netherlands/6/2008 (H5N2)	2008-12-30	KX978329	KX978609
A/Greater white-fronted goose/Netherlands/6/2009 (H6N8)	2009-01-04	KX978349	KX977708
A/Greater white-fronted goose/Netherlands/6/2010 (H6N2)	2010-11-23	KX978734	KX978786
A/Greater white-fronted goose/Netherlands/6/2011 (H6N8)	2011-12-14	KX978828	KX977722
A/Greater white-fronted goose/Netherlands/8/2009 (H6N2)	2009-12-30	KX978319	KX978626
A/Greater white-fronted goose/Netherlands/9/2009 (H6N2)	2009-12-30	KX978339	KX978180
A/Mallard duck/Netherlands/1/2007 (H10N7)	2007-01-01*	KX978192	KX977605
A/Mallard duck/Netherlands/1/2008 (H5N3)	2008-01-30	KX977798	KX978667
A/Mallard duck/Netherlands/1/2009 (H10N7)	2009-02-04	KX977889	KX979271
A/Mallard duck/Netherlands/1/2011 (H7N7)	2011-02-23	KX979524	KX978358
A/Mallard duck/Netherlands/1/2012 (H10N7)	2012-01-14	KX979240	KX978542
A/Mallard duck/Netherlands/1/2013 (H7N7)	2013-01-26	KX978524	KX978579
A/Mallard duck/Netherlands/1/2014 (H10N7)	2014-02-17	KX979229	KX978673
A/Mallard duck/Netherlands/10/2012 (H6N1)	2012-09-05	KX979411	KX978507
A/Mallard duck/Netherlands/12/2006 (H5N2)	2006-09-16	KX977669	KX979415
A/Mallard duck/Netherlands/12/2009 (H11N9)	2009-09-02	KX978958	KX978900
A/Mallard duck/Netherlands/12/2012 (H6N1)	2012-10-18	KX978330	KX979308
A/Mallard duck/Netherlands/13/2007 (H6N5)	2007-11-01	KX977641	KX977833
A/Mallard duck/Netherlands/13/2012 (H3N8)	2012-10-01	KX979517	KX979121
A/Mallard duck/Netherlands/13/2013 (H7N7)	2013-12-10	KX979399	KX978080
A/Mallard duck/Netherlands/14/2006 (H8N4)	2006-01-01*	KX977927	KX979046
A/Mallard duck/Netherlands/15/2007 (H6N8)	2007-11-09	KX979354	KX979328
A/Mallard duck/Netherlands/15/2009 (H6N8)	2009-10-02	KX978489	KX978378
A/Mallard duck/Netherlands/15/2011 (H6N8)	2011-09-14	KX978593	KX978920
A/Mallard duck/Netherlands/16/2006 (H10N7)	2006-01-14	KX978236	KX978605
A/Mallard duck/Netherlands/16/2007 (H6N8)	2007-11-15	KX978196	KX978905
A/Mallard duck/Netherlands/16/2009 (H1N1)	2009-09-30	KX978081	KX977672
A/Mallard duck/Netherlands/17/2009 (H6N8)	2009-10-06	KX978322	KX978549
A/Mallard duck/Netherlands/17/2011 (H3N8)	2011-09-20	KX978036	KX978560
A/Mallard duck/Netherlands/18/2007 (H4N6)	2007-11-22	KX979234	KX978376
A/Mallard duck/Netherlands/18/2009 (H6N2)	2009-10-08	KX978564	KX979049
A/Mallard duck/Netherlands/18/2010 (H6N8)	2010-09-03	KX979113	KX977957
A/Mallard duck/Netherlands/19/2007 (H6N2)	2007-12-07	KX978966	KX978008
A/Mallard duck/Netherlands/19/2009 (H5N3)	2009-10-26	KX978032	KX978963
A/Mallard duck/Netherlands/19/2012 (H4N6)	2012-09-07	KX979134	KX978671
A/Mallard duck/Netherlands/2/2007 (H10N7)	2007-01-01*	KX978494	KX979021
A/Mallard duck/Netherlands/2/2008 (H10N7)	2008-01-07	KX978964	KX978237
A/Mallard duck/Netherlands/2/2009 (H7N7)	2009-01-09	KX978114	KX978763
A/Mallard duck/Netherlands/2/2011 (H10N7)	2011-03-08	KX978624	KX979161
A/Mallard duck/Netherlands/2/2015 (H7N7)	2015-01-16	KX979185	KX978353
A/Mallard duck/Netherlands/20/2009 (H5N3)	2009-10-26	KX978388	KX977757
A/Mallard duck/Netherlands/20/2011 (H6N8)	2011-09-24	KX978022	KX979447
A/Mallard duck/Netherlands/22/2010 (H10N7)	2010-09-07	KX978006	KX978632
A/Mallard duck/Netherlands/23/2006 (H1N1)	2006-08-25	KX979325	KX978811
A/Mallard duck/Netherlands/23/2012 (H11N9)	2012-09-27	KX977778	KX977684
A/Mallard duck/Netherlands/24/2009 (H6N1)	2009-12-01	KX978844	KX978238
A/Mallard duck/Netherlands/24/2013 (H4N6)	2013-09-16	KX978385	KX978890
A/Mallard duck/Netherlands/26/2010 (H4N6)	2010-09-13	KX979423	KX979292
A/Mallard duck/Netherlands/26/2011 (H11N9)	2011-10-10	KX979452	KX978692
A/Mallard duck/Netherlands/27/2009 (H4N6)	2009-12-21	KX978295	KX978169
A/Mallard duck/Netherlands/28/2008 (H5N6)	2008-09-22	KX977852	KX978060
A/Mallard duck/Netherlands/28/2009 (H1N1)	2009-12-21	KX977807	KX978631
A/Mallard duck/Netherlands/28/2010 (H5N2)	2010-09-17	KX979224	KX977947
A/Mallard duck/Netherlands/29/2008 (H11N9)	2008-09-22	KX978267	KX978971
A/Mallard duck/Netherlands/29/2009 (H11N9)	2009-12-21	KX977705	KX977840

Isolate name	Collection date	Genbank HA segment ID	Genbank NA segment ID
A/Mallard duck/Netherlands/3/2008 (H6N2)	2008-01-07	KX977797	KX978336
A/Mallard duck/Netherlands/3/2015 (H7N7)	2015-01-24	KX978067	KX979143
A/Mallard duck/Netherlands/30/2008 (H5N3)	2008-09-23	KX978877	KX978452
A/Mallard duck/Netherlands/30/2010 (H3N8)	2010-09-21	KX978097	KX979363
A/Mallard duck/Netherlands/30/2011 (H6N4)	2011-10-19	KX979356	KX979148
A/Mallard duck/Netherlands/30/2014 (H4N6)	2014-07-21	KX978886	KX979350
A/Mallard duck/Netherlands/31/2008 (H6N1)	2008-09-22	KX978655	KX978400
A/Mallard duck/Netherlands/31/2012 (H7N1)	2012-01-10	KX977922	KX978711
A/Mallard duck/Netherlands/31/2013 (H10N7)	2013-05-20	KX978193	KX977867
A/Mallard duck/Netherlands/32/2011 (H5N2)	2011-10-20	KX979009	KX978469
A/Mallard duck/Netherlands/32/2013 (H10N7)	2013-08-05	KX977924	KX978875
A/Mallard duck/Netherlands/33/2014 (H10N7)	2014-12-16	KX978314	KX979578
A/Mallard duck/Netherlands/34/2006 (H3N8)	2006-09-11	KX979215	KX979577
A/Mallard duck/Netherlands/36/2006 (H5N3)	2006-09-11	KX979003	KX979553
A/Mallard duck/Netherlands/36/2008 (H11N9)	2008-09-23	KX978075	KX978903
A/Mallard duck/Netherlands/37/2011 (H1N1)	2011-12-08	KX977882	KX979536
A/Mallard duck/Netherlands/40/2006 (H6N8)	2006-09-16	KX977839	KX978188
A/Mallard duck/Netherlands/43/2006 (H5N2)	2006-09-18	KX979501	KX978545
A/Mallard duck/Netherlands/43/2011 (H7N1)	2011-12-24	KX977677	KX978462
A/Mallard duck/Netherlands/44/2011 (H7N1)	2011-12-24	KX978740	KX977735
A/Mallard duck/Netherlands/47/2010 (H10N7)	2010-11-26	KX978640	KX977904
A/Mallard duck/Netherlands/5/2008 (H6N8)	2008-09-10	KX979551	KX977649
A/Mallard duck/Netherlands/5/2009 (H5N2)	2009-02-02	KX977767	KX978896
A/Mallard duck/Netherlands/5/2012 (H10N7)	2012-05-12	KX978848	KX979065
A/Mallard duck/Netherlands/5/2013 (H1N1)	2013-09-16	KX979268	KX978982
A/Mallard duck/Netherlands/51/2010 (H1N1)	2010-12-03	KX978723	KX978328
A/Mallard duck/Netherlands/52/2008 (H5N2)	2008-10-09	KX979499	KX977925
A/Mallard duck/Netherlands/53/2010 (H1N1)	2010-12-08	KX978255	KX977899
A/Mallard duck/Netherlands/55/2008 (H4N6)	2008-10-06	KX978110	KX978178
A/Mallard duck/Netherlands/6/2006 (H4N6)	2006-06-03	KX977697	KX979028
A/Mallard duck/Netherlands/6/2013 (H3N8)	2013-10-01	KX979335	KX979409
A/Mallard duck/Netherlands/6/2015 (H10N7)	2015-03-04	KX978577	KX978040
A/Mallard duck/Netherlands/60/2008 (H7N1)	2008-10-15	KX978337	KX979011
A/Mallard duck/Netherlands/64/2006 (H1N1)	2006-09-22	KX978552	KX977762
A/Mallard duck/Netherlands/67/2008 (H10N7)	2008-12-13	KX979437	KX977963
A/Mallard duck/Netherlands/7/2008 (H3N8)	2008-08-22	KX978132	KX978054
A/Mallard duck/Netherlands/7/2009 (H7N7)	2009-02-02	KX977692	KX978598
A/Mallard duck/Netherlands/7/2011 (H4N6)	2011-08-15	KX978670	KX977749
A/Mallard duck/Netherlands/7/2012 (H1N1)	2012-07-09	KX978074	KX978447
A/Mallard duck/Netherlands/7/2014 (H6N2)	2014-10-01	KX978776	KX979466
A/Mallard duck/Netherlands/76/2008 (H6N8)	2008-11-27	KX978130	KX978245
A/Mallard duck/Netherlands/77/2008 (H7N1)	2008-12-29	KX977881	KX978814
A/Mallard duck/Netherlands/78/2006 (H6N8)	2006-10-21	KX978159	KX977724
A/Mallard duck/Netherlands/8/2008 (H6N1)	2008-09-27	KX978779	KX977984
A/Mallard duck/Netherlands/8/2012 (H1N1)	2012-10-23	KX979169	KX977770
A/Mallard duck/Netherlands/82/2008 (H7N7)	2008-12-17	KX979315	KX979225
A/Mallard duck/Netherlands/9/2013 (H6N8)	2013-10-15	KX979493	KX979138
A/Mute swan/Netherlands/2/2006 (H4N6)	2006-01-01*	KX978073	KX979448
A/Mute swan/Netherlands/3/2006 (H4N6)	2006-01-01*	KX977958	KX978909
A/Turnstone/Netherlands/1/2010 (H3N8)	2010-10-09	KX979124	KX977713
A/Turnstone/Netherlands/2/2007 (H3N8)	2007-07-31	KX979144	KX978279
A/Turnstone/Netherlands/3/2008 (H3N8)	2008-10-31	KX978665	KX979101
A/Turnstone/Netherlands/4/2008 (H3N8)	2008-11-01	KX978661	KX979097

* date unknown, set to 1st of January

S2 TABLE. GISAID ACCESSION NUMBERS OF POULTRY VIRUS SEQUENCES.

GISAID's EpiFlu database accession numbers of hemagglutinin (HA) and neuraminidase (NA) gene segment sequences of low pathogenic avian influenza (LPAI) viruses from poultry, detected as part of the national avian influenza (AI) surveillance program in the Netherlands, January 2006-September 2016.

Isolate name	Collection date	GISAID HA segment ID	GISAID NA segment ID
A/Chicken/Netherlands/06022003/2006 (H7N7)	2006-08-01	EPI1229818	EPI1229817
A/Chicken/Netherlands/09006942/2009 (H10N7)	2009-04-15	EPI1229826	EPI1229825
A/Chicken/Netherlands/10007882/2010 (H7N4)	2010-05-14	EPI1229834	EPI1229833
A/Chicken/Netherlands/10008427/2010 (H10N7)	2010-05-20	EPI966037	EPI966050
A/Chicken/Netherlands/10009401/2010 (H8N4)	2010-06-04	EPI1229842	EPI1229841
A/Chicken/Netherlands/10010413/2010 (H6N1)	2010-06-21	EPI1229850	EPI1229849
A/Chicken/Netherlands/10012103/2010 (H6N1)	2010-07-19	EPI1229858	EPI1229857
A/Chicken/Netherlands/10020245/2010 (H9N2)	2010-12-10	EPI1229866	EPI1229865
A/Chicken/Netherlands/11004004/2011 (H8N4)	2011-03-09	EPI1229874	EPI1229873
A/Chicken/Netherlands/11004875/2011 (H7N1)	2011-03-22	EPI1229882	EPI1229881
A/Chicken/Netherlands/11008325/2011 (H8N4)	2011-05-10	EPI1229890	EPI1229889
A/Chicken/Netherlands/11008327/2011 (H7N7)	2011-05-12	EPI1230758	EPI1230760
A/Chicken/Netherlands/11009919/2011 (H1N1)	2011-05-30	EPI1229898	EPI1229897
A/Chicken/Netherlands/11011392/2011 (H7N7)	2011-06-22	EPI1229906	EPI1229905
A/Chicken/Netherlands/12002495-001-005/2012 (H10N9)	2012-02-06	EPI1229914	EPI1229913
A/Chicken/Netherlands/12014794/2012 (H7N7)	2012-08-09	EPI1229922	EPI1229921
A/Chicken/Netherlands/13003601/2013 (H7N7)	2013-03-12	EPI1229930	EPI1229929
A/Chicken/Netherlands/13003983/2013 (H7N7)	2013-03-18	EPI1229938	EPI1229937
A/Chicken/Netherlands/13015884/2013 (H5N3)	2013-11-29	EPI1229946	EPI1229945
A/Chicken/Netherlands/13016263-031-035/2013 (H5N3)	2013-12-10	EPI1229954	EPI1229953
A/Chicken/Netherlands/14002541/2014 (H5N1)	2014-02-25	EPI1229962	EPI1229961
A/Chicken/Netherlands/14003005/2014 (H2N7)	2014-03-05	EPI1229970	EPI1229969
A/Chicken/Netherlands/14003323/2014 (H5N2)	2014-03-12	EPI1229978	EPI1229977
A/Chicken/Netherlands/14004070/2014 (H9N1)	2014-03-26	EPI1229986	EPI1229985
A/Chicken/Netherlands/15005968-056060/2015 (H5N2)	2015-04-02	EPI1230063	EPI1230062
A/Chicken/Netherlands/15007212/2015 (H10N7)	2015-04-28	EPI774514	EPI774516
A/Chicken/Netherlands/16007311-037041/2016 (H7N9)	2016-06-08	EPI773765	EPI773767
A/Chicken/Netherlands/16010778-021-025/2016 (H2N3)	2016-08-30	EPI1230079	EPI1230078
A/Chicken/NL-Barneveld/15004745-001-005/2015 (H7N7)	2015-03-11	EPI629344	EPI629341
A/Duck/Netherlands/06027358/2006 (H3N8)	2006-09-27	EPI1230087	EPI1230086
A/Duck/Netherlands/14015610/2014 (H6N2)	2014-11-17	EPI1230095	EPI1230094
A/Duck/Netherlands/14016168/2014 (H6N8)	2014-11-25	EPI1230103	EPI1230102
A/Duck/Netherlands/14016396/2014 (H6N2)	2014-11-25	EPI1230111	EPI1230110
A/Turkey/Netherlands/06001571/2006 (H6N5)	2006-01-24	EPI1229994	EPI1229993
A/Turkey/Netherlands/07014290/2007 (H1N5)	2007-05-31	EPI1230002	EPI1230001
A/Turkey/Netherlands/07016245/2007 (H1N5)	2007-06-22	EPI1230010	EPI1230009
A/Turkey/Netherlands/09006938/2009 (H10N7)	2009-04-14	EPI1230018	EPI1230017
A/Turkey/Netherlands/11011530/2011 (H7N7)	2011-06-25	EPI1230026	EPI1230025
A/Turkey/Netherlands/11015452/2011 (H9N2)	2011-08-31	EPI1230034	EPI1230033
A/Turkey/Netherlands/12004763-001-004/2012 (H10N9)	2012-03-05	EPI1230042	EPI1230041
A/Turkey/Netherlands/12005615/2012 (H10N9)	2012-03-13	EPI1230050	EPI1230049
A/Turkey/Netherlands/13001007/2013 (H8N4)	2013-01-18	EPI1230127	EPI1230126

S3 TABLE. COLLECTION AND SUBTYPING OF WILD BIRD SAMPLES.

Number of wild birds sampled and number of subtyped cases of low pathogenic avian influenza (LPAI) virus detections per wild bird species as part of the national avian influenza (AI) surveillance program in the Netherlands, January 2006-September 2016. A case is considered subtyped if the hemagglutinin (HA) or neuraminidase (NA) subtype of the virus is determined.

Order	Group	Species	Latin name	Number of birds sampled	Number of subtyped cases
Anseriformes	Ducks	Mallard	*Anas platyrhynchos*	46939	446
		Eurasian Wigeon	*Anas penelope*	10039	22
		Gadwall	*Anas strepera*	1317	5
		Common Teal	*Anas crecca*	1209	8
		Northern Pintail	*Anas acuta*	372	0
		Northern Shoveler	*Anas clypeata*	355	1
		Common Eider	*Somateria mollissima*	99	2
		Other ducks		312	0
	Geese	White-fronted Goose	*Anser albifrons*	12001	31
		Barnacle Goose	*Branta leucopsis*	4933	8
		Greylag Goose	*Anser anser*	1924	1
		Egyptian Goose	*Alopochen aegyptiacus*	1159	0
		Bean Goose	*Anser fabalis*	1095	3
		Brent Goose	*Branta bernicla*	858	1
		Canada Goose	*Branta canadensis*	326	0
		Pink-footed Goose	*Anser brachyrhynchus*	206	1
		Other geese		110	0
	Swans	Mute Swan	*Cygnus olor*	2126	3
		Bewick's Swan	*Cygnus bewickii*	250	14
		Other swans		46	0
Charadriiformes	Gulls	Black-headed Gull	*Chroicocephalus ridibundus*	14170	401
		Common Gull	*Larus canus*	2087	0
		Herring Gull	*Larus argentatus*	1024	10
		Lesser Black-backed Gull	*Larus fuscus*	894	4
		Mediterranean Gull	*Larus Melanocephalus*	207	1
		Great Black-backed Gull	*Larus marinus*	31	1
		Other gulls		51	0
	Waders	Turnstone	*Arenaria interpres*	911	14
		Dunlin	*Calidris alpina*	654	4
		Oystercatcher	*Haematopus ostralegus*	724	0
		Red Knot	*Calidris canutus*	163	0
		Other waders		177	0
	Other *Charadriiformes*			351	0
Other				2172	0
Unknown				1822	0
Total				111114	981

S4 TABLE. COLLECTION AND SUBTYPING OF POULTRY SAMPLES.

Number of serologically tested poultry farms and number of subtyped cases of low pathogenic avian influenza (LPAI) virus detections per poultry type as part of the national avian influenza (AI) surveillance program in the Netherlands, January 2006-September 2016. A case is considered if the hemagglutinin (HA) or neuraminidase (NA) subtype of the virus or the subtype-specificity of the influenza virus-specific antibodies is determined.

Poultry type		Housing system	Number of farms tested	Number of subtyped cases
Chicken	Layer	Indoor	18883	20
		Outdoor	11797	120
		Unknown		8
	Broiler		7309	9
	Unknown			10
Turkey			2269	33
Duck			889	13
Unknown			662	7
Total			41769	220

S5 TABLE. POULTRY VIRUSES AND THEIR MOST IDENTICAL WILD BIRD VIRUS.

Low pathogenic avian influenza (LPAI) viruses isolated from poultry, detected as part of the national avian influenza (AI) surveillance program in the Netherlands, January 2006-September 2016, with their genetically most identical wild bird virus based on the hemagglutinin (HA) and neuraminidase (NA) gene segment sequences as determined by BLAST. The time interval between collection days and the percentage of nucleotide (nt) sequence identity between the poultry virus and the most identical wild bird virus are shown. We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu database on which this research is based. All submitters of data may be contacted directly via the GISAID website (http://www.gisaid.org).

Year	Poultry virus	Location poultry virus (province – city)	Seg-ment	Most identical wild bird virus	Location wild bird virus (continent – country – province)	Time interval (days)	Sequence identity (%)
2006	A/Turkey/Netherlands/06001571/2006 (H6N5)	Noord Brabant - Dinteloord	HA	A/Greater white-fronted goose/Netherlands/ 1/2006 (H6N2)	EU - NL - Noord Brabant	10	99.5%
			NA	A/Mallard/Sweden/343/2002 (H12N5)	EU - Sweden	1181	98.7%
	A/Chicken/Netherlands/06022003/2006 (H7N7)	Gelderland - Voorthuizen	HA	A/Mallard Duck/Netherlands/ 60/2008 (H7N1)	EU - NL - Noord Holland	-806	99.5%
			NA	A/Mallard/Sweden/5927/2005 (H7N7)	EU - Sweden	251	98.7%
				A/Mallard/Sweden/5944/2005 (H7N7)	EU - Sweden	248	
				A/Mallard/Sweden/95/2005 (H7N7)	EU - Sweden	577	
	A/Duck/Netherlands/06027358/2006 (H3N8)	Gelderland - Nijmegen	HA	A/Mallard Duck/Netherlands/ 60/2006 (H3N8)	EU - NL - Noord Holland	9	99.8%
			NA	A/Mallard Duck/Netherlands/ 60/2006 (H3N8)	EU - NL - Noord Holland	9	99.8%
2007	A/Turkey/Netherlands/07014290/2007 (H1N5)	Limburg - Nederweert	HA	A/Bewicks swan/Netherlands/ 1/2007 (H1N5)	EU - NL - Friesland	150	98.9%
			NA	A/Black-headed gull/Netherlands/ 1/2006 (H4N5)	EU - NL - Gelderland	515	98.5%
	A/Turkey/Netherlands/07016245/2007 (H1N5)	Limburg - Weert	HA	A/Bewicks swan/Netherlands/ 1/2007 (H1N5)	EU - NL - Friesland	172	99.0%
			NA	A/Black-headed gull/Netherlands/ 1/2006 (H4N5)	EU - NL - Gelderland	537	98.5%
2009	A/Turkey/Netherlands/09006938/2009 (H10N7)	Noord Brabant - Deurne	HA	A/Mallard/Sweden/64476/2007 (H10N4)	EU - Sweden	677	98.9%
			NA	A/Anas_platyrhynchos/Camargue/091863/09 (H10N7)	EU - France	-36	99.8%
				A/Mallard Duck/Netherlands/ 82/2008 (H7N7)	EU - NL - Zuid Holland	118	
	A/Chicken/Netherlands/09006942/2009 (H10N7)	Noord Brabant - Deurne	HA	A/Mallard/Sweden/64476/2007 (H10N4)	EU - Sweden	678	98.8%
				A/Mallard/Sweden/93475/2009 (H10N6)	EU - Sweden	-130	
			NA	A/Anas_platyrhynchos/Camargue/091863/09 (H10N7)	EU - France	-35	99.8%
				A/Mallard Duck/Netherlands/ 82/2008 (H7N7)	EU - NL - Zuid Holland	119	

2010	A/Chicken/Netherlands/10007882/2010 (H7N4)	Noord Brabant - Deurne	HA	A/Mallard Duck/Netherlands/ 60/2008 (H7N1)	EU - NL - Noord Holland	576	99.1%
			NA	A/Teal/Chany/7119/2008 (H15N4)	EU - Russia	622	99.1%
	A/Chicken/Netherlands/10008427/2010 (H10N7)	Friesland – Drachtstercompagnie	HA	A/Mallard/Netherlands/ 67/2008 (H10N7)	EU - NL - Friesland	523	99.0%
			NA	A/Mallard/Netherlands/ 67/2008 (H10N7)	EU - NL - Friesland	523	99.0%
				A/Mallard/Netherlands/ 82/2008 (H10N7)	EU - NL - Friesland	519	
				A/Mallard/Netherlands/ 1/2009 (H10N7)	EU - NL - Friesland	470	
	A/Chicken/Netherlands/10009401/2010 (H8N4)	Friesland - Hiaure	HA	A/Mallard/Sweden/99377/2009 (H8N4)	EU - Sweden	274	98.5%
			NA	A/Teal/Chany/7119/2008 (H15N4)	EU - Russia	643	99.2%
	A/Chicken/Netherlands/10010413/2010 (H6N1)	Friesland - Idsegahuizum	HA	A/Mallard Duck/Netherlands/ 18/2010 (H6N8)	EU - NL - Zuid Holland	-74	98.8%
			NA	A/Mallard/Bavaria/185-8/2008 (H1N1)	EU - Germany	637	99.2%
	A/Chicken/Netherlands/10012103/2010 (H6N1)	Friesland - Parrega	HA	A/Mallard Duck/Netherlands/ 18/2010 (H6N8)	EU - NL - Zuid Holland	-46	98.8%
			NA	A/Mallard/Bavaria/185-8/2008 (H1N1)	EU - Germany	665	99.1%
	A/Chicken/Netherlands/10020245/2010 (H9N2)	Zuid Holland - Pijnacker	HA	A/Bewicks swan/Netherlands/ 4/2006 (H9N2)	EU - NL - Noord Holland	1484	96.6%
			NA	A/Mallard Duck/Netherlands/ 53/2006 (H4N2)	EU - NL - Noord Holland	1544	98.0%
				A/Mallard/Netherlands/ 7/2007 (H4N2)	EU - NL - Zuid Holland	1439	
2011	A/Chicken/Netherlands/11004004/2011 (H8N4)	Utrecht - Vreeland	HA	A/Common Teal/Netherlands/ 12002960/2012 (H8N4)	EU - NL - Noord Holland	-359	99.2%
			NA	A/Common Teal/Netherlands/ 12002960/2012 (H8N4)	EU - NL - Noord Holland	-359	99.5%
	A/Chicken/Netherlands/11004875/2011 (H7N1)	Zeeland - Schore	HA	A/Swan/Czech Republic/5416/2011 (H7N7)	EU - Czech Republic	-15	99.5%
			NA	A/Mallard Duck/Netherlands/ 51/2010 (H1N1)	EU - NL - Zuid Holland	109	99.6%
	A/Chicken/Netherlands/11008325/2011 (H8N4)	Gelderland - Lunteren	HA	A/Mallard/Sweden/101165/2009 (H8N4)	EU - Sweden	551	99.2%
			NA	A/Mallard/Sweden/133546/2011 (H10N4)	EU - Sweden	-197	99.4%
	A/Chicken/Netherlands/11008327/2011 (H7N7)	Gelderland - Kootwijkerbroek	HA	A/Mallard Duck/Netherlands/ 1/2011 (H7N7)	EU - NL - Noord Holland	78	99.4%
			NA	A/Mallard Duck/Netherlands/ 1/2011 (H7N7)	EU - NL - Noord Holland	78	98.8%
	A/Chicken/Netherlands/11009919/2011 (H1N1)	Zuid Holland - Stolwijk	HA	A/Greater white-fronted goose/Netherlands/ 4/2011 (H1N1)	EU - NL - Noord Brabant	133	98.8%
			NA	A/Greater white-fronted goose/Netherlands/ 4/2011 (H1N1)	EU - NL - Noord Brabant	133	99.9%
	A/Chicken/Netherlands/11011392/2011 (H7N7) A/Chicken/Netherlands/11011326/2011 (H7N7)*	Flevoland - Creil	HA	A/Mallard Duck/Netherlands/ 1/2011 (H7N7)	EU - NL - Noord Holland	120	99.3%
			NA	A/Mallard Duck/Netherlands/ 1/2011 (H7N7)	EU - NL - Noord Holland	120	98.8%
	A/Turkey/Netherlands/11011530/2011 (H7N7)	Flevoland - Creil	HA	A/Mallard Duck/Netherlands/ 1/2011 (H7N7)	EU - NL - Noord Holland	122	99.3%
			NA	A/Mallard Duck/Netherlands/ 1/2011 (H7N7)	EU - NL - Noord Holland	122	98.7%
	A/Turkey/Netherlands/11015452/2011 (H9N2)	Noord Brabant - Deurne	HA	A/Anas platyrhynchos/Belgium/24311pcs5/2012 (H9N2)	EU - Belgium	-375	99.2%
			NA	A/Mallard Duck/Netherlands/ 16/2012 (H3N2)	EU - NL - Zuid Holland	-352	99.1%
2012	A/Chicken/Netherlands/12002495-001-005/2012 (H10N9)	Noord Brabant - Heusden	HA	A/Mallard/Sweden/105186/2009 (H10N1)	EU - Sweden	800	98.2%
				A/Mallard/Sweden/105254/2009 (H10N1)	EU - Sweden	799	
				A/Mallard/Sweden/105259/2009 (H10N1)	EU - Sweden	799	
				A/Mallard/Sweden/105364/2009 (H10N1)	EU - Sweden	798	
				A/Mallard/Sweden/105365/2009 (H10N1)	EU - Sweden	798	

				A/Mallard/Sweden/105474/2009 (H10N1)	EU - Sweden	796	
				A/Mallard/Sweden/107688/2009 (H10N1)	EU - Sweden	794	
			NA	A/Mallard/Sweden/100878/2009 (H11N9)	EU - Sweden	827	99.0%
				A/Mallard/Sweden/101011/2009 (H11N9)	EU - Sweden	825	
				A/Mallard/Sweden/50980/2006 (H11N9)	EU - Sweden	1946	
	A/Turkey/Netherlands/12004763-001-004/2012 (H10N9)	Limburg - Ospel	HA	A/Mallard/Sweden/105186/2009 (H10N1)	EU - Sweden	828	98.2%
				A/Mallard/Sweden/105254/2009 (H10N1)	EU - Sweden	827	
				A/Mallard/Sweden/105259/2009 (H10N1)	EU - Sweden	827	
				A/Mallard/Sweden/105364/2009 (H10N1)	EU - Sweden	826	
				A/Mallard/Sweden/105365/2009 (H10N1)	EU - Sweden	826	
				A/Mallard/Sweden/105474/2009 (H10N1)	EU - Sweden	824	
				A/Mallard/Sweden/107688/2009 (H10N1)	EU - Sweden	822	
			NA	A/Mallard/Sweden/100878/2009 (H11N9)	EU - Sweden	855	98.9%
				A/Mallard/Sweden/101011/2009 (H11N9)	EU - Sweden	853	
				A/Mallard/Sweden/50980/2006 (H11N9)	EU - Sweden	1974	
	A/Turkey/Netherlands/12005615/2012 (H10N9)	Limburg - Nederweert	HA	A/Mallard/Sweden/105186/2009 (H10N1)	EU - Sweden	836	98.2%
				A/Mallard/Sweden/105254/2009 (H10N1)	EU - Sweden	835	
				A/Mallard/Sweden/105259/2009 (H10N1)	EU - Sweden	835	
				A/Mallard/Sweden/105364/2009 (H10N1)	EU - Sweden	834	
				A/Mallard/Sweden/105365/2009 (H10N1)	EU - Sweden	834	
				A/Mallard/Sweden/105474/2009 (H10N1)	EU - Sweden	832	
				A/Mallard/Sweden/107688/2009 (H10N1)	EU - Sweden	830	
			NA	A/Mallard/Sweden/100878/2009 (H11N9)	EU - Sweden	863	98.8%
				A/Mallard/Sweden/101011/2009 (H11N9)	EU - Sweden	861	
				A/Mallard/Sweden/50980/2006 (H11N9)	EU - Sweden	1982	
	A/Chicken/Netherlands/12014794/2012 (H7N7)	Utrecht - Hagestein	HA	A/Anas platyrhynchos/Belgium/23852cls33/2012 (H7N7)	EU - Belgium	-34	99.4%
			NA	A/Swan/Czech Republic/5416/2011 (H7N7)	EU - Czech Republic	491	98.7%
2013	A/Turkey/Netherlands/13001007/2013 (H8N4)	Noord Brabant - Helmond	HA	A/Mallard/Sweden/101165/2009 (H8N4)	EU - Sweden	1170	98.2%
			NA	A/Mallard/Sweden/99377/2009 (H8N4)	EU - Sweden	1233	98.4%
	A/Chicken/Netherlands/13003601/2013 (H7N7)	Gelderland - Lochem	HA	A/Anas platyrhynchos/Belgium/23852cls33/2012 (H7N7)	EU - Belgium	181	99.2%
				A/Swan/Czech Republic/5416/2011 (H7N7)	EU - Czech Republic	706	
			NA	A/Anas platyrhynchos/Belgium/23852cls33/2012 (H7N7)	EU - Belgium	181	99.2%
	A/Chicken/Netherlands/13003983/2013 (H7N7)	Flevoland - Zeewolde	HA	A/Swan/Czech Republic/5416/2011 (H7N7)	EU - Czech Republic	712	98.9%
			NA	A/Mallard Duck/Netherlands/ 5/2012 (H10N7)	EU - NL - Zuid Holland	310	98.7%
	A/Chicken/Netherlands/13015884/2013 (H5N3)	Groningen - Sint Annen	HA	A/Razorbill/Scotland/7343/14 (H5Nx)	EU - United Kingdom	-94	98.5%
			NA	A/Mallard/Netherlands/ 29/2010 (H2N3)	EU - NL - Noord Holland	1428	98.7%
				A/Mallard/Sweden/101900/2009 (H4N3)	EU - Sweden	1476	

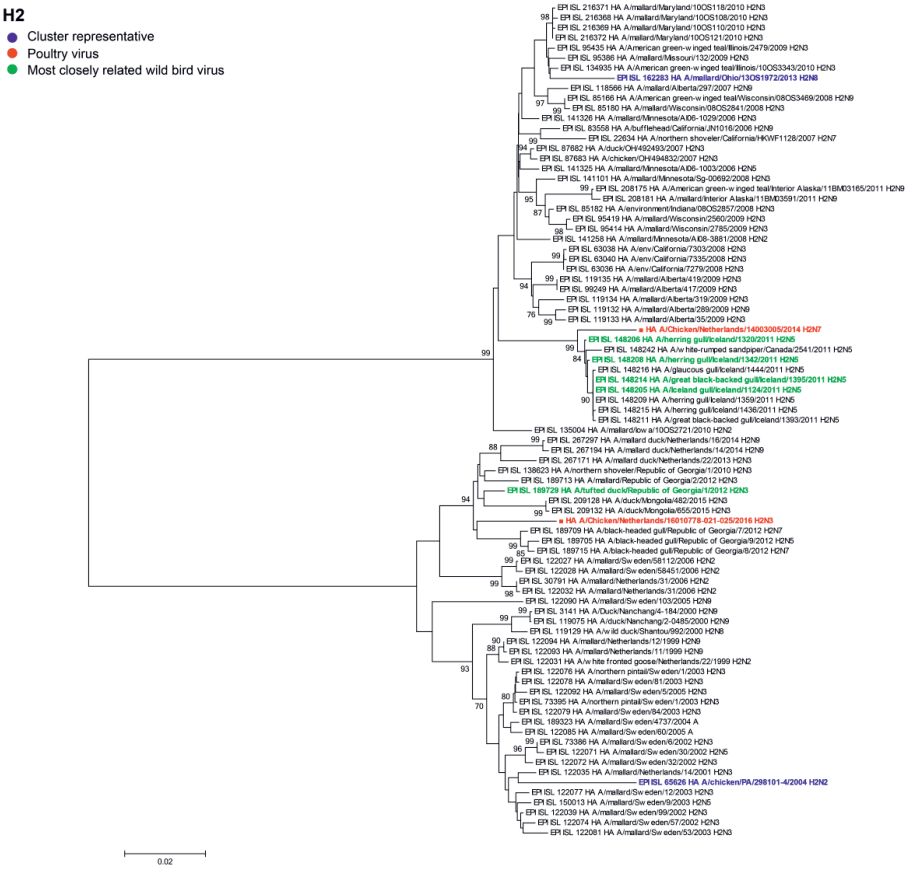
	A/Chicken/Netherlands/13016263-031-035/2013 (H5N3)	Groningen - Scheemda	HA	A/Razorbill/Scotland/7343/14 (H5Nx)	EU - United Kingdom	-83	98.5%
			NA	A/Mallard/Netherlands/ 29/2010 (H2N3)	EU - NL - Noord Holland	1439	98.8%
				A/Mallard/Sweden/101900/2009 (H4N3)	EU - Sweden	1487	
2014	A/Chicken/Netherlands/14002541/2014 (H5N1)	Flevoland - Swifterbant	HA	A/Razorbill/Scotland/7343/14 (H5Nx)	EU - United Kingdom	-6	98.8%
			NA	A/Mallard/Republic of Georgia/4/2012 (H1N1)	EU - Georgia	433	98.7%
	A/Chicken/Netherlands/14003005/2014 (H2N7)	Overijssel - Witharen	HA	A/Great black-backed gull/Iceland/1395/2011 (H2N5)	EU - Iceland	862	98.3%
				A/Herring gull/Iceland/1320/2011 (H2N5)	EU - Iceland	864	
				A/Herring gull/Iceland/1342/2011 (H2N5)	EU - Iceland	862	
				A/Iceland gull/Iceland/1124/2011 (H2N5)	EU - Iceland	871	
			NA	A/Mallard Duck/Netherlands/ 21/2010 (H10N7)	EU - NL - Noord Holland	1275	98.3%
	A/Chicken/Netherlands/14003323/2014 (H5N2)	Gelderland - Bruchem	HA	A/Duck/Hunan/S4120/2011 (H5N2)	AS - China	852	97.6%
			NA	A/Wild bird/Korea/L60-2/2008 (H5N2)	AS - Republic of Korea	1924	97.7%
	A/Chicken/Netherlands/14004070/2014 (H9N1)	Groningen - Uithuizermeeden	HA	A/Teal/Finland/10529/2010 (H9N2)	EU - Finland	1545	99.3%
			NA	A/Mallard/Sweden/816/2014 (H1N1)	EU - Sweden	-264	99.4%
	A/Duck/Netherlands/14015610/2014 (H6N2)	Utrecht - Lopik	HA	A/Mallard Duck/Netherlands/ 10/2012 (H6N1)	EU - NL - Noord Holland	803	99.0%
			NA	A/Swan/Netherlands/ 14000281/2014 (H5N2)	EU - NL - Zuid Holland	313	99.7%
	A/Duck/Netherlands/14016396/2014 (H6N2)	Gelderland - Hierden	HA	A/Mallard Duck/Netherlands/ 10/2012 (H6N1)	EU - NL - Noord Holland	811	99.0%
			NA	A/Swan/Netherlands/ 14000281/2014 (H5N2)	EU - NL - Zuid Holland	321	99.6%
	A/Duck/Netherlands/14016168/2014 (H6N8)	Gelderland - Putten	HA	A/Mallard Duck/Netherlands/ 15/2011 (H6N8)	EU - NL - Noord Holland	1168	97.8%
				A/Mallard Duck/Netherlands/ 20/2011 (H6N8)	EU - NL - Noord Holland	1158	
			NA	A/Mallard Duck/Netherlands/ 15/2011 (H6N8)	EU - NL - Noord Holland	1168	98.2%
2015	A/Chicken/NL-Barneveld/15004745-001-005/2015 (H7N7)	Gelderland - Barneveld	HA	A/Mallard Duck/Netherlands/ 23/2013 (H7N3)	EU - NL - Zuid Holland	701	98.5%
			NA	A/Mallard Duck/Netherlands/ 13/2013 (H7N7)	EU - NL - Zuid Holland	456	98.8%
	A/Chicken/Netherlands/15005968-056060/2015 (H5N2)	Noord Brabant - Milheeze	HA	A/Razorbill/Scotland/7343/14 (H5Nx)	EU - United Kingdom	395	96.6%
			NA	A/Mallard Duck/Netherlands/ 9/2014 (H6N2)	EU - NL - Zuid Holland	156	99.4%
	A/Chicken/Netherlands/15007212/2015 (H10N7)	Noord Brabant - Heusden	HA	A/Mallard Duck/Netherlands/ 31/2013 (H10N7)	EU - NL - Zuid Holland	708	97.9%
				A/Mallard Duck/Netherlands/ 32/2013 (H10N7)	EU - NL - Zuid Holland	631	
			NA	A/Mallard Duck/Netherlands/ 5/2012 (H10N7)	EU - NL - Zuid Holland	1081	98.1%
2016	A/Chicken/Netherlands/16007311-037041/2016 (H7N9)	Friesland - Hiaure	HA	A/Mallard Duck/Netherlands/ 24/2014 (H7N3)	EU - NL - Noord Holland	617	99.4%
			NA	A/Duck/Bangladesh/26980/2015 (H7N9)	AS - Bangladesh	181	97.3%
				A/Duck/Bangladesh/26992/2015 (H7N9)	AS - Bangladesh	181	
				A/Duck/Bangladesh/27042/2015 (H7N9)	AS - Bangladesh	181	
	A/Chicken/Netherlands/16010778-021-025/2016 (H2N3)	Overijssel - Bathmen	HA	A/Tufted Duck/Georgia/1/2012 (H2N3)	EU - Georgia	1347	97.2%
			NA	A/Mallard Duck/Netherlands/ 24/2014 (H7N3)	EU - NL - Noord Holland	700	98.9%

AS, Asia; EU, Europe; NL, The Netherlands;
* two viruses were isolated from a single poultry farm

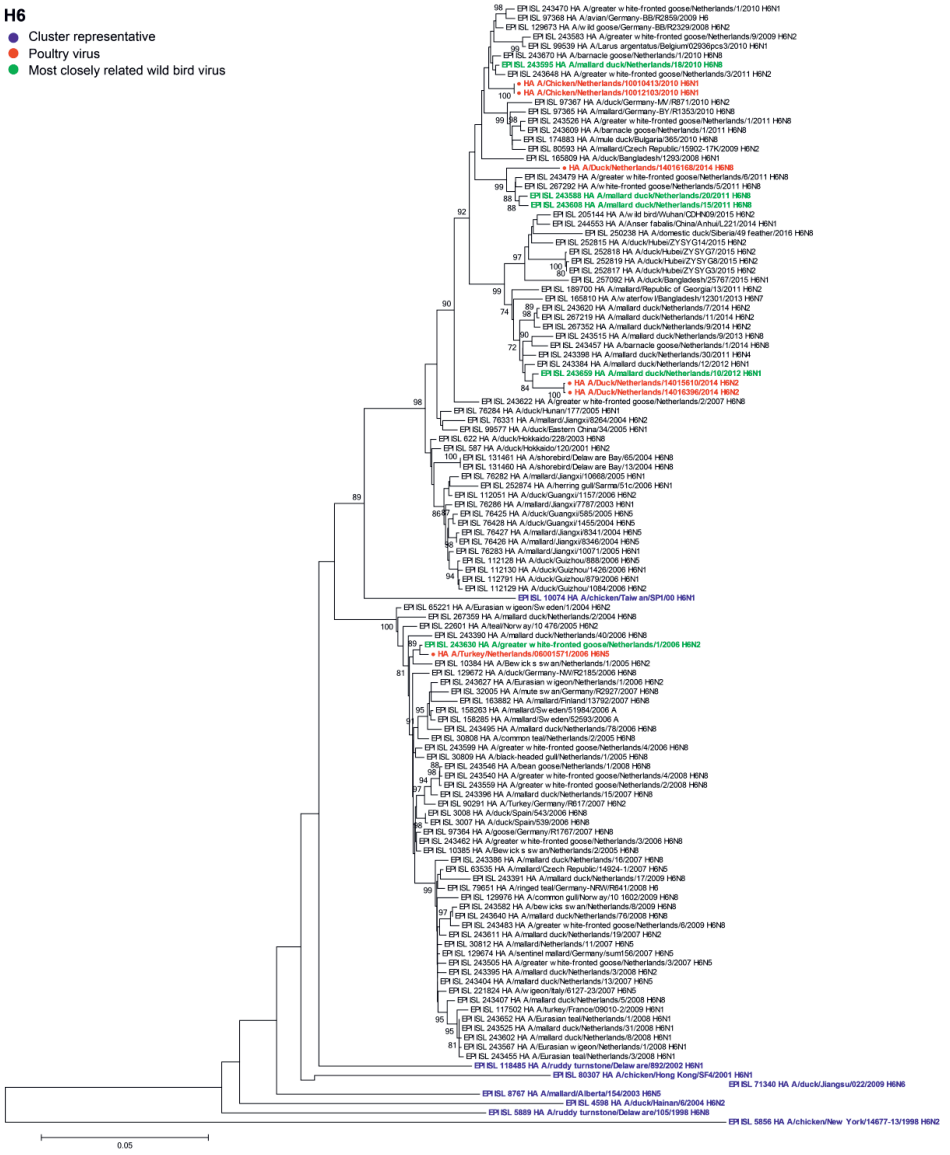
S1 FIGURE. PHYLOGENETIC TREES OF HA GENES.

Neighbour joining (NJ) phylogenetic trees of the hemagglutinin (HA) gene segments of low pathogenic avian influenza (LPAI) viruses from poultry, detected as part of the national avian influenza (AI) surveillance program in the Netherlands, January 2006-September 2016. Nucleotide (nt) sequences of cluster representatives, poultry viruses and top 50 BLAST hits were aligned for each HA separately: H1 (1659 nt), H2 (1679 nt), H3 (1699 nt), H5 (1571 nt), H6 (1596 nt), H7 (1494 nt), (H8 1626 nt), H9 (1567 nt) and H10 (1585 nt). Phylogenetic trees were generated using the Tamura-Nei substitution model with a gamma distribution (shape parameter = 1) for rate variation within the MEGA7 software package. Bootstrap support values above 70 (1,000 replicates) are shown at the branches. Colours represent cluster representatives (blue), poultry viruses (red), and most identical wild bird viruses as determined by BLAST (red). Symbols represent poultry viruses most identical to wild bird viruses isolated in the Netherlands (dots), other European countries (squares) and Asia (triangles). We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu database on which this research is based. All submitters of data may be contacted directly via the GISAID website (http://www.gisaid.org).


H1
Cluster representative
Poultry virus
Most closely related wild bird virus
EPI ISL 243610 HA A/mallard duck/Netherlands/7/2012 H1N1
EPI ISL 267284 HA A/mallard duck/Netherlands/6/2012 H1N1
EPI ISL 243537 HA A/mallard duck/Netherlands/5/2013 H1N1
EPI ISL 267197 HA A/mallard duck/Netherlands/20/2012 H1N1
EPI ISL 243570 HA A/mallard duck/Netherlands/37/2011 H1N1
EPI ISL 243580 HA A/mallard duck/Netherlands/53/2010 H1N1
EPI ISL 243387 HA A/mallard duck/Netherlands/8/2012 H1N1
EPI ISL 144349 HA A/mallard/Sweden/104803/2009 H1N1
EPI ISL 243468 HA A/greater w hite-fronted goose/Netherlands/4/2011 H1N1
EPI ISL 243654 HA A/greater w hite-fronted goose/Netherlands/1/2012 H1N1
• HA A/Chicken/Netherlands/11009919/2011 H1N1
EPI ISL 243594 HA A/mallard duck/Netherlands/51/2010 H1N1
EPI ISL 165792 HA A/duck/Bangladesh/1584/2010 H1N3
EPI ISL 165788 HA A/duck/Bangladesh/1687/2010 H1N1
EPI ISL 165789 HA A/duck/Bangladesh/1352/2009 H1N1
EPI ISL 96133 HA A/duck/Zhejiang/0224-6/2011 H1N2
EPI ISL 149872 HA A/tufted duck/Mongolia/1409/2010 H1N1
EPI ISL 149861 HA A/common goldeneye/Mongolia/1216/2010 H1N1
EPI ISL 149859 HA A/common goldeneye/Mongolia/1238/2010 H1N1
EPI ISL 165790 HA A/duck/Bangladesh/1592/2010 H1N1
EPI ISL 209380 HA A/shelduck/Yasnopolyanskie/4-08-02/2011 H1N1
EPI ISL 138651 HA A/mallard/Republic of Georgia/1/2011 H1N1
EPI ISL 189702 HA A/mallard/Republic of Georgia/1/2012 H1N1
EPI ISL 208811 HA A/duck/Hainan/Q47/2012 H1N2
EPI ISL 189708 HA A/mallard/Republic of Georgia/4/2012 H1N1
EPI ISL 267256 HA A/mallard duck/Netherlands/18/2014 H1N1
EPI ISL 267317 HA A/mallard duck/Netherlands/26/2014 H1N1
EPI ISL 142936 HA A/duck/Guangxi/GXd-1/2011 H1N2
EPI ISL 142938 HA A/duck/Guangxi/GXd-4/2011 H1N2
EPI ISL 142935 HA A/chicken/Guangxi/GXc-1/2011 H1N2
EPI ISL 151927 HA A/w ild w aterfow l/Dongting/C2383/2012 H1N2
EPI ISL 208808 HA A/duck/Yunan/V286/2012 H1N2
EPI ISL 208812 HA A/duck/Anhui/AB10/2011 H1N2
EPI ISL 142939 HA A/duck/Guangxi/GXd-2/2012 H1N2
EPI ISL 142937 HA A/sparrow /Guangxi/GXs-1/2012 H1N2
EPI ISL 142965 HA A/Muscovy duck/Guangxi/GXd-3/2012 H1N2
EPI ISL 85906 HA A/mallard/Italy/432-21/08 H1N1
EPI ISL 120191 HA A/shoveler/Egypt/00134-NAMRU3/2005 H1N1
EPI ISL 243566 HA A/brent goose/Netherlands/1/2006 H1N1
EPI ISL 267427 HA A/mallard duck/Netherlands/12/2005 H1N1
EPI ISL 120208 HA A/teal/Egypt/01351-NAMRU3/2007 H1N1
EPI ISL 85897 HA A/duck/Italy/1447/05 H1N1
EPI ISL 25672 HA A/duck/Italy/281904/2006 H1N1
EPI ISL 28841 HA A/duck/Italy/69238/2007 H1N1
EPI ISL 85904 HA A/mallard/Italy/378-49/06 H1N1
EPI ISL 85903 HA A/pintail/Italy/2703-25/06 H1N1
EPI ISL 267436 HA A/common teal/Netherlands/3/2005 H1N1
EPI ISL 120216 HA A/teal/Egypt/00677-NAMRU3/2004 H1N1
EPI ISL 25673 HA A/goose/Italy/296426/2003 H1N1
EPI ISL 29997 HA A/Goose/Italy/6117/2004 H1N1
EPI ISL 120184 HA A/teal/Egypt/20431-NAMRU3/2003 H1N2
EPI ISL 243445 HA A/greater w hite-fronted goose/Netherlands/1/2007 H1N1
EPI ISL 71602 HA A/mallard/Germany/WV355/07 H1N1
EPI ISL 243653 HA A/bew icks sw an/Netherlands/6/2006 H1N1
EPI ISL 243669 HA A/barnacle goose/Netherlands/1/2006 H1N1
EPI ISL 71601 HA A/w ild duck/Germany/WV30/06 H1N1
EPI ISL 144380 HA A/mallard/Sw eden/99859/2009 H1N2
EPI ISL 144355 HA A/mallard/Sw eden/99791/2009 H1N1
EPI ISL 144378 HA A/mallard/Sw eden/99857/2009 H1N1
EPI ISL 144365 HA A/mallard/Sw eden/99842/2009 H1N1
EPI ISL 144357 HA A/mallard/Sw eden/99818/2009 H1N1
EPI ISL 243518 HA A/mallard duck/Netherlands/64/2006 H1N1
EPI ISL 267245 HA A/mallard duck/Netherlands/46/2006 H1N1
EPI ISL 243539 HA A/bew icks sw an/Netherlands/3/2006 H1N1
EPI ISL 63666 HA A/mallard/Germany/R2843/06 H1N1
EPI ISL 71603 HA A/w ild duck/Germany-NW/R04/08 H1N1
EPI ISL 163876 HA A/mallard/Finland/14740/2008 H1N1
EPI ISL 84561 HA A/Bew icks sw an/Netherlands/1/2007 H1N5
EPI ISL 243497 HA A/bean goose/Netherlands/1/2007 H1N1
• HA A/Turkey/Netherlands/07014290/2007 H1N5
• HA A/Turkey/Netherlands/07016245/2007 H1N5
EPI ISL 158244 HA A/mallard/Sw eden/51588/2006 A
EPI ISL 158182 HA A/mallard/Sw eden/51880/2006 A
EPI ISL 158271 HA A/mallard/Sw eden/52209/2006 A
EPI ISL 91542 HA A/duck/Shimane/188/1999 H1N1
EPI ISL 73384 HA A/mallard/Sw eden/57/2003 H1N1
EPI ISL 84552 HA A/mallard/Netherlands/30/2006 H1N4
EPI ISL 243438 HA A/mallard duck/Netherlands/23/2006 H1N1
EPI ISL 243508 HA A/Eurasian w igeon/Netherlands/6/2007 H1N1
EPI ISL 243383 HA A/Eurasian w igeon/Netherlands/2/2007 H1N1
EPI ISL 80760 HA A/mallard/Bavaria/185-26/2008 H1N1
EPI ISL 243543 HA A/mallard duck/Netherlands/28/2009 H1N1
EPI ISL 267277 HA A/mallard duck/Netherlands/25/2009 H1N1
EPI ISL 8799 HA A/mallard/ALB/201/1998 H1N1
EPI ISL 63571 HA A/northern pintail/Interior Alaska/1/2007 H1N1
0.02


H2
Cluster representative
Poultry virus
Most closely related wild bird virus
EPI ISL 216371 HA A/mallard/Maryland/10OS118/2010 H2N3
EPI ISL 216368 HA A/mallard/Maryland/10OS108/2010 H2N3
EPI ISL 216369 HA A/mallard/Maryland/10OS110/2010 H2N3
EPI ISL 216372 HA A/mallard/Maryland/10OS121/2010 H2N3
EPI ISL 95435 HA A/American green-w inged teal/Illinois/2479/2009 H2N3
EPI ISL 95386 HA A/mallard/Missouri/132/2009 H2N3
EPI ISL 134935 HA A/American green-w inged teal/Illinois/10OS3343/2010 H2N3
EPI ISL 162283 HA A/mallard/Ohio/13OS1972/2013 H2N8
EPI ISL 118566 HA A/mallard/Alberta/297/2007 H2N9
EPI ISL 85166 HA A/American green-w inged teal/Wisconsin/08OS3469/2008 H2N9
EPI ISL 85180 HA A/mallard/Wisconsin/08OS2641/2008 H2N3
EPI ISL 141326 HA A/mallard/Minnesota/AI06-1029/2006 H2N3
EPI ISL 83558 HA A/bufflehead/California/JN1016/2006 H2N9
EPI ISL 22634 HA A/northern shoveler/California/HKWF1128/2007 H2N7
EPI ISL 87682 HA A/duck/OH/492493/2007 H2N3
EPI ISL 87683 HA A/chicken/OH/494832/2007 H2N3
EPI ISL 141325 HA A/mallard/Minnesota/AI06-1003/2006 H2N5
EPI ISL 141101 HA A/mallard/Minnesota/Sg-00692/2008 H2N3
EPI ISL 208175 HA A/American green-w inged teal/Interior Alaska/11BM03165/2011 H2N9
EPI ISL 208181 HA A/mallard/Interior Alaska/11BM03591/2011 H2N9
EPI ISL 85182 HA A/environment/Indiana/08OS2857/2008 H2N3
EPI ISL 95419 HA A/mallard/Wisconsin/2560/2009 H2N3
EPI ISL 95414 HA A/mallard/Wisconsin/2785/2009 H2N3
EPI ISL 141258 HA A/mallard/Minnesota/AI08-3881/2008 H2N2
EPI ISL 63038 HA A/env/California/7303/2008 H2N3
EPI ISL 63040 HA A/env/California/7335/2008 H2N3
EPI ISL 63036 HA A/env/California/7279/2008 H2N3
EPI ISL 119135 HA A/mallard/Alberta/419/2009 H2N3
EPI ISL 99249 HA A/mallard/Alberta/417/2009 H2N3
EPI ISL 119134 HA A/mallard/Alberta/319/2009 H2N3
EPI ISL 119132 HA A/mallard/Alberta/289/2009 H2N9
EPI ISL 119133 HA A/mallard/Alberta/35/2009 H2N3
HA A/Chicken/Netherlands/14003005/2014 H2N7
EPI ISL 148206 HA A/herring gull/Iceland/1320/2011 H2N5
EPI ISL 148242 HA A/w hite-rumped sandpiper/Canada/2541/2011 H2N5
EPI ISL 148208 HA A/herring gull/Iceland/1342/2011 H2N5
EPI ISL 148216 HA A/glaucous gull/Iceland/1444/2011 H2N5
EPI ISL 148214 HA A/great black-backed gull/Iceland/1395/2011 H2N5
EPI ISL 148205 HA A/Iceland gull/Iceland/1124/2011 H2N5
EPI ISL 148209 HA A/herring gull/Iceland/1359/2011 H2N5
EPI ISL 148215 HA A/herring gull/Iceland/1436/2011 H2N5
EPI ISL 148211 HA A/great black-backed gull/Iceland/1393/2011 H2N5
EPI ISL 135004 HA A/mallard/low a/10OS2721/2010 H2N2
EPI ISL 267297 HA A/mallard duck/Netherlands/16/2014 H2N9
EPI ISL 267194 HA A/mallard duck/Netherlands/14/2014 H2N9
EPI ISL 267171 HA A/mallard duck/Netherlands/22/2013 H2N3
EPI ISL 138623 HA A/northern shoveler/Republic of Georgia/1/2010 H2N3
EPI ISL 189713 HA A/mallard/Republic of Georgia/2/2012 H2N3
EPI ISL 189729 HA A/tufted duck/Republic of Georgia/1/2012 H2N3
EPI ISL 209128 HA A/duck/Mongolia/482/2015 H2N3
EPI ISL 209132 HA A/duck/Mongolia/655/2015 H2N3
HA A/Chicken/Netherlands/16010778-021-025/2016 H2N3
EPI ISL 189709 HA A/black-headed gull/Republic of Georgia/7/2012 H2N7
EPI ISL 189705 HA A/black-headed gull/Republic of Georgia/9/2012 H2N5
EPI ISL 189715 HA A/black-headed gull/Republic of Georgia/8/2012 H2N7
EPI ISL 122027 HA A/mallard/Sw eden/58112/2006 H2N2
EPI ISL 122028 HA A/mallard/Sw eden/58451/2006 H2N2
EPI ISL 30791 HA A/mallard/Netherlands/31/2006 H2N2
EPI ISL 122032 HA A/mallard/Netherlands/31/2006 H2N2
EPI ISL 122090 HA A/mallard/Sw eden/103/2005 H2N9
EPI ISL 3141 HA A/Duck/Nanchang/4-184/2000 H2N9
EPI ISL 119075 HA A/duck/Nanchang/2-0485/2000 H2N9
EPI ISL 119129 HA A/w ild duck/Shantou/992/2000 H2N8
EPI ISL 122094 HA A/mallard/Netherlands/12/1999 H2N9
EPI ISL 122093 HA A/mallard/Netherlands/11/1999 H2N9
EPI ISL 122031 HA A/w hite fronted goose/Netherlands/22/1999 H2N2
EPI ISL 122076 HA A/northern pintail/Sw eden/1/2003 H2N3
EPI ISL 122078 HA A/mallard/Sw eden/81/2003 H2N3
EPI ISL 122092 HA A/mallard/Sw eden/5/2005 H2N3
EPI ISL 73395 HA A/northern pintail/Sw eden/1/2003 H2N3
EPI ISL 122079 HA A/mallard/Sw eden/84/2003 H2N3
EPI ISL 189323 HA A/mallard/Sw eden/4737/2004 A
EPI ISL 122085 HA A/mallard/Sw eden/60/2005 A
EPI ISL 73386 HA A/mallard/Sw eden/6/2002 H2N3
EPI ISL 122071 HA A/mallard/Sw eden/30/2002 H2N5
EPI ISL 122072 HA A/mallard/Sw eden/32/2002 H2N3
EPI ISL 122035 HA A/mallard/Netherlands/14/2001 H2N3
EPI ISL 65626 HA A/chicken/PA/298101-4/2004 H2N2
EPI ISL 122077 HA A/mallard/Sw eden/12/2003 H2N3
EPI ISL 150013 HA A/mallard/Sw eden/9/2003 H2N5
EPI ISL 122039 HA A/mallard/Sw eden/99/2002 H2N3
EPI ISL 122074 HA A/mallard/Sw eden/57/2002 H2N3
EPI ISL 122081 HA A/mallard/Sw eden/53/2003 H2N3
0.02
H3
Cluster representative
Poultry virus
Most closely related wild bird virus
EPI ISL 26267 HA A/Anas platyrhynchos/Belgium/12827/2007 H3N8
HA A/Duck/Netherlands/06027358/2006 H3N8
EPI ISL 243606 HA A/gadwall duck/Netherlands/3/2006 H3N8
EPI ISL 267207 HA A/mallard duck/Netherlands/60/2006 H3N8
EPI ISL 33850 HA A/mallard/Netherlands/1/2007 H3N2
EPI ISL 84553 HA A/mallard/Netherlands/28/2006 H3N1
EPI ISL 267236 HA A/mallard duck/Netherlands/51/2008 H3N2
EPI ISL 267203 HA A/mallard duck/Netherlands/14/2011 H3N2
EPI ISL 243527 HA A/mallard duck/Netherlands/17/2011 H3N8
EPI ISL 64024 HA A/Anas platyrhynchos/Spain/0454/2006 H3N8
EPI ISL 25671 HA A/duck/Italy/194659/2006 H3N2
EPI ISL 85901 HA A/duck/Italy/3139-2/06 H3N8
EPI ISL 120213 HA A/mallard/Ukraine/05842-NMRLB/2006 H3N8
EPI ISL 243492 HA A/mallard duck/Netherlands/7/2008 H3N8
EPI ISL 267342 HA A/mallard duck/Netherlands/2/2013 H3N6
EPI ISL 79645 HA A/mallard/Germany-RR232/07 H3N8
EPI ISL 85900 HA A/duck/Italy/6207/08 H3N6
EPI ISL 79642 HA A/mallard/Germany-BW/SR871/2008 H3N8
EPI ISL 257070 HA A/duck/Bangladesh/26918/2015 H3N6
EPI ISL 257089 HA A/duck/Bangladesh/26974/2015 H3N6
EPI ISL 267386 HA A/mallard duck/Netherlands/34/2010 H3N2
EPI ISL 240672 HA A/shoveler/Chany/82K/2014 H3N8
EPI ISL 149873 HA A/common shelduck/Mongolia/2076/2011 H3N8
EPI ISL 209111 HA A/duck/Mongolia/30/2015 H3N8
EPI ISL 209134 HA A/duck/Mongolia/777/2015 H3N8
EPI ISL 209135 HA A/duck/Mongolia/996/2015 H3N8
EPI ISL 86980 HA A/mallard/Czech Republic/13577-24K/2010 H3N8
EPI ISL 189686 HA A/common Teal/Republic of Georgia/1/2011 H3N8
EPI ISL 31484 HA A/mallard/Hungary/19616/2007 H3N8
EPI ISL 158324 HA A/mallard/Sw eden/79230/2008 A
EPI ISL 189613 HA A/mallard/Sw eden/79389/2008 A
EPI ISL 32007 HA A/mallard/Germany/WV437/2004 H3N2
EPI ISL 189687 HA A/mallard/Republic of Georgia/3/2011 H3N8
EPI ISL 63529 HA A/mallard/Czech Republic/14516/2007 H3N8
EPI ISL 158261 HA A/mallard/Sw eden/51976/2006 A
EPI ISL 243402 HA A/dunlin/Netherlands/2/2007 H3N8
EPI ISL 243636 HA A/dunlin/Netherlands/3/2007 H3N8
EPI ISL 30805 HA A/turnstone/Netherlands/1/2007 H3N8
EPI ISL 267251 HA A/mallard duck/Netherlands/1/2003 H3N8
EPI ISL 267401 HA A/mallard duck/Netherlands/1/2004 H3N8
EPI ISL 73383 HA A/common teal/Sw eden/1/2003 H3N3
EPI ISL 189612 HA A/mallard/Sw eden/1636/2002 H3N8
EPI ISL 267224 HA A/mallard duck/Netherlands/16/2012 H3N2
EPI ISL 30546 HA A/chicken/Laos/A0573/2007 H3N8
EPI ISL 190031 HA A/duck/Guangxi/175D12/2014 H3N6
EPI ISL 25506 HA A/northern shoveler/California/HKWF848/2007 H3N7
EPI ISL 30020 HA A/mallard/Interior Alaska/8/2007 H3N8
0.02

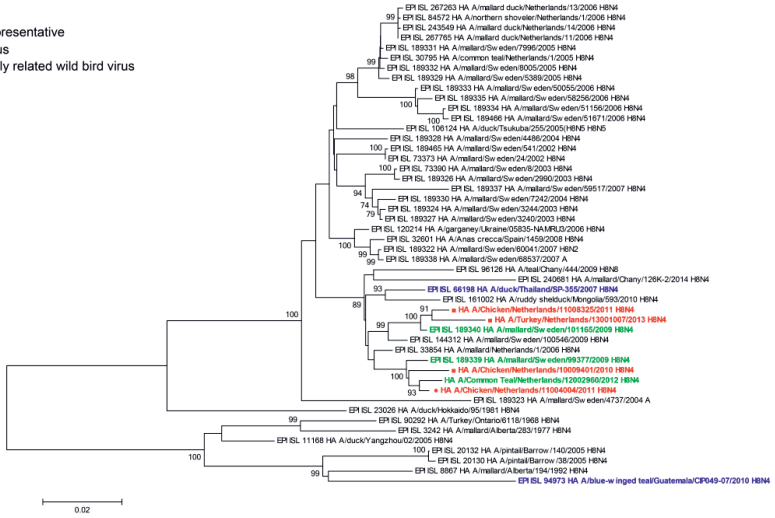

H5
Cluster representative
Poultry virus
Most closely related wild bird virus
HA A/Chicken/Netherlands/13015884/2013 H5N3
HA A/Chicken/Netherlands/13016283-031-035/2013 H5N3
HA A/Chicken/Netherlands/14002541/2014 H5N1
EPI ISL 158945 HA A/razorbill/Scotland/87343/14 H5
EPI ISL 205833 HA A/chicken/Scotland/532/2016 H5N1
HA A/Chicken/Netherlands/15005968-056060/2015 H5N2
EPI ISL 165808 HA A/duck/Bangladesh/1559/2009 H5N2
EPI ISL 98885 HA A/duck/Mongolia/195/2011 H5N3
EPI ISL 144490 HA A/tundra sw an/Shimane/3211A001/2011 H5N2
EPI ISL 158779 HA A/w ild duck/Korea/PSC18-20/2010 H5N8
EPI ISL 158778 HA A/w ild duck/Korea/PSC18-15/2010 H5N8
EPI ISL 256570 HA A/aquatic bird/Korea/CN2/2009 H5N2
EPI ISL 131280 HA A/baikal teal/Xianghai/426/2011 H5N2
EPI ISL 131281 HA A/spot-billed duck/Xianghai/427/2011 H5N2
EPI ISL 131283 HA A/little grebe/Xianghai/429/2011 H5N2
EPI ISL 131284 HA A/green-w inged teal/Xianghai/430/2011 H5N2
EPI ISL 131282 HA A/canvasback/Xianghai/428/2011 H5N2
EPI ISL 156345 HA A/duck/Thailand/CU-12205C/2012 H5N8
EPI ISL 243385 HA A/greater w hite-fronted goose/Netherlands/5/2010 H5N3
EPI ISL 148203 HA A/great black-backed gull/Iceland/1110/2011 H5N2
EPI ISL 116138 HA A/mallard/Czech Republic/15008-11K/2009 H5N3
EPI ISL 243485 HA A/mallard duck/Netherlands/28/2010 H5N2
EPI ISL 243592 HA A/Eurasian w igeon/Netherlands/1/2010 H5N2
EPI ISL 252419 HA A/guinea fow l/France/150207n/2015 H5N9
EPI ISL 234918 HA A/chicken/France/150169a/2015 H5N1
EPI ISL 205110 HA A/chicken/Wuhan/WHUF/2014 H5N2
EPI ISL 63709 HA A/teal/Crimea/97/2008 H5N2
EPI ISL 87809 HA A/spur-w inged goose/Nigeria/226/2008 H5N2
EPI ISL 87807 HA A/spur-w inged goose/Nigeria/2/2008 H5N2
EPI ISL 116137 HA A/mallard/Czech Republic/14602-37K/2011 H5N2
EPI ISL 243542 HA A/greater w hite-fronted goose/Netherlands/2/2010 H5N2
EPI ISL 267289 HA A/w hite-fronted goose/Netherlands/3/2010 H5N2
EPI ISL 71224 HA A/w ild duck/Germany/R2892/08 H5N3
EPI ISL 156215 HA A/duck/Moscow /4182/2010 H5N3
EPI ISL 99818 HA A/w hooper sw an/Germany-HER635/2009 H5N2
EPI ISL 243617 HA A/mallard duck/Netherlands/19/2009 H5N3
EPI ISL 243514 HA A/mallard duck/Netherlands/20/2009 H5N3
EPI ISL 144326 HA A/mallard/Sw eden/101226/2009 H5N9
EPI ISL 144327 HA A/mallard/Sw eden/101288/2009 H5N9
EPI ISL 144325 HA A/mallard/Sw eden/101224/2009 H5N9
EPI ISL 243601 HA A/greater w hite-fronted goose/Netherlands/2/2009 H5N3
EPI ISL 243646 HA A/mallard duck/Netherlands/30/2008 H5N3
EPI ISL 158321 HA A/mallard/Sw eden/79167/2008 A
EPI ISL 267166 HA A/mallard duck/Netherlands/38/2008 H5N3
EPI ISL 243465 HA A/Eurasian w igeon/Netherlands/1/2009 H5N2
EPI ISL 74838 HA A/Anas platyrhynchos/Belgium/09-762-P1/2008 H5N1
EPI ISL 74837 HA A/Anas platyrhynchos/09-884/2008 H5N3
EPI ISL 10102 HA A/mallard/Bavaria/1/2005 H5N2
EPI ISL 7159 HA A/teal/Italy/3931-38/2005 H5N2
EPI ISL 60062 HA A/mallard/Italy/5582-31/2007 H5N2
EPI ISL 120183 HA A/shoveler/Egypt/20313-NAMRU3/2003 H5N2
EPI ISL 7160 HA A/teal/Italy/3812/2005 H5N3
EPI ISL 68242 HA A/mallard/Bavaria/40/2006 H5
EPI ISL 24789 HA A/mallard/Italy/4223-2/2006 H5N2
EPI ISL 243585 HA A/mallard duck/Netherlands/36/2006 H5N3
EPI ISL 77445 HA A/mallard/PT/28006/2007 H5N3
EPI ISL 77448 HA A/mallard/PT/28003/2007 H5N3
EPI ISL 77447 HA A/mallard/PT/37369/2007 H5N3
EPI ISL 243405 HA A/mallard duck/Netherlands/1/2008 H5N3
EPI ISL 68237 HA A/mallard/Bavaria/35/2006 H5
EPI ISL 68240 HA A/mallard/Bavaria/38/2006 H5N3
EPI ISL 243561 HA A/mallard duck/Netherlands/28/2008 H5N2
EPI ISL 243475 HA A/bew ick's sw an/Netherlands/5/2008 H5N2
EPI ISL 243655 HA A/greater w hite-fronted goose/Netherlands/4/2009 H5N2
EPI ISL 243521 HA A/mallard duck/Netherlands/5/2009 H5N2
EPI ISL 7494 HA A/duck/Italy/775/2004 H5N3
EPI ISL 84556 HA A/mallard/Sw eden/74/2003 H5N2
EPI ISL 158394 HA A/mallard/Sw eden/101782/2009 A
EPI ISL 243557 HA A/greater w hite-fronted goose/Netherlands/5/2008 H5N2
EPI ISL 243589 HA A/greater w hite-fronted goose/Netherlands/6/2008 H5N2
EPI ISL 7029 HA A/turkey/Italy/1258/2005 H5N2
EPI ISL 25703 HA A/sw an/Mangystau/3/2006 H5N1
EPI ISL 62729 HA A/mallard/Hokkaido/24/2009 H5N1
EPI ISL 143806 HA A/w ild bird/Korea/A344-2/2009 H5N1
EPI ISL 134108 HA A/w ild duck/Korea/SNU50-5/2009 H5N1
EPI ISL 134109 HA A/w ild duck/Korea/SNU50-5/2009 H5N1
EPI ISL 190641 HA A/chicken/Vietnam/NCVD-03/2008 H5N1
EPI ISL 104408 HA A/great crested grebe/Qinghai/1/2010 H5N1
EPI ISL 169632 HA A/domestic duck/Germany-NIR3468/2014 H5N8
EPI ISL 175337 HA A/Taiw an/DV413/2006 H5N2
Asian strains <1985
EPI ISL 69991 HA A/turkey/England/N28/1973 H5N2
EPI ISL 84881 HA A/duck/Victoria/26/1981 H5N2
EPI ISL 168490 HA A/chicken/Scotland/1959 H5N1
EPI ISL 24631 HA A/chicken/Texas/167280-4/2002 H5N3
EPI ISL 3706 HA A/chicken/Chiapas/15224/97 H5N2
EPI ISL 25500 HA A/cinnamon teal/California/HKWF1111/2007 H5N7
EPI ISL 30685 HA A/duck/Tsukuba/168/2005 H5N2
EPI ISL 69393 HA A/duck/Korea/GJ54/2004 H5N2
HA A/Chicken/Netherlands/14003323/2014 H5N2
EPI ISL 139369 HA A/Eurasian Woodcock/Vietnam/8/2007 H5N2
EPI ISL 77203 HA A/w ild bird/Korea/L60-2/2008 H5N2
EPI ISL 143942 HA A/duck/Hunan/S4101/2011 H5N2
EPI ISL 143944 HA A/duck/Hunan/S4120/2011 H5N2
EPI ISL 143945 HA A/duck/Hunan/S4124/2011 H5N2
0.02


H6
Cluster representative
Poultry virus
Most closely related wild bird virus
EPI ISL 243470 HA A/greater w hite-fronted goose/Netherlands/1/2010 H6N1
EPI ISL 97368 HA A/avian/Germany-BB/R2859/2009 H6
EPI ISL 129673 HA A/w ild goose/Germany-BB/R2329/2008 H6N2
EPI ISL 243583 HA A/greater w hite-fronted goose/Netherlands/9/2009 H6N2
EPI ISL 99539 HA A/Larus argentatus/Belgium/02936pcs3/2010 H6N1
EPI ISL 243670 HA A/barnacle goose/Netherlands/1/2010 H6N8
EPI ISL 243595 HA A/mallard duck/Netherlands/18/2010 H6N8
EPI ISL 243648 HA A/greater w hite-fronted goose/Netherlands/3/2011 H6N2
HA A/Chicken/Netherlands/10010413/2010 H6N1
HA A/Chicken/Netherlands/10012103/2010 H6N1
EPI ISL 97367 HA A/duck/Germany-NM/R671/2010 H6N2
EPI ISL 97365 HA A/mallard/Germany-SY/R1353/2010 H6N8
EPI ISL 243526 HA A/greater w hite-fronted goose/Netherlands/1/2011 H6N8
EPI ISL 243609 HA A/barnacle goose/Netherlands/1/2011 H6N8
EPI ISL 174883 HA A/mule duck/Bulgaria/365/2010 H6N8
EPI ISL 80593 HA A/mallard/Czech Republic/15902-17K/2009 H6N2
EPI ISL 165809 HA A/duck/Bangladesh/1293/2008 H6N1
HA A/Duck/Netherlands/14016168/2014 H6N8
EPI ISL 243479 HA A/greater w hite-fronted goose/Netherlands/6/2011 H6N8
EPI ISL 267292 HA A/w hite-fronted goose/Netherlands/5/2011 H6N8
EPI ISL 243588 HA A/mallard duck/Netherlands/20/2011 H6N8
EPI ISL 243608 HA A/mallard duck/Netherlands/15/2011 H6N8
EPI ISL 205144 HA A/w ild bird/Wuhan/CH-N09/2015 H6N2
EPI ISL 244553 HA A/Anser fabalis/China/Anhui/L221/2014 H6N1
EPI ISL 250238 HA A/domestic duck/Siberia/49 feather/2016 H6N8
EPI ISL 252815 HA A/duck/Hubei/ZYSYG14/2015 H6N2
EPI ISL 252818 HA A/duck/Hubei/ZYSYG7/2015 H6N2
EPI ISL 252819 HA A/duck/Hubei/ZYSYG8/2015 H6N2
EPI ISL 252817 HA A/duck/Hubei/ZYSYG3/2015 H6N2
EPI ISL 257092 HA A/duck/Bangladesh/25767/2015 H6N1
EPI ISL 189700 HA A/mallard/Republic of Georgia/13/2011 H6N2
EPI ISL 165810 HA A/w aterfow l/Bangladesh/12301/2013 H6N7
EPI ISL 243620 HA A/mallard duck/Netherlands/7/2014 H6N2
EPI ISL 267219 HA A/mallard duck/Netherlands/11/2014 H6N2
EPI ISL 267352 HA A/mallard duck/Netherlands/9/2014 H6N2
EPI ISL 243515 HA A/mallard duck/Netherlands/9/2013 H6N8
EPI ISL 243457 HA A/barnacle goose/Netherlands/1/2014 H6N8
EPI ISL 243398 HA A/mallard duck/Netherlands/30/2011 H6N4
EPI ISL 243384 HA A/mallard duck/Netherlands/12/2012 H6N1
EPI ISL 243659 HA A/mallard duck/Netherlands/10/2012 H6N1
HA A/Duck/Netherlands/14015610/2014 H6N2
HA A/Duck/Netherlands/14015396/2014 H6N2
EPI ISL 243622 HA A/greater w hite-fronted goose/Netherlands/2/2007 H6N8
EPI ISL 76284 HA A/duck/Hunan/177/2005 H6N1
EPI ISL 76331 HA A/mallard/Jiangxi/8264/2004 H6N2
EPI ISL 99577 HA A/duck/Eastern China/34/2005 H6N1
EPI ISL 622 HA A/duck/Hokkaido/228/2003 H6N8
EPI ISL 587 HA A/duck/Hokkaido/120/2001 H6N2
EPI ISL 131461 HA A/shorebird/Delaw are Bay/65/2004 H6N8
EPI ISL 131460 HA A/shorebird/Delaw are Bay/13/2004 H6N8
EPI ISL 76282 HA A/mallard/Jiangxi/10668/2005 H6N1
EPI ISL 252874 HA A/herring gull/Sarma/51c/2006 H6N1
EPI ISL 112051 HA A/duck/Guangxi/1157/2006 H6N2
EPI ISL 76288 HA A/mallard/Jiangxi/7787/2003 H6N1
EPI ISL 76425 HA A/duck/Guangxi/585/2005 H6N5
EPI ISL 76428 HA A/duck/Guangxi/1455/2004 H6N5
EPI ISL 76427 HA A/mallard/Jiangxi/8341/2004 H6N5
EPI ISL 76426 HA A/mallard/Jiangxi/8346/2004 H6N5
EPI ISL 76283 HA A/mallard/Jiangxi/10071/2005 H6N1
EPI ISL 112128 HA A/duck/Guizhou/888/2006 H6N6
EPI ISL 112130 HA A/duck/Guizhou/1426/2006 H6N1
EPI ISL 112791 HA A/duck/Guizhou/879/2006 H6N1
EPI ISL 112129 HA A/duck/Guizhou/1084/2006 H6N2
EPI ISL 10074 HA A/chicken/Taiw an/SPI/00 H6N1
EPI ISL 65221 HA A/Eurasian w igeon/Sw eden/1/2004 H6N2
EPI ISL 267359 HA A/mallard duck/Netherlands/2/2004 H6N8
EPI ISL 22601 HA A/teal/Norw ay/10 476/2005 H6N2
EPI ISL 243390 HA A/mallard duck/Netherlands/40/2006 H6N8
EPI ISL 243630 HA A/greater w hite-fronted goose/Netherlands/1/2006 H6N2
HA A/Turkey/Netherlands/06001571/2006 H6N5
EPI ISL 10384 HA A/Bew ick s sw an/Netherlands/1/2005 H6N2
EPI ISL 129672 HA A/duck/Germany-NW/R2185/2006 H6N8
EPI ISL 243627 HA A/Eurasian w igeon/Netherlands/1/2008 H6N2
EPI ISL 32005 HA A/mute sw an/Germany/R2927/2007 H6N8
EPI ISL 163882 HA A/mallard/Finland/13792/2007 H6N8
EPI ISL 158263 HA A/mallard/Sw eden/51984/2006 A
EPI ISL 158285 HA A/mallard/Sw eden/52593/2006 A
EPI ISL 243495 HA A/mallard duck/Netherlands/78/2006 H6N8
EPI ISL 30808 HA A/common teal/Netherlands/2/2005 H6N8
EPI ISL 243599 HA A/greater w hite-fronted goose/Netherlands/4/2006 H6N8
EPI ISL 30809 HA A/black-headed gull/Netherlands/1/2005 H6N8
EPI ISL 243546 HA A/bean goose/Netherlands/1/2008 H6N8
EPI ISL 243540 HA A/greater w hite-fronted goose/Netherlands/4/2008 H6N8
EPI ISL 243559 HA A/greater w hite-fronted goose/Netherlands/2/2008 H6N8
EPI ISL 243396 HA A/mallard duck/Netherlands/15/2007 H6N8
EPI ISL 90291 HA A/Turkey/Germany/R617/2007 H6N2
EPI ISL 3008 HA A/duck/Spain/543/2006 H6N8
EPI ISL 3007 HA A/duck/Spain/539/2006 H6N8
EPI ISL 97364 HA A/goose/Germany/R1767/2007 H6N8
EPI ISL 243462 HA A/greater w hite-fronted goose/Netherlands/3/2006 H6N8
EPI ISL 10385 HA A/Bew ick s sw an/Netherlands/2/2005 H6N8
EPI ISL 243386 HA A/mallard duck/Netherlands/16/2007 H6N8
EPI ISL 63535 HA A/mallard/Czech Republic/14924-1/2007 H6N5
EPI ISL 243391 HA A/mallard duck/Netherlands/17/2009 H6N8
EPI ISL 79651 HA A/ringed teal/Germany-NRW/R641/2008 H6
EPI ISL 129976 HA A/common gull/Norw ay/10 1602/2009 H6N8
EPI ISL 243582 HA A/bew icks sw an/Netherlands/8/2009 H6N8
EPI ISL 243640 HA A/mallard duck/Netherlands/76/2008 H6N8
EPI ISL 243483 HA A/greater w hite-fronted goose/Netherlands/6/2009 H6N8
EPI ISL 243611 HA A/mallard duck/Netherlands/19/2007 H6N2
EPI ISL 30812 HA A/mallard/Netherlands/11/2007 H6N5
EPI ISL 129674 HA A/sentinel mallard/Germany/sum156/2007 H6N5
EPI ISL 243505 HA A/greater w hite-fronted goose/Netherlands/3/2007 H6N5
EPI ISL 243395 HA A/mallard duck/Netherlands/3/2008 H6N2
EPI ISL 243404 HA A/mallard duck/Netherlands/13/2007 H6N5
EPI ISL 221824 HA A/w igeon/Italy/6127-23/2007 H6N5
EPI ISL 243407 HA A/mallard duck/Netherlands/5/2008 H6N8
EPI ISL 117502 HA A/turkey/France/09010-2/2009 H6N1
EPI ISL 243652 HA A/Eurasian teal/Netherlands/1/2008 H6N1
EPI ISL 243525 HA A/mallard duck/Netherlands/31/2008 H6N1
EPI ISL 243602 HA A/mallard duck/Netherlands/8/2008 H6N1
EPI ISL 243567 HA A/Eurasian w igeon/Netherlands/1/2008 H6N1
EPI ISL 243455 HA A/Eurasian teal/Netherlands/3/2008 H6N1
EPI ISL 118485 HA A/ruddy turnstone/Delaw are/892/2002 H6N1
EPI ISL 80307 HA A/chicken/Hong Kong/SF4/2001 H6N1
EPI ISL 71340 HA A/duck/Jiangsu/022/2009 H6N6
EPI ISL 8767 HA A/mallard/Alberta/154/2003 H6N5
EPI ISL 4598 HA A/duck/Hainan/6/2004 H6N2
EPI ISL 5889 HA A/ruddy turnstone/Delaw are/105/1998 H6N8
EPI ISL 5856 HA A/chicken/New York/14677-13/1998 H6N2
0.05


H7
Cluster representative
Poultry virus
Most closely related wild bird virus
HA A/Chicken/Netherlands/11011392/2011 H7N7
HA A/Turkey/Netherlands/11011530/2011 H7N7
HA A/Chicken/Netherlands/11011326/2011 H7N7
HA A/Chicken/Netherlands/11008327/2011 H7N7
EPI ISL 151743 HA A/chicken/Germany/R1362/11 H7N7
HA A/mallard duck/Netherlands/1/2011 H7N7
EPI ISL 213840 HA A/Anas platyrhynchos/Belgium/25576cls41/2012 H7N7
EPI ISL 99175 HA A/swan/Czech Republic/5416/2011 H7N7
HA A/Chicken/Netherlands/11004875/2011 H7N1
EPI ISL 86043 HA A/chicken/Germany-N/R874/2010 H7N7
EPI ISL 74018 HA A/mallard/Poland/446/09 H7N7
EPI ISL 109745 HA A/Branta canadensis/Belgium/13000-9-2/2010 H7N7
EPI ISL 143739 HA A/chicken/Spain/1664/2013 H7N1
HA A/Chicken/Netherlands/13003601/2013 H7N7
HA A/Chicken/Netherlands/13003983/2013 H7N7
EPI ISL 151692 HA A/mallard/Denmark/303878-15/13 H7N7
HA A/Chicken/Netherlands/12014794/2012 H7N7
EPI ISL 213839 HA A/Anas platyrhynchos/Belgium/23852cls33/2012 H7N7
EPI ISL 151693 HA A/mallard/Denmark/58-62-KLSV-119/09 H7N1
EPI ISL 60708 HA A/mallard/Poland/41/09 H7N7
EPI ISL 163883 HA A/teal/Finland/9201/2010 H7N3
EPI ISL 25597 HA A/mallard/Italy/6104-14/2007 H7N3
EPI ISL 25593 HA A/mallard/Italy/6103-5/2007 H7N1
EPI ISL 20096 HA A/mallard/Italy/6103-12/2007 H7N3
EPI ISL 25598 HA A/shoveler/Italy/6104-30/2007 H7N3
EPI ISL 189718 HA A/mallard/Republic of Georgia/2/2010 H7N7
EPI ISL 189724 HA A/yellow -legged gull/Republic of Georgia/1/2012 A
EPI ISL 151747 HA A/mallard/Italy/11VIR-540/11 H7N7
EPI ISL 151748 HA A/teal/Italy/11VIR-792/11 H7N6
EPI ISL 216878 HA A/mallard/Nizhnegorsk/3-06-10/2010 H7N7
EPI ISL 216877 HA A/mallard/Nevorsy/tailvka/10-23-12/2010 H7N7
EPI ISL 216879 HA A/mallard/Askania-Nova/39-15-02/2011 H7N7
EPI ISL 243579 HA A/mallard duck/Netherlands/1/2013 H7N7
EPI ISL 146472 HA A/turkey/Germany-N/R534/2013 H7N7
EPI ISL 267186 HA A/mallard duck/Netherlands/8/2014 H7N5
EPI ISL 243563 HA A/mallard duck/Netherlands/3/2015 H7N7
EPI ISL 243439 HA A/mallard duck/Netherlands/13/2013 H7N7
EPI ISL 171121 HA A/chicken/England/2830/2015 H7N7
EPI ISL 260528 HA A/mallard/Netherlands/20/2015 H7N8
HA A/Chicken/Netherlands/16007311-037041/2016 H7N9
EPI ISL 267389 HA A/mallard duck/Netherlands/24/2014 H7N3
EPI ISL 120206 HA A/shoveler/Egypt/00017-NAMRU3/2007 H7N3
EPI ISL 120202 HA A/shoveler/Egypt/00241-NAMRU3/2007 H7N3
EPI ISL 99642 HA A/duck/Turkey/55/Cetinkaya/49/2006 H7N1
EPI ISL 243590 HA A/mallard duck/Netherlands/2/2009 H7N7
EPI ISL 243466 HA A/mallard duck/Netherlands/7/2009 H7N7
EPI ISL 60937 HA A/goose/Czech Republic/1848/2009 H7N9
EPI ISL 243661 HA A/mallard duck/Netherlands/82/2008 H7N7
EPI ISL 200923 HA A/ostrich/South Africa/IMP2013 H7N7
EPI ISL 25594 HA A/mallard/Italy/3397-65/2008 H7N1
EPI ISL 120195 HA A/shoveler/Egypt/14879-NAMRU3/2006 H7N1
EPI ISL 63708 HA A/teal/Crimea/2027/2008 H7N8
EPI ISL 26339 HA A/mallard/Poland/01/08 H7N7
EPI ISL 25596 HA A/mallard/Italy/794-18/2008 H7N1
EPI ISL 25595 HA A/teal/Italy/794-3/2008 H7N1
EPI ISL 32602 HA A/Anas crecca/Spain/1460/2008 H7N9
EPI ISL 60058 HA A/guinea fow l/Italy/407/2008 H7N1
EPI ISL 151750 HA A/mallard/Italy/731/09 H7N1
EPI ISL 267268 HA A/mallard duck/Netherlands/23/2013 H7N3
A/Chicken/NL-Barneveld/15004745-001-005/2015 H7N7
EPI ISL 260526 HA A/mallard/Netherlands/15/2015 H7N7
EPI ISL 260529 HA A/mallard/Netherlands/12/2015 H7
EPI ISL 260523 HA A/mallard/Netherlands/16/2015 H7N7
EPI ISL 120199 HA A/Egyptian goose/Egypt/05588-NAMRU3/2006 H7N7
EPI ISL 120185 HA A/shoveler/Egypt/00597-NAMRU3/2004 H7N1
EPI ISL 120188 HA A/teal/Egypt/00835-NAMRU3/2004 H7N7
EPI ISL 151744 HA A/pochard/Germany/R916/06 H7N7
EPI ISL 12738 HA A/chicken/Wales/1306/2007 H7N2
EPI ISL 151746 HA A/swan/Germany/R57/06 H7N7
EPI ISL 186320 HA A/mallard/Sweden/5994/2005 H7N7
EPI ISL 84571 HA A/mallard/Sweden/95/2005 H7N7
EPI ISL 11490 HA A/chicken/England/4054/2006 H7N3
EPI ISL 11489 HA A/chicken/England/4266/2006 H7N3
EPI ISL 84564 HA A/mallard/Netherlands/9/2005 H7N7
EPI ISL 77441 HA A/duck/Denmark/53-147-B/2008 H7N1
EPI ISL 30813 HA A/mallard/Netherlands/33/2006 H7N8
EPI ISL 33852 HA A/mallard/Netherlands/29/2006 H7N2
EPI ISL 33853 HA A/mallard/Netherlands/22/2007 H7N1
EPI ISL 94068 HA A/shoveler/Italy/2698-27/2006 H7N7
EPI ISL 151745 HA A/swan/Germany/R736/06 H7N4
EPI ISL 31483 HA A/mute swan/Hungary/5973/2007 H7N7
EPI ISL 77444 HA A/tufted duck/PT/13771/2006 H7N3
EPI ISL 20095 HA A/chicken/Italy/2837-58/2007 H7N3
EPI ISL 20094 HA A/chicken/Italy/2837-54/2007 H7N3
HA A/Chicken/Netherlands/06022003/2006 H7N7
EPI ISL 267189 HA A/mallard duck/Netherlands/4/2010 H7N3
EPI ISL 243568 HA A/mallard duck/Netherlands/60/2008 H7N1
EPI ISL 243581 HA A/bewicks swan/Netherlands/7/2008 H7N1
HA A/Chicken/Netherlands/10007882/2010 H7N4
EPI ISL 243569 HA A/mallard duck/Netherlands/77/2008 H7N1
EPI ISL 174478 HA A/Anas platyrhynchos/Spain/1877/2009 H7N2
EPI ISL 243668 HA A/bean goose/Netherlands/1/2009 H7N1
EPI ISL 213838 HA A/Tadorna tadorna/Belgium/3441-P3/2009 H7N1
EPI ISL 13574 HA A/mallard/Italy/199/01 H7N3
EPI ISL 3911 HA A/mallard/Italy/43/2001 H7N3
EPI ISL 166868 HA A/ostrich/Italy/8856/2002 H7N3
EPI ISL 21059 HA A/turkey/Italy/8535/02 H7N3
EPI ISL 20081 HA A/chicken/Italy/8093/2002 H7N3
EPI ISL 7493 HA A/turkey/Italy/8000/2002 H7N3
EPI ISL 94075 HA A/turkey/Italy/8534/2002 H7N3
EPI ISL 6917 HA A/turkey/Italy/251/2003 H7N3
EPI ISL 3910 HA A/turkey/Italy/21464/02 H7N3
EPI ISL 8070 HA A/turkey/Italy/9742/2002 H7N3
EPI ISL 20088 HA A/turkey/Italy/9369/2002 H7N3
EPI ISL 7011 HA A/turkey/Italy/1010/2003 H7N3
EPI ISL 6919 HA A/turkey/Italy/8912/2002 H7N3
EPI ISL 24788 HA A/chicken/Italy/682/2003 H7N3
EPI ISL 68417 HA A/mallard/Netherlands/12/2000 H7N3
EPI ISL 200122 HA A/pigeon/Wuxi/0405007/2013 H7N9
EPI ISL 76068 HA A/chicken/Chaiwa/NARC-148/2004 H7N3
EPI ISL 22568 HA A/chicken/PA/19241/1997 H7N2
EPI ISL 65193 HA A/chicken/British Columbia/04 H7N3
EPI ISL 18347 HA A/chicken/British Columbia/NS-01827-4/2004 H7N3
0.05

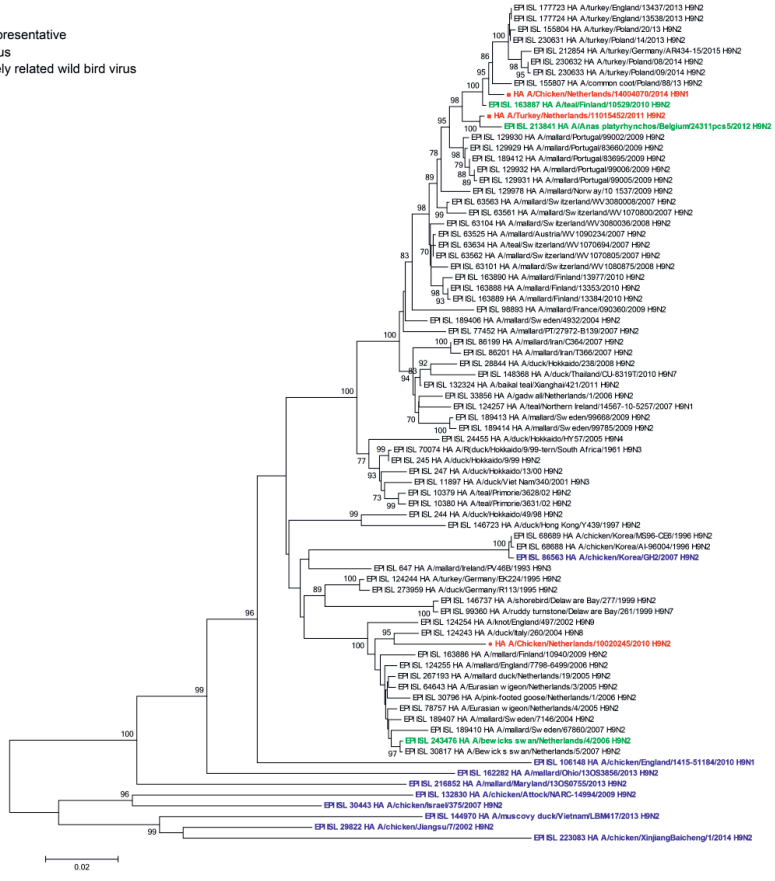
H8

- Cluster representative
- Poultry virus
- Most closely related wild bird virus



H9

- Cluster representative
- Poultry virus
- Most closely related wild bird virus




H10
Cluster representative
Poultry virus
Most closely related wild bird virus
EPI ISL 167906 HA Seal/Sweden/SVA0824/2014 /H10N7 H10N7
EPI ISL 166971 HA A/harbour seal/Germany/1/2014 H10N7
EPI ISL 166972 HA A/harbour seal/Germany/1/2014 H10N7
EPI ISL 166244 HA A/harbor seal/Denmark/14-5061-1lu/2014-07 H10N7
EPI ISL 167225 HA A/Seal/Sweden/SVA0546/2014 H10N7
EPI ISL 243453 HA A/mallard duck/Netherlands/31/2013 H10N7
EPI ISL 243491 HA A/mallard duck/Netherlands/32/2013 H10N7
EPI ISL 169274 HA A/mallard/Netherlands/1/2014 H10N7
EPI ISL 243434 HA A/mallard duck/Netherlands/33/2014 H10N7
EPI ISL 243393 HA A/mallard duck/Netherlands/6/2015 H10N7
HA A/Chicken/Netherlands/15007212/2015 H10N7
EPI ISL 189392 HA A/mallard/Sweden/133546/2011 H10N4
EPI ISL 166243 HA A/mallard/Denmark/16109-4/2011-11-14 H10N6
EPI ISL 243574 HA A/mallard duck/Netherlands/2/2011 H10N7
EPI ISL 169275 HA A/mallard/Netherlands/50/2010 H10N7
EPI ISL 243500 HA A/mallard duck/Netherlands/47/2010 H10N7
EPI ISL 169276 HA A/mallard/Netherlands/47/2010 H10N7
EPI ISL 189714 HA A/mallard/Republic of Georgia/14/2011 H10N7
EPI ISL 189685 HA A/domestic duck/Republic of Georgia/1/2010 H10N7
HA A/Turkey/Netherlands/12005615/2012 H10N8
HA A/Turkey/Netherlands/12004763-001-004/2012 H10N9
HA A/Chicken/Netherlands/12002495-001-005/2012 H10N9
EPI ISL 243456 HA A/mallard duck/Netherlands/5/2012 H10N7
EPI ISL 243534 HA A/mallard duck/Netherlands/1/2012 H10N7
H10N1 Mallard Sweden 2009
EPI ISL 182003 HA A/duck/Jiangxi/26141/2009 H10N7
EPI ISL 182005 HA A/duck/Jiangxi/26281/2009 H10N7
EPI ISL 182004 HA A/duck/Jiangxi/26182/2009 H10N7
EPI ISL 182006 HA A/duck/Jiangxi/26331/2009 H10N7
EPI ISL 125924 HA A/mallard/Korea/1242/2010 H10N8
EPI ISL 149883 HA A/surf scooter/Mongolia/878V/2009 H10N8
EPI ISL 252872 HA A/duck/Shanxi/3180/2010 H10N7
EPI ISL 181970 HA A/duck/Jiangxi/24570/2009 A
EPI ISL 177495 HA A/duck/Hunan/S3137/2009 H10N8
EPI ISL 182002 HA A/migratory duck/Jiangxi/21248/2009 H10N8
EPI ISL 252870 HA A/duck/Shanghai/602/2009 H10N8
EPI ISL 146352 HA A/common teal/Hong Kong/MPK630/2009 H10N9
EPI ISL 125926 HA A/mallard/Korea/1203/2010 H10N8
EPI ISL 125923 HA A/wild bird/Korea/A12/2010 H10N1
EPI ISL 125922 HA A/wild bird/Korea/A13/2010 H10N1
EPI ISL 138758 HA A/avian/Israel/543/2008 H10N7
EPI ISL 177497 HA A/duck/Hunan/S4280/2009 H10N8
EPI ISL 120200 HA A/shoveler/Egypt/01198-NAMRU3/2007 H10N7
EPI ISL 120207 HA A/shoveler/Egypt/01574-NAMRU3/2007 H10N4
EPI ISL 120189 HA A/shoveler/Egypt/09782-NAMRU3/2004 H10N7
EPI ISL 138759 HA A/avian/Israel/824/2005 H10N2
EPI ISL 29998 HA A/Duck/Italy/62330/2006 H10N7
EPI ISL 29228 HA A/duck/Italy/73383/2006 H10N7
EPI ISL 10383 HA A/mallard/Bavaria/3/2006 H10N7
EPI ISL 243649 HA A/mallard duck/Netherlands/2/2008 H10N7
EPI ISL 120210 HA A/teal/Egypt/12908-NAMRU3/2005 H10N1
EPI ISL 120203 HA A/teal/Egypt/01207-NAMRU3/2007 H10N7
EPI ISL 120205 HA A/shoveler/Egypt/00006-NAMRU3/2007 H10N1
EPI ISL 120204 HA A/shoveler/Egypt/00004-NAMRU3/2007 H10N9
HA A/Chicken/Netherlands/09006942/2009 H10N7
HA A/Turkey/Netherlands/09006938/2009 H10N7
EPI ISL 189372 HA A/mallard/Sweden/65475/2009 H10N6
EPI ISL 189368 HA A/mallard/Sweden/64475/2007 H10N4
EPI ISL 75563 HA A/mallard/Switzerland/WV 1090023/2009 H10
EPI ISL 189348 HA A/mallard/Sweden/1417/2002 H10N7
EPI ISL 33857 HA A/mallard/Sweden/4/2005 H10N4
EPI ISL 189469 HA A/mallard/Sweden/936/2002 H10N9
EPI ISL 189349 HA A/mallard/Sweden/3151/2003 H10N7
EPI ISL 390 HA A/duck/Mongolia/149/03 H10N5
EPI ISL 181414 HA A/migratory duck/Jiangxi/6847/2003 H10N5
EPI ISL 120186 HA A/shoveler/Egypt/00600-NAMRU3/2004 H10N7
EPI ISL 189346 HA A/mallard/Sweden/831/2002 H10N2
EPI ISL 78790 HA A/mallard/Sweden/51/2002 H10N2
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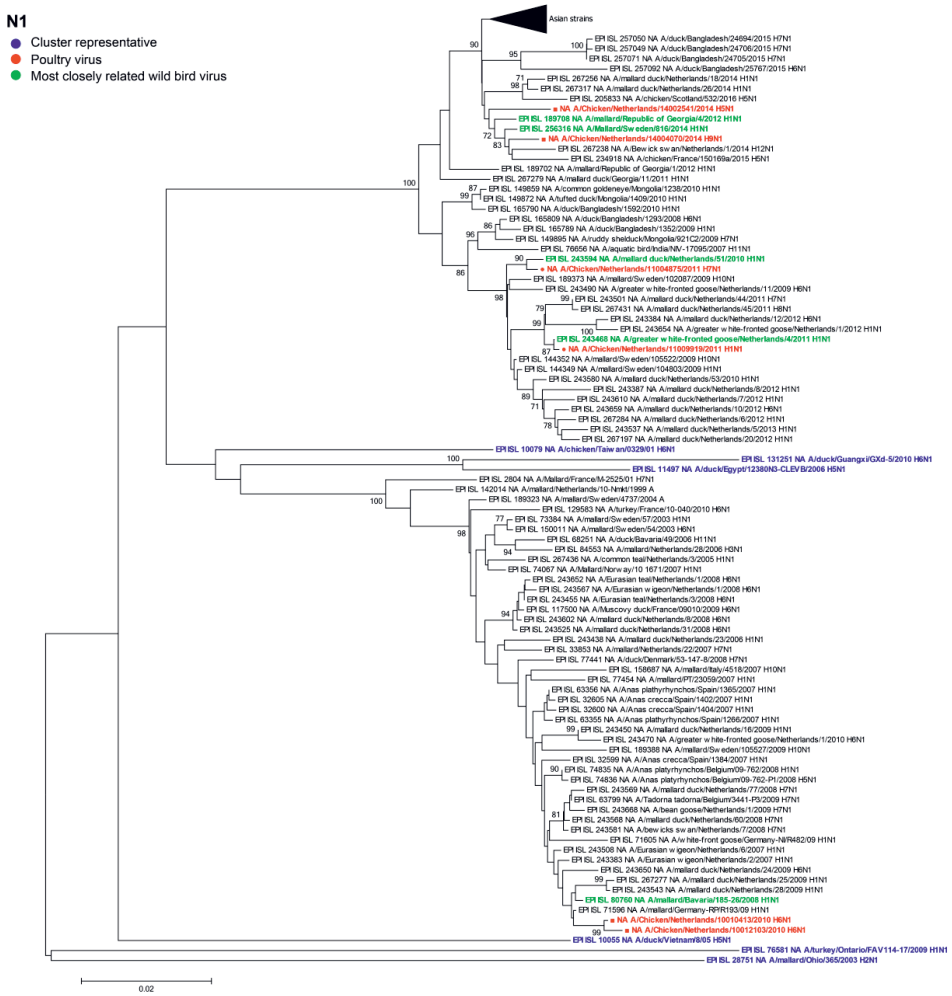
EPI ISL 189352 HA A/mallard/Sweden/4411/2004 H10N4
EPI ISL 138754 HA A/avian/Israel/297/2001 H10N7
EPI ISL 189473 HA A/mallard/Sweden/8004/2005 H10N4
EPI ISL 189471 HA A/mallard/Sweden/6039/2005 H10N4
EPI ISL 189357 HA A/mallard/Sweden/8023/2005 H10N4
EPI ISL 189360 HA A/mallard/Sweden/51619/2006 H10N4
EPI ISL 189356 HA A/mallard/Sweden/6148/2005 H10N7
EPI ISL 189354 HA A/mallard/Sweden/5824/2005 H10N7
EPI ISL 189355 HA A/mallard/Sweden/5933/2005 A
EPI ISL 129928 HA A/mallard/Portugal/79940/2009 H10
EPI ISL 129926 HA A/mallard/Portugal/79906/2009 H10
EPI ISL 95121 HA A/Anas platyrhynchos/Camargue/091863/09 H10N7
EPI ISL 189388 HA A/mallard/Sweden/105527/2009 H10N1
EPI ISL 138652 HA A/mallard/Republic of Georgia/12/2011 H10N4
EPI ISL 189719 HA A/mallard/Republic of Georgia/1/2010 H10N7
EPI ISL 138621 HA A/mallard/Republic of Georgia/1/2010 H10N1
EPI ISL 243394 HA A/mallard duck/Netherlands/22/2010 H10N7
EPI ISL 267180 HA A/mallard duck/Netherlands/21/2010 H10N7
HA H10 A/Chicken/Netherlands/10008427/2010 (H10N7)
EPI ISL 243382 HA A/mallard duck/Netherlands/67/2008 H10N7
EPI ISL 243575 HA A/mallard duck/Netherlands/1/2008 H10N7
EPI ISL 177508 HA A/redhead/Ohio/13OS0391/2013 H10N7
EPI ISL 20857 HA A/chicken/California/755/1999 H10N7
0.02

S2 FIGURE. PHYLOGENETIC TREES OF NA GENES.

Neighbour joining (NJ) phylogenetic trees of the neuraminidase (NA) gene segments of low pathogenic avian influenza (LPAI) viruses from poultry, detected as part of the national avian influenza (AI) surveillance program in the Netherlands, January 2006-September 2016. Nucleotide (nt) sequences of cluster representatives, poultry viruses and top 50 BLAST hits were aligned for each NA gene segment separately: N1 (1221 nt), N2 (1245 nt), N3 (1343 nt), N4 (1337 nt), N5 (1232 nt), N7 (1225 nt), N8 (1376 nt) and N9 (1342 nt). Phylogenetic trees were generated using the Tamura-Nei substitution model with a gamma distribution (shape parameter = 1) for rate variation within the MEGA7 software package. Bootstrap support values above 70 (1,000 replicates) are shown at the branches. Colours represent cluster representatives (blue), poultry viruses (red), and most identical wild bird viruses as determined by BLAST (red). Symbols represent poultry viruses most identical to wild bird viruses isolated in the Netherlands (dots), other European countries (squares) and Asia (triangles). We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu database on which this research is based. All submitters of data may be contacted directly via the GISAID website (http://www.gisaid.org).


N1
Cluster representative
Poultry virus
Most closely related wild bird virus
Asian strains
EPI ISL 257050 NA A/duck/Bangladesh/24694/2015 H7N1
EPI ISL 257049 NA A/duck/Bangladesh/24706/2015 H7N1
EPI ISL 257071 NA A/duck/Bangladesh/24705/2015 H7N1
EPI ISL 257092 NA A/duck/Bangladesh/25767/2015 H6N1
EPI ISL 267256 NA A/mallard duck/Netherlands/18/2014 H1N1
EPI ISL 267317 NA A/mallard duck/Netherlands/26/2014 H1N1
EPI ISL 205833 NA A/chicken/Scotland/532/2016 H5N1
NA A/Chicken/Netherlands/14002541/2014 H5N1
EPI ISL 189708 NA A/mallard/Republic of Georgia/4/2012 H1N1
EPI ISL 256316 NA A/Mallard/Sweden/616/2014 H1N1
NA A/Chicken/Netherlands/14004070/2014 H9N1
EPI ISL 267238 NA A/Bewicks swan/Netherlands/1/2014 H12N1
EPI ISL 234918 NA A/chicken/France/150169a/2015 H5N1
EPI ISL 189702 NA A/mallard/Republic of Georgia/1/2012 H1N1
EPI ISL 267279 NA A/mallard duck/Georgia/11/2011 H1N1
EPI ISL 149859 NA A/common goldeneye/Mongolia/1238/2010 H1N1
EPI ISL 149872 NA A/tufted duck/Mongolia/1409/2010 H1N1
EPI ISL 165790 NA A/duck/Bangladesh/1592/2010 H1N1
EPI ISL 165809 NA A/duck/Bangladesh/1293/2008 H6N1
EPI ISL 165789 NA A/duck/Bangladesh/1352/2009 H1N1
EPI ISL 149895 NA A/ruddy shelduck/Mongolia/921C2/2009 H7N1
EPI ISL 78656 NA A/aquatic bird/India/NIV-17095/2007 H11N1
EPI ISL 243594 NA A/mallard duck/Netherlands/51/2010 H1N1
NA A/Chicken/Netherlands/11004875/2011 H7N1
EPI ISL 189373 NA A/mallard/Sweden/102087/2009 H10N1
EPI ISL 243490 NA A/greater w hite-fronted goose/Netherlands/11/2009 H6N1
EPI ISL 243501 NA A/mallard duck/Netherlands/44/2011 H7N1
EPI ISL 267431 NA A/mallard duck/Netherlands/45/2011 H6N1
EPI ISL 243384 NA A/mallard duck/Netherlands/12/2012 H6N1
EPI ISL 243654 NA A/greater w hite-fronted goose/Netherlands/1/2012 H1N1
EPI ISL 243468 NA A/greater w hite-fronted goose/Netherlands/4/2011 H1N1
NA A/Chicken/Netherlands/11009919/2011 H1N1
EPI ISL 144352 NA A/mallard/Sweden/105522/2009 H10N1
EPI ISL 144349 NA A/mallard/Sweden/104803/2009 H10N1
EPI ISL 243580 NA A/mallard duck/Netherlands/53/2010 H1N1
EPI ISL 243387 NA A/mallard duck/Netherlands/8/2012 H1N1
EPI ISL 243610 NA A/mallard duck/Netherlands/7/2012 H1N1
EPI ISL 243659 NA A/mallard duck/Netherlands/10/2012 H6N1
EPI ISL 267284 NA A/mallard duck/Netherlands/6/2012 H1N1
EPI ISL 243537 NA A/mallard duck/Netherlands/5/2013 H1N1
EPI ISL 267197 NA A/mallard duck/Netherlands/20/2012 H1N1
EPI ISL 10079 NA A/chicken/Taiwan/0329/01 H6N1
EPI ISL 131251 NA A/duck/Guangxi/GXd-5/2010 H6N1
EPI ISL 11497 NA A/duck/Egypt/12380N3-CLEVB/2006 H5N1
EPI ISL 2804 NA A/Mallard/France/M-2525/01 H7N1
EPI ISL 142014 NA A/mallard/Netherlands/10-Nmkt/1999 A
EPI ISL 189323 NA A/mallard/Sweden/4737/2004 A
EPI ISL 129583 NA A/turkey/France/10-040/2010 H6N1
EPI ISL 73384 NA A/mallard/Sweden/57/2003 H1N1
EPI ISL 150011 NA A/mallard/Sweden/54/2003 H6N1
EPI ISL 68251 NA A/duck/Bavaria/49/2006 H11N1
EPI ISL 84553 NA A/mallard/Netherlands/28/2006 H3N1
EPI ISL 267436 NA A/common teal/Netherlands/3/2005 H1N1
EPI ISL 74067 NA A/Mallard/Norway/10 1671/2007 H1N1
EPI ISL 243652 NA A/Eurasian teal/Netherlands/1/2008 H6N1
EPI ISL 243567 NA A/Eurasian w igeon/Netherlands/1/2008 H6N1
EPI ISL 243455 NA A/Eurasian teal/Netherlands/3/2008 H6N1
EPI ISL 117500 NA A/Muscovy duck/France/09010/2009 H6N1
EPI ISL 243602 NA A/mallard duck/Netherlands/8/2008 H6N1
EPI ISL 243525 NA A/mallard duck/Netherlands/31/2008 H6N1
EPI ISL 243438 NA A/mallard duck/Netherlands/23/2006 H1N1
EPI ISL 33853 NA A/mallard/Netherlands/22/2007 H7N1
EPI ISL 77441 NA A/duck/Denmark/53-147-8/2008 H7N1
EPI ISL 158667 NA A/mallard/Italy/4518/2007 H10N1
EPI ISL 77454 NA A/mallard/PT/23059/2007 H1N1
EPI ISL 63356 NA A/Anas platyrhynchos/Spain/1365/2007 H1N1
EPI ISL 32605 NA A/Anas crecca/Spain/1402/2007 H1N1
EPI ISL 32600 NA A/Anas crecca/Spain/1404/2007 H1N1
EPI ISL 63355 NA A/Anas platyrhynchos/Spain/1266/2007 H1N1
EPI ISL 243450 NA A/mallard duck/Netherlands/16/2009 H1N1
EPI ISL 243470 NA A/greater w hite-fronted goose/Netherlands/1/2010 H6N1
EPI ISL 189388 NA A/mallard/Sweden/105527/2009 H10N1
EPI ISL 32599 NA A/Anas crecca/Spain/1384/2007 H1N1
EPI ISL 74835 NA A/Anas platyrhynchos/Belgium/09-762/2008 H1N1
EPI ISL 74836 NA A/Anas platyrhynchos/Belgium/09-762-P1/2008 H5N1
EPI ISL 243569 NA A/mallard duck/Netherlands/77/2008 H7N1
EPI ISL 63799 NA A/Tadorna tadorna/Belgium/3441-P9/2009 H7N1
EPI ISL 243688 NA A/bean goose/Netherlands/1/2009 H7N1
EPI ISL 243568 NA A/mallard duck/Netherlands/80/2008 H7N1
EPI ISL 243581 NA A/bewicks swan/Netherlands/7/2008 H7N1
EPI ISL 71605 NA A/white-front goose/Germany-NI/R482/09 H1N1
EPI ISL 243508 NA A/Eurasian w igeon/Netherlands/6/2007 H1N1
EPI ISL 243383 NA A/Eurasian w igeon/Netherlands/2/2007 H1N1
EPI ISL 243650 NA A/mallard duck/Netherlands/24/2009 H6N1
EPI ISL 267277 NA A/mallard duck/Netherlands/25/2009 H1N1
EPI ISL 243543 NA A/mallard duck/Netherlands/28/2009 H1N1
EPI ISL 80760 NA A/mallard/Bavaria/185-26/2008 H1N1
EPI ISL 71596 NA A/mallard/Germany-RPR193/09 H1N1
NA A/Chicken/Netherlands/10010413/2010 H6N1
NA A/Chicken/Netherlands/10012103/2010 H6N1
EPI ISL 10055 NA A/duck/Vietnam/8/05 H5N1
EPI ISL 76581 NA A/turkey/Ontario/FAV114-17/2009 H1N1
EPI ISL 28751 NA A/mallard/Ohio/365/2003 H2N1
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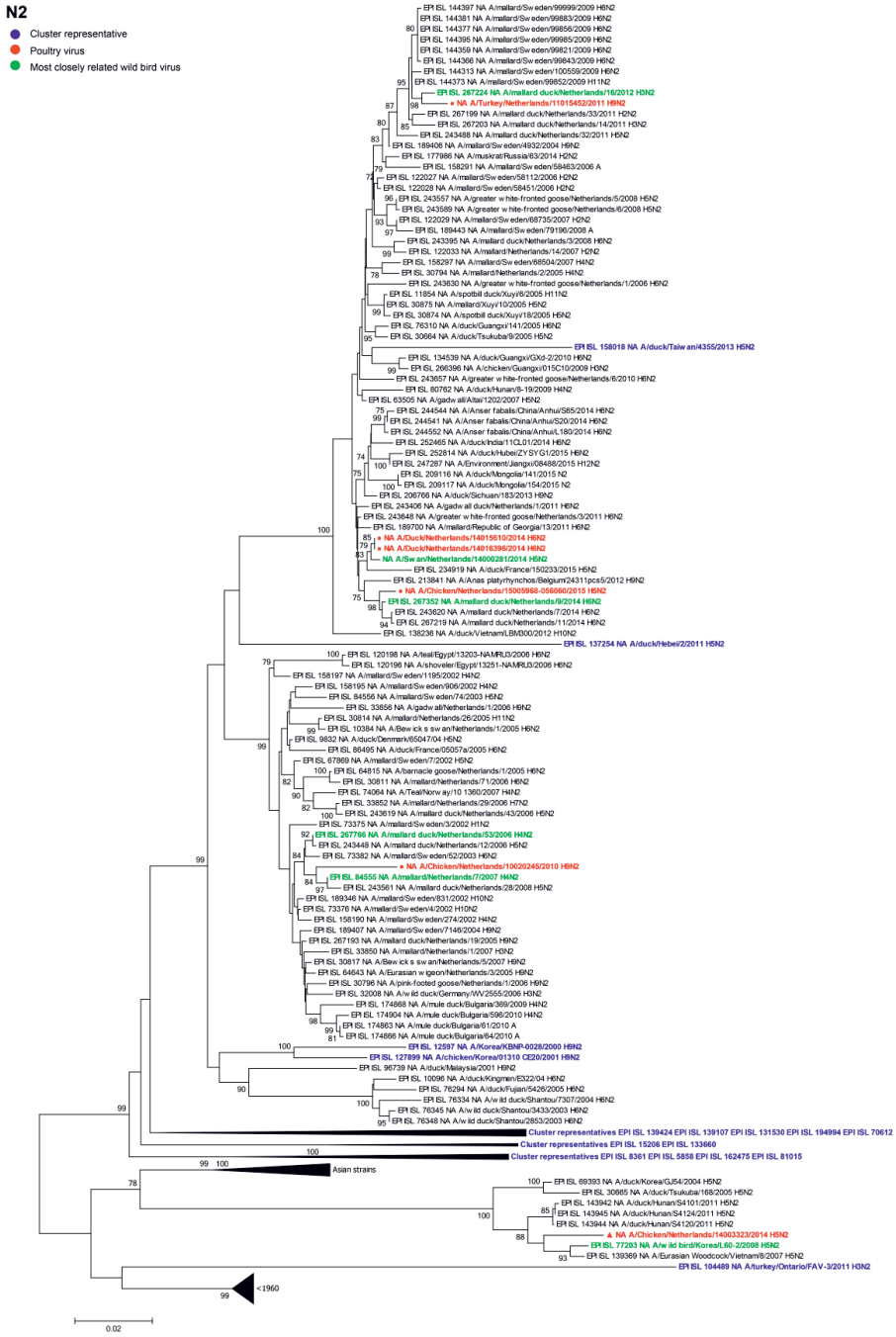
N2
Cluster representative
Poultry virus
Most closely related wild bird virus
EPI ISL 144397 NA A/mallard/Sweden/99999/2009 H6N2
EPI ISL 144381 NA A/mallard/Sweden/99883/2009 H6N2
EPI ISL 144377 NA A/mallard/Sweden/99856/2009 H6N2
EPI ISL 144395 NA A/mallard/Sweden/99985/2009 H6N2
EPI ISL 144359 NA A/mallard/Sweden/99821/2009 H6N2
EPI ISL 144366 NA A/mallard/Sweden/99843/2009 H6N2
EPI ISL 144313 NA A/mallard/Sweden/100559/2009 H6N2
EPI ISL 144373 NA A/mallard/Sweden/99852/2009 H11N2
EPI ISL 267224 NA A/mallard duck/Netherlands/16/2012 H3N2
NA A/Turkey/Netherlands/11015452/2011 H8N2
EPI ISL 267199 NA A/mallard duck/Netherlands/33/2011 H2N2
EPI ISL 267203 NA A/mallard duck/Netherlands/14/2011 H3N2
EPI ISL 243488 NA A/mallard duck/Netherlands/32/2011 H5N2
EPI ISL 189406 NA A/mallard/Sweden/4932/2004 H6N2
EPI ISL 177986 NA A/muskrat/Russia/63/2014 H2N2
EPI ISL 158291 NA A/mallard/Sweden/58463/2006 A
EPI ISL 122027 NA A/mallard/Sweden/58112/2006 H2N2
EPI ISL 122028 NA A/mallard/Sweden/58451/2006 H2N2
EPI ISL 243557 NA A/greater white-fronted goose/Netherlands/5/2008 H5N2
EPI ISL 243589 NA A/greater white-fronted goose/Netherlands/6/2008 H6N2
EPI ISL 122029 NA A/mallard/Sweden/68735/2007 H2N2
EPI ISL 189443 NA A/mallard/Sweden/79196/2008 A
EPI ISL 243595 NA A/mallard duck/Netherlands/3/2008 H6N2
EPI ISL 122033 NA A/mallard/Netherlands/14/2007 H2N2
EPI ISL 158297 NA A/mallard/Sweden/68504/2007 H4N2
EPI ISL 30794 NA A/mallard/Netherlands/2/2005 H4N2
EPI ISL 243630 NA A/greater white-fronted goose/Netherlands/1/2006 H6N2
EPI ISL 11854 NA A/spotbill duck/Xuyi/6/2005 H11N2
EPI ISL 30875 NA A/mallard/Xuyi/10/2005 H5N2
EPI ISL 30874 NA A/spotbill duck/Xuyi/18/2005 H5N2
EPI ISL 76310 NA A/duck/Guangxi/141/2005 H6N2
EPI ISL 30664 NA A/duck/Tsukuba/9/2005 H5N2
EPI ISL 158018 NA A/duck/Taiwan/4355/2013 H5N2
EPI ISL 134539 NA A/duck/Guangxi/GXd-2/2010 H6N2
EPI ISL 266396 NA A/chicken/Guangxi/015C10/2009 H3N2
EPI ISL 243657 NA A/greater white-fronted goose/Netherlands/6/2010 H6N2
EPI ISL 60762 NA A/duck/Hunan/8-19/2009 H4N2
EPI ISL 63505 NA A/gadwall/Altai/1202/2007 H5N2
EPI ISL 244544 NA A/Anser fabalis/China/Anhui/S65/2014 H6N2
EPI ISL 244541 NA A/Anser fabalis/China/Anhui/S20/2014 H6N2
EPI ISL 244552 NA A/Anser fabalis/China/Anhui/L180/2014 H6N2
EPI ISL 252465 NA A/duck/India/11CL01/2014 H6N2
EPI ISL 252814 NA A/duck/Hubei/ZYSYG1/2015 H6N2
EPI ISL 247287 NA A/Environment/Jiangxi/08488/2015 H12N2
EPI ISL 209116 NA A/duck/Mongolia/141/2015 N2
EPI ISL 209117 NA A/duck/Mongolia/154/2015 N2
EPI ISL 206766 NA A/duck/Sichuan/183/2013 H6N2
EPI ISL 243406 NA A/gadwall duck/Netherlands/1/2011 H6N2
EPI ISL 243648 NA A/greater white-fronted goose/Netherlands/3/2011 H6N2
EPI ISL 189700 NA A/mallard/Republic of Georgia/13/2011 H6N2
NA A/Duck/Netherlands/14015610/2014 H6N2
NA A/Duck/Netherlands/14016396/2014 H6N2
NA A/Swan/Netherlands/14000281/2014 H3N2
EPI ISL 234919 NA A/duck/France/150233/2015 H5N2
EPI ISL 213841 NA A/Anas platyrhynchos/Belgium/24311pcs5/2012 H9N2
NA A/Chicken/Netherlands/15005968-056060/2015 H5N2
EPI ISL 267352 NA A/mallard duck/Netherlands/9/2014 H6N2
EPI ISL 243620 NA A/mallard duck/Netherlands/7/2014 H6N2
EPI ISL 267219 NA A/mallard duck/Netherlands/11/2014 H6N2
EPI ISL 138236 NA A/duck/Vietnam/LBM300/2012 H10N2
EPI ISL 137254 NA A/duck/Hebei/2/2011 H5N2
EPI ISL 120198 NA A/teal/Egypt/13203-NAMRU3/2006 H6N2
EPI ISL 120196 NA A/shoveler/Egypt/13251-NAMRU3/2006 H6N2
EPI ISL 158197 NA A/mallard/Sweden/1195/2002 H4N2
EPI ISL 158195 NA A/mallard/Sweden/906/2002 H4N2
EPI ISL 84556 NA A/mallard/Sweden/74/2003 H6N2
EPI ISL 33856 NA A/gadwall/Netherlands/1/2006 H6N2
EPI ISL 30814 NA A/mallard/Netherlands/26/2005 H11N2
EPI ISL 10384 NA A/Bewick swan/Netherlands/1/2005 H6N2
EPI ISL 9832 NA A/duck/Denmark/65047/04 H5N2
EPI ISL 86495 NA A/duck/France/05057a/2005 H6N2
EPI ISL 67869 NA A/mallard/Sweden/7/2002 H5N2
EPI ISL 64815 NA A/barnacle goose/Netherlands/1/2005 H6N2
EPI ISL 30811 NA A/mallard/Netherlands/71/2006 H6N2
EPI ISL 74084 NA A/Teal/Norway/10 1360/2007 H4N2
EPI ISL 33852 NA A/mallard/Netherlands/29/2006 H7N2
EPI ISL 243619 NA A/mallard duck/Netherlands/43/2006 H5N2
EPI ISL 73375 NA A/mallard/Sweden/3/2002 H1N2
EPI ISL 267766 NA A/mallard duck/Netherlands/53/2006 H4N2
EPI ISL 243448 NA A/mallard duck/Netherlands/12/2006 H6N2
EPI ISL 73382 NA A/mallard/Sweden/52/2003 H6N2
NA A/Chicken/Netherlands/10020245/2010 H3N2
EPI ISL 84555 NA A/mallard/Netherlands/7/2007 H4N2
EPI ISL 243561 NA A/mallard duck/Netherlands/28/2008 H5N2
EPI ISL 189346 NA A/mallard/Sweden/831/2002 H10N2
EPI ISL 73376 NA A/mallard/Sweden/4/2002 H10N2
EPI ISL 158190 NA A/mallard/Sweden/274/2002 H4N2
EPI ISL 189407 NA A/mallard/Sweden/7148/2004 H6N2
EPI ISL 267193 NA A/mallard duck/Netherlands/19/2005 H8N2
EPI ISL 33850 NA A/mallard/Netherlands/1/2007 H3N2
EPI ISL 30817 NA A/Bewick swan/Netherlands/5/2007 H6N2
EPI ISL 64643 NA A/Eurasian wigeon/Netherlands/3/2005 H6N2
EPI ISL 30796 NA A/pink-footed goose/Netherlands/1/2006 H6N2
EPI ISL 32008 NA A/wild duck/Germany/WV2555/2006 H3N2
EPI ISL 174868 NA A/mule duck/Bulgaria/369/2009 H4N2
EPI ISL 174904 NA A/mule duck/Bulgaria/596/2010 H4N2
EPI ISL 174883 NA A/mule duck/Bulgaria/81/2010 A
EPI ISL 174866 NA A/mule duck/Bulgaria/64/2010 A
EPI ISL 12597 NA A/Korea/KBNP-0028/2000 H9N2
EPI ISL 127899 NA A/chicken/Korea/01310 CE20/2001 H9N2
EPI ISL 96739 NA A/duck/Malaysia/2001 H9N2
EPI ISL 10096 NA A/duck/Kingmen/E322/04 H6N2
EPI ISL 76294 NA A/duck/Fujian/5426/2005 H6N2
EPI ISL 76334 NA A/wild duck/Shantou/7307/2004 H6N2
EPI ISL 76345 NA A/wild duck/Shantou/3433/2003 H6N2
EPI ISL 76348 NA A/wild duck/Shantou/2853/2003 H6N2
Cluster representatives EPI ISL 139424 EPI ISL 139107 EPI ISL 131530 EPI ISL 194994 EPI ISL 70612
Cluster representatives EPI ISL 15206 EPI ISL 133660
Cluster representatives EPI ISL 8361 EPI ISL 5858 EPI ISL 162475 EPI ISL 81015
Asian strains
EPI ISL 69393 NA A/duck/Korea/GJ54/2004 H5N2
EPI ISL 30695 NA A/duck/Tsukuba/168/2005 H5N2
EPI ISL 143942 NA A/duck/Hunan/S4101/2011 H5N2
EPI ISL 143945 NA A/duck/Hunan/S4124/2011 H5N2
EPI ISL 143944 NA A/duck/Hunan/S4120/2011 H5N2
NA A/Chicken/Netherlands/14003323/2014 H5N2
EPI ISL 77203 NA A/wild bird/Korea/L60-2/2008 H5N2
EPI ISL 139369 NA A/Eurasian Woodcock/Vietnam/8/2007 H5N2
EPI ISL 104489 NA A/turkey/Ontario/FAV-3/2011 H3N2
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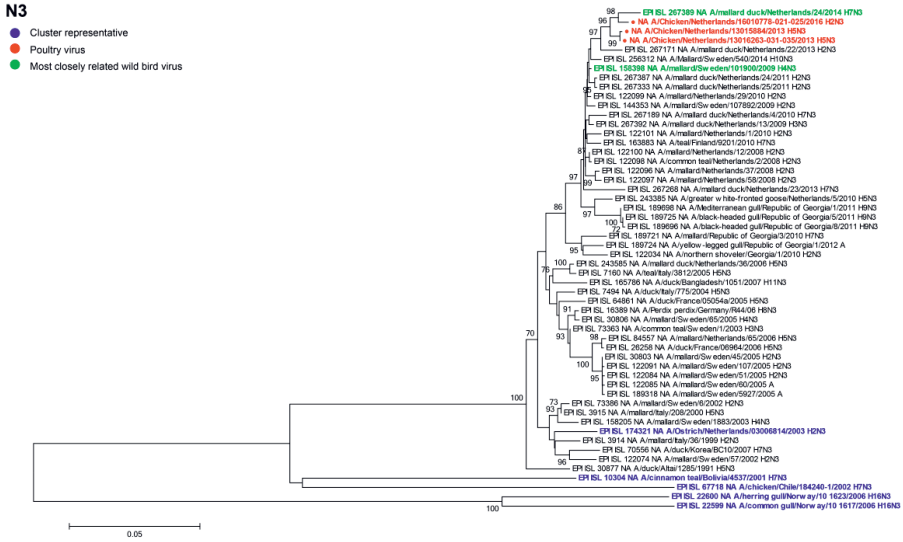
N3
Cluster representative
Poultry virus
Most closely related wild bird virus
EPI ISL 267389 NA A/mallard duck/Netherlands/24/2014 H7N3
NA A/Chicken/Netherlands/16010778-021-025/2016 H2N3
NA A/Chicken/Netherlands/13015884/2013 H5N3
NA A/Chicken/Netherlands/13016263-031-035/2013 H5N3
EPI ISL 267171 NA A/mallard duck/Netherlands/22/2013 H2N3
EPI ISL 256312 NA A/Mallard/Sweden/540/2014 H10N3
EPI ISL 158398 NA A/mallard/Sweden/101800/2009 H4N3
EPI ISL 267387 NA A/mallard duck/Netherlands/24/2011 H2N3
EPI ISL 267333 NA A/mallard duck/Netherlands/25/2011 H2N3
EPI ISL 122099 NA A/mallard/Netherlands/29/2010 H2N3
EPI ISL 144353 NA A/mallard/Sweden/107892/2009 H2N3
EPI ISL 267189 NA A/mallard duck/Netherlands/4/2010 H7N3
EPI ISL 267392 NA A/mallard duck/Netherlands/13/2009 H3N3
EPI ISL 122101 NA A/mallard/Netherlands/1/2010 H2N3
EPI ISL 163883 NA A/teal/Finland/9201/2010 H7N3
EPI ISL 122100 NA A/mallard/Netherlands/12/2008 H2N3
EPI ISL 122098 NA A/common teal/Netherlands/2/2008 H2N3
EPI ISL 122096 NA A/mallard/Netherlands/37/2008 H2N3
EPI ISL 122097 NA A/mallard/Netherlands/58/2008 H2N3
EPI ISL 267268 NA A/mallard duck/Netherlands/23/2013 H7N3
EPI ISL 243385 NA A/greater w hite-fronted goose/Netherlands/5/2010 H5N3
EPI ISL 189698 NA A/Mediterranean gull/Republic of Georgia/1/2011 H8N3
EPI ISL 189725 NA A/black-headed gull/Republic of Georgia/5/2011 H8N3
EPI ISL 189696 NA A/black-headed gull/Republic of Georgia/8/2011 H8N3
EPI ISL 189721 NA A/mallard/Republic of Georgia/3/2010 H7N3
EPI ISL 189724 NA A/yellow -legged gull/Republic of Georgia/1/2012 A
EPI ISL 122034 NA A/northern shoveler/Georgia/1/2010 H2N3
EPI ISL 243585 NA A/mallard duck/Netherlands/36/2006 H6N3
EPI ISL 7160 NA A/teal/Italy/3812/2005 H5N3
EPI ISL 165786 NA A/duck/Bangladesh/1051/2007 H11N3
EPI ISL 7494 NA A/duck/Italy/775/2004 H5N3
EPI ISL 64861 NA A/duck/France/05054a/2005 H5N3
EPI ISL 16389 NA A/Perdix perdix/Germany/R44/06 H6N3
EPI ISL 30806 NA A/mallard/Sweden/65/2005 H4N3
EPI ISL 73363 NA A/common teal/Sweden/1/2003 H3N3
EPI ISL 84557 NA A/mallard/Netherlands/65/2006 H5N3
EPI ISL 26258 NA A/duck/France/06964/2006 H5N3
EPI ISL 30803 NA A/mallard/Sweden/45/2005 H2N3
EPI ISL 122091 NA A/mallard/Sweden/107/2005 H2N3
EPI ISL 122084 NA A/mallard/Sweden/51/2005 H2N3
EPI ISL 122085 NA A/mallard/Sweden/60/2005 A
EPI ISL 189318 NA A/mallard/Sweden/5927/2005 A
EPI ISL 73386 NA A/mallard/Sweden/6/2002 H2N3
EPI ISL 3915 NA A/mallard/Italy/208/2000 H5N3
EPI ISL 158205 NA A/mallard/Sweden/1883/2003 H4N3
EPI ISL 174321 NA A/Ostrich/Netherlands/03006814/2003 H2N3
EPI ISL 3914 NA A/mallard/Italy/36/1999 H2N3
EPI ISL 70556 NA A/duck/Korea/BC10/2007 H7N3
EPI ISL 122074 NA A/mallard/Sweden/57/2002 H2N3
EPI ISL 30877 NA A/duck/Altai/1285/1991 H5N3
EPI ISL 10304 NA A/cinnamon teal/Bolivia/4537/2001 H7N3
EPI ISL 67718 NA A/chicken/Chile/184240-1/2002 H7N3
EPI ISL 22600 NA A/herring gull/Norway/10 1623/2006 H16N3
EPI ISL 22599 NA A/common gull/Norway/10 1617/2006 H16N3
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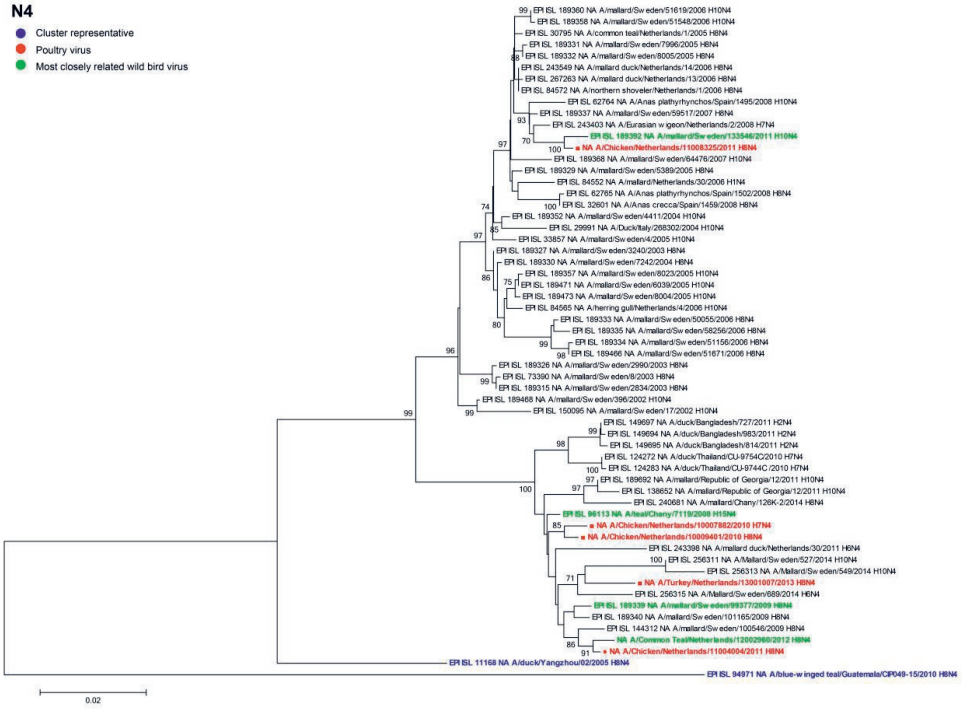
N4
Cluster representative
Poultry virus
Most closely related wild bird virus
EPI ISL 189360 NA A/mallard/Sweden/51619/2006 H10N4
EPI ISL 189358 NA A/mallard/Sweden/51549/2006 H10N4
EPI ISL 30795 NA A/common teal/Netherlands/1/2005 H8N4
EPI ISL 189331 NA A/mallard/Sweden/7996/2005 H8N4
EPI ISL 189332 NA A/mallard/Sweden/8005/2005 H8N4
EPI ISL 243549 NA A/mallard duck/Netherlands/14/2006 H8N4
EPI ISL 267263 NA A/mallard duck/Netherlands/13/2006 H8N4
EPI ISL 84572 NA A/northern shoveler/Netherlands/1/2006 H8N4
EPI ISL 62764 NA A/Anas platyrhynchos/Spain/1495/2008 H10N4
EPI ISL 189337 NA A/mallard/Sweden/59517/2007 H8N4
EPI ISL 243403 NA A/Eurasian w igeon/Netherlands/2/2008 H7N4
EPI ISL 189392 NA A/mallard/Sweden/133546/2011 H10N4
NA A/Chicken/Netherlands/11008325/2011 H8N4
EPI ISL 189368 NA A/mallard/Sweden/64476/2007 H10N4
EPI ISL 189329 NA A/mallard/Sweden/5389/2005 H8N4
EPI ISL 84552 NA A/mallard/Netherlands/30/2006 H1N4
EPI ISL 62765 NA A/Anas platyrhynchos/Spain/1502/2008 H8N4
EPI ISL 32601 NA A/Anas crecca/Spain/1458/2008 H8N4
EPI ISL 189352 NA A/mallard/Sweden/4411/2004 H10N4
EPI ISL 29991 NA A/Duck/Italy/268302/2004 H10N4
EPI ISL 33857 NA A/mallard/Sweden/4/2005 H10N4
EPI ISL 189327 NA A/mallard/Sweden/3240/2003 H8N4
EPI ISL 189330 NA A/mallard/Sweden/7242/2004 H8N4
EPI ISL 189357 NA A/mallard/Sweden/8023/2005 H10N4
EPI ISL 189471 NA A/mallard/Sweden/6039/2005 H10N4
EPI ISL 189473 NA A/mallard/Sweden/8004/2005 H10N4
EPI ISL 84565 NA A/herring gull/Netherlands/4/2006 H10N4
EPI ISL 189333 NA A/mallard/Sweden/50055/2006 H8N4
EPI ISL 189335 NA A/mallard/Sweden/58256/2006 H8N4
EPI ISL 189334 NA A/mallard/Sweden/51156/2006 H8N4
EPI ISL 189466 NA A/mallard/Sweden/51671/2006 H8N4
EPI ISL 189326 NA A/mallard/Sweden/2990/2003 H8N4
EPI ISL 73390 NA A/mallard/Sweden/8/2003 H8N4
EPI ISL 189315 NA A/mallard/Sweden/2834/2003 H8N4
EPI ISL 189468 NA A/mallard/Sweden/396/2002 H10N4
EPI ISL 150095 NA A/mallard/Sweden/17/2002 H10N4
EPI ISL 149697 NA A/duck/Bangladesh/727/2011 H2N4
EPI ISL 149694 NA A/duck/Bangladesh/983/2011 H2N4
EPI ISL 149695 NA A/duck/Bangladesh/814/2011 H2N4
EPI ISL 124272 NA A/duck/Thailand/CU-9754C/2010 H7N4
EPI ISL 124283 NA A/duck/Thailand/CU-9744C/2010 H7N4
EPI ISL 189692 NA A/mallard/Republic of Georgia/12/2011 H10N4
EPI ISL 138652 NA A/mallard/Republic of Georgia/12/2011 H10N4
EPI ISL 240681 NA A/mallard/Chany/126K-2/2014 H8N4
EPI ISL 96113 NA A/teal/Chany/7119/2008 H15N4
NA A/Chicken/Netherlands/10007802/2010 H7N4
NA A/Chicken/Netherlands/10009401/2010 H8N4
EPI ISL 243398 NA A/mallard duck/Netherlands/30/2011 H6N4
EPI ISL 256311 NA A/Mallard/Sweden/527/2014 H10N4
EPI ISL 256313 NA A/Mallard/Sweden/549/2014 H10N4
NA A/Turkey/Netherlands/13001007/2013 H8N4
EPI ISL 256315 NA A/Mallard/Sweden/689/2014 H6N4
EPI ISL 189339 NA A/mallard/Sweden/99377/2009 H8N4
EPI ISL 189340 NA A/mallard/Sweden/101165/2009 H8N4
EPI ISL 144312 NA A/Mallard/Sweden/100546/2009 H8N4
NA A/Common Teal/Netherlands/12002960/2012 H8N4
NA A/Chicken/Netherlands/11004004/2011 H8N4
EPI ISL 11168 NA A/duck/Yangzhou/02/2005 H8N4
EPI ISL 94971 NA A/blue-w inged teal/Guatemala/CIP049-15/2010 H8N4
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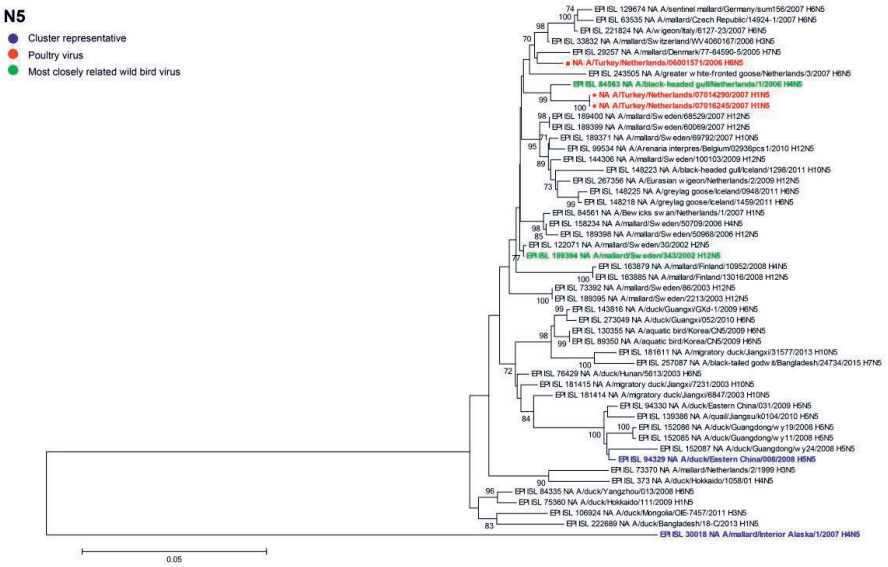
N5
Cluster representative
Poultry virus
Most closely related wild bird virus
EPI ISL 129674 NA A/sentinel mallard/Germany/sum156/2007 H6N5
EPI ISL 63535 NA A/mallard/Czech Republic/14924-1/2007 H6N5
EPI ISL 221824 NA A/wigeon/Italy/6127-23/2007 H6N5
EPI ISL 33832 NA A/mallard/Switzerland/WV4060167/2006 H3N5
EPI ISL 29257 NA A/mallard/Denmark/77-64590-5/2005 H7N5
NA A/Turkey/Netherlands/06001571/2006 H6N5
EPI ISL 243505 NA A/greater white-fronted goose/Netherlands/3/2007 H6N5
EPI ISL 84563 NA A/black-headed gull/Netherlands/1/2006 H4N5
NA A/Turkey/Netherlands/07014290/2007 H1N5
NA A/Turkey/Netherlands/07016245/2007 H1N5
EPI ISL 189400 NA A/mallard/Sweden/68529/2007 H12N5
EPI ISL 189399 NA A/mallard/Sweden/60069/2007 H12N5
EPI ISL 189371 NA A/mallard/Sweden/69792/2007 H10N5
EPI ISL 99534 NA A/Arenaria interpres/Belgium/02936pcs1/2010 H12N5
EPI ISL 144306 NA A/mallard/Sweden/100103/2009 H12N5
EPI ISL 148223 NA A/black-headed gull/Iceland/1298/2011 H10N5
EPI ISL 267356 NA A/Eurasian wigeon/Netherlands/2/2009 H12N5
EPI ISL 148225 NA A/greylag goose/Iceland/0948/2011 H6N5
EPI ISL 148218 NA A/greylag goose/Iceland/1459/2011 H6N5
EPI ISL 84561 NA A/Bewicks swan/Netherlands/1/2007 H1N5
EPI ISL 158234 NA A/mallard/Sweden/50709/2006 H4N5
EPI ISL 189398 NA A/mallard/Sweden/50968/2006 H12N5
EPI ISL 122071 NA A/mallard/Sweden/30/2002 H2N5
EPI ISL 189394 NA A/mallard/Sweden/343/2002 H12N5
EPI ISL 163879 NA A/mallard/Finland/10952/2008 H4N5
EPI ISL 163885 NA A/mallard/Finland/13016/2008 H12N5
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EPI ISL 89350 NA A/aquatic bird/Korea/CN5/2009 H6N5
EPI ISL 181611 NA A/migratory duck/Jiangxi/31577/2013 H10N5
EPI ISL 257087 NA A/black-tailed godwit/Bangladesh/24734/2015 H7N5
EPI ISL 76429 NA A/duck/Hunan/5613/2003 H6N5
EPI ISL 181415 NA A/migratory duck/Jiangxi/7231/2003 H10N5
EPI ISL 181414 NA A/migratory duck/Jiangxi/6847/2003 H10N5
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EPI ISL 139386 NA A/quail/Jiangsu/k0104/2010 H5N5
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EPI ISL 152085 NA A/duck/Guangdong/wy11/2008 H5N5
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EPI ISL 94329 NA A/duck/Eastern China/008/2008 H5N5
EPI ISL 73370 NA A/mallard/Netherlands/2/1999 H3N5
EPI ISL 373 NA A/duck/Hokkaido/1058/01 H4N5
EPI ISL 84335 NA A/duck/Yangzhou/013/2008 H6N5
EPI ISL 75360 NA A/duck/Hokkaido/111/2009 H1N5
EPI ISL 106924 NA A/duck/Mongolia/OIE-7457/2011 H3N5
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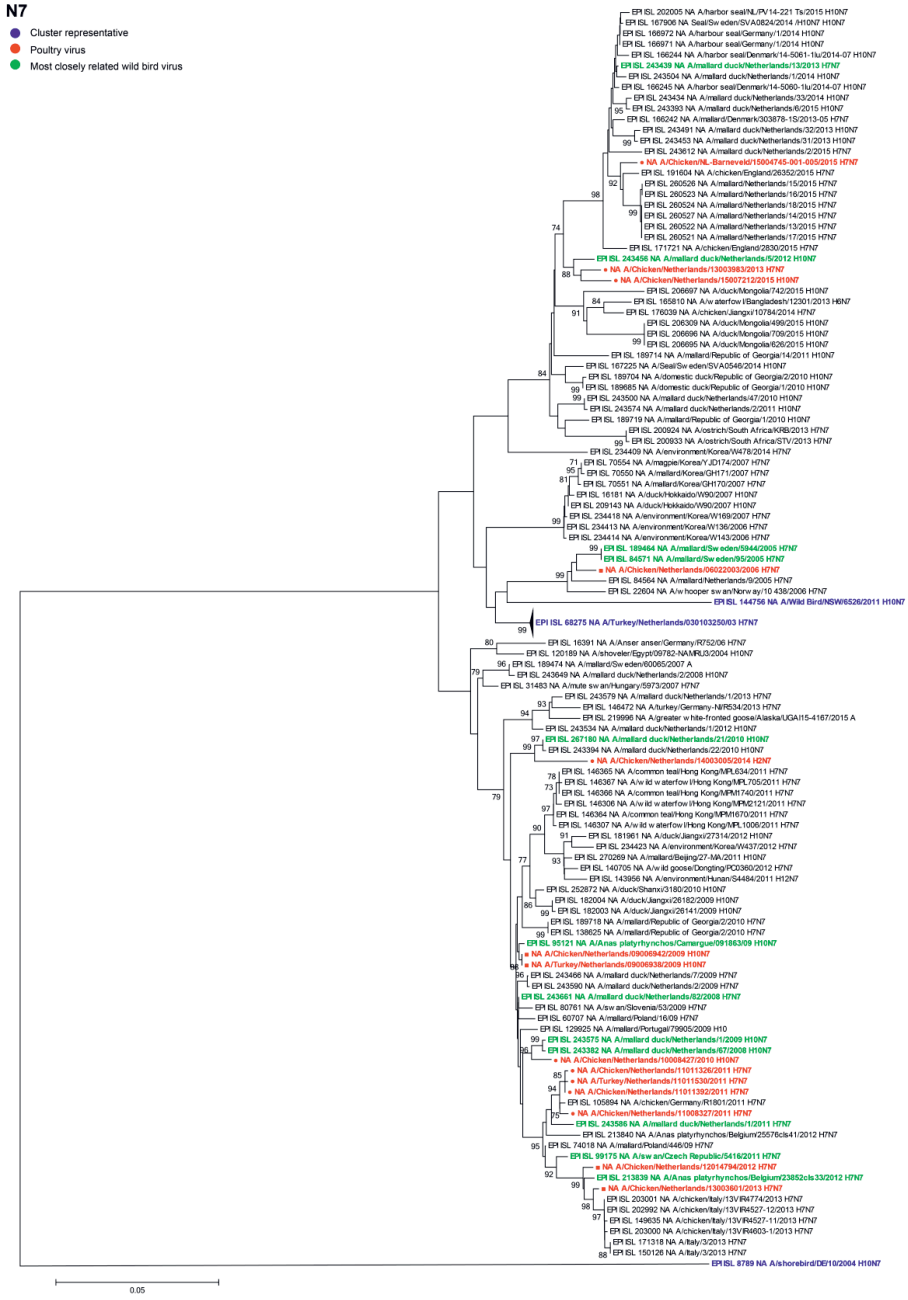
N7
Cluster representative
Poultry virus
Most closely related wild bird virus
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EPI ISL 166244 NA A/harbor seal/Denmark/14-5061-1lu/2014-07 H10N7
EPI ISL 243439 NA A/mallard duck/Netherlands/13/2013 H7N7
EPI ISL 243504 NA A/mallard duck/Netherlands/1/2014 H10N7
EPI ISL 166245 NA A/harbor seal/Denmark/14-5060-1lu/2014-07 H10N7
EPI ISL 243434 NA A/mallard duck/Netherlands/33/2014 H10N7
EPI ISL 243393 NA A/mallard duck/Netherlands/6/2015 H10N7
EPI ISL 166242 NA A/mallard/Denmark/303878-15/2013-05 H7N7
EPI ISL 243491 NA A/mallard duck/Netherlands/32/2013 H10N7
EPI ISL 243453 NA A/mallard duck/Netherlands/31/2013 H10N7
EPI ISL 243612 NA A/mallard duck/Netherlands/2/2015 H7N7
NA A/Chicken/NL-Barneveld/15004745-001-005/2015 H7N7
EPI ISL 191604 NA A/chicken/England/26352/2015 H7N7
EPI ISL 260526 NA A/mallard/Netherlands/15/2015 H7N7
EPI ISL 260523 NA A/mallard/Netherlands/16/2015 H7N7
EPI ISL 260524 NA A/mallard/Netherlands/18/2015 H7N7
EPI ISL 260527 NA A/mallard/Netherlands/14/2015 H7N7
EPI ISL 260522 NA A/mallard/Netherlands/13/2015 H7N7
EPI ISL 260521 NA A/mallard/Netherlands/17/2015 H7N7
EPI ISL 171721 NA A/chicken/England/2830/2015 H7N7
EPI ISL 243466 NA A/mallard duck/Netherlands/5/2012 H10N7
NA A/Chicken/Netherlands/13003983/2013 H7N7
NA A/Chicken/Netherlands/15007212/2015 H10N7
EPI ISL 206697 NA A/duck/Mongolia/742/2015 H10N7
EPI ISL 165810 NA A/w aterfow l/Bangladesh/12301/2013 H6N7
EPI ISL 176039 NA A/chicken/Jiangxi/10784/2014 H7N7
EPI ISL 206309 NA A/duck/Mongolia/499/2015 H10N7
EPI ISL 206696 NA A/duck/Mongolia/709/2015 H10N7
EPI ISL 206695 NA A/duck/Mongolia/626/2015 H10N7
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EPI ISL 167225 NA A/Seal/Sweden/SVA0546/2014 H10N7
EPI ISL 189704 NA A/domestic duck/Republic of Georgia/2/2010 H10N7
EPI ISL 189685 NA A/domestic duck/Republic of Georgia/1/2010 H10N7
EPI ISL 243500 NA A/mallard duck/Netherlands/47/2010 H10N7
EPI ISL 243574 NA A/mallard duck/Netherlands/2/2011 H10N7
EPI ISL 189719 NA A/mallard/Republic of Georgia/1/2010 H10N7
EPI ISL 200924 NA A/ostrich/South Africa/KRB/2013 H7N7
EPI ISL 200933 NA A/ostrich/South Africa/STV/2013 H7N7
EPI ISL 234409 NA A/environment/Korea/W478/2014 H7N7
EPI ISL 70554 NA A/magpie/Korea/YJD174/2007 H7N7
EPI ISL 70550 NA A/mallard/Korea/GH171/2007 H7N7
EPI ISL 70551 NA A/mallard/Korea/GH170/2007 H7N7
EPI ISL 16181 NA A/duck/Hokkaido/W90/2007 H10N7
EPI ISL 209143 NA A/duck/Hokkaido/W90/2007 H10N7
EPI ISL 234418 NA A/environment/Korea/W169/2007 H7N7
EPI ISL 234413 NA A/environment/Korea/W136/2006 H7N7
EPI ISL 234414 NA A/environment/Korea/W143/2006 H7N7
EPI ISL 189464 NA A/mallard/Sweden/5944/2005 H7N7
EPI ISL 84571 NA A/mallard/Sweden/95/2005 H7N7
NA A/Chicken/Netherlands/06022003/2006 H7N7
EPI ISL 84564 NA A/mallard/Netherlands/9/2005 H7N7
EPI ISL 22604 NA A/w hooper swan/Norway/10 438/2006 H7N7
EPI ISL 144756 NA A/Wild Bird/NSW/6526/2011 H10N7
EPI ISL 68275 NA A/Turkey/Netherlands/03010350/03 H7N7
EPI ISL 16391 NA A/Anser anser/Germany/R752/06 H7N7
EPI ISL 120189 NA A/shoveler/Egypt/09782-NAMRU3/2004 H10N7
EPI ISL 189474 NA A/mallard/Sweden/60065/2007 A
EPI ISL 243649 NA A/mallard duck/Netherlands/2/2008 H10N7
EPI ISL 31483 NA A/mute swan/Hungary/5973/2007 H7N7
EPI ISL 243579 NA A/mallard duck/Netherlands/1/2013 H7N7
EPI ISL 146472 NA A/turkey/Germany-NR/634/2013 H7N7
EPI ISL 219996 NA A/greater white-fronted goose/Alaska/UGA15-4167/2015 A
EPI ISL 243534 NA A/mallard duck/Netherlands/1/2012 H10N7
EPI ISL 267186 NA A/mallard duck/Netherlands/21/2010 H10N7
EPI ISL 243394 NA A/mallard duck/Netherlands/22/2010 H10N7
NA A/Chicken/Netherlands/14003005/2014 H2N7
EPI ISL 146365 NA A/common teal/Hong Kong/MPL634/2011 H7N7
EPI ISL 146367 NA A/w ild w aterfow l/Hong Kong/MPL705/2011 H7N7
EPI ISL 146366 NA A/common teal/Hong Kong/MPM740/2011 H7N7
EPI ISL 146306 NA A/w ild w aterfow l/Hong Kong/MPM1121/2011 H7N7
EPI ISL 146364 NA A/common teal/Hong Kong/MPM1670/2011 H7N7
EPI ISL 146307 NA A/w ild w aterfow l/Hong Kong/MPL1006/2011 H7N7
EPI ISL 181961 NA A/duck/Jiangxi/27314/2012 H10N7
EPI ISL 234423 NA A/environment/Korea/W437/2012 H7N7
EPI ISL 270269 NA A/mallard/Beijing/27-MA/2011 H10N7
EPI ISL 140705 NA A/w ild goose/Dongting/PC0360/2012 H7N7
EPI ISL 143956 NA A/environment/Hunan/S4484/2011 H12N7
EPI ISL 252872 NA A/duck/Shanxi/3180/2010 H10N7
EPI ISL 182004 NA A/duck/Jiangxi/26182/2009 H10N7
EPI ISL 182003 NA A/duck/Jiangxi/26141/2009 H10N7
EPI ISL 189718 NA A/mallard/Republic of Georgia/2/2010 H7N7
EPI ISL 138625 NA A/mallard/Republic of Georgia/2/2010 H7N7
EPI ISL 95121 NA A/Anas platyrhynchos/Camargue/09186309 H10N7
NA A/Chicken/Netherlands/09006942/2009 H10N7
NA A/Turkey/Netherlands/09006938/2009 H10N7
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EPI ISL 243590 NA A/mallard duck/Netherlands/2/2009 H7N7
EPI ISL 243661 NA A/mallard duck/Netherlands/82/2008 H7N7
EPI ISL 80761 NA A/swan/Slovenia/53/2009 H7N7
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EPI ISL 129925 NA A/mallard/Portugal/79905/2009 H10
EPI ISL 243575 NA A/mallard duck/Netherlands/1/2009 H10N7
EPI ISL 243382 NA A/mallard duck/Netherlands/67/2008 H10N7
NA A/Chicken/Netherlands/10008427/2010 H10N7
NA A/Chicken/Netherlands/11011326/2011 H7N7
NA A/Turkey/Netherlands/11011530/2011 H7N7
NA A/Chicken/Netherlands/11011392/2011 H7N7
EPI ISL 105894 NA A/chicken/Germany/R1801/2011 H7N7
NA A/Chicken/Netherlands/11008327/2011 H7N7
EPI ISL 243586 NA A/mallard duck/Netherlands/1/2011 H7N7
EPI ISL 213840 NA A/Anas platyrhynchos/Belgium/25576cls41/2012 H7N7
EPI ISL 74018 NA A/mallard/Poland/446/09 H7N7
EPI ISL 99175 NA A/swan/Czech Republic/5416/2011 H7N7
NA A/Chicken/Netherlands/12014794/2012 H7N7
EPI ISL 213839 NA A/Anas platyrhynchos/Belgium/23852cls33/2012 H7N7
NA A/Chicken/Netherlands/13003601/2013 H7N7
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EPI ISL 202992 NA A/chicken/Italy/13VIR4527-12/2013 H7N7
EPI ISL 149635 NA A/chicken/Italy/13VIR4527-11/2013 H7N7
EPI ISL 203000 NA A/chicken/Italy/13VIR4603-1/2013 H7N7
EPI ISL 171318 NA A/Italy/3/2013 H7N7
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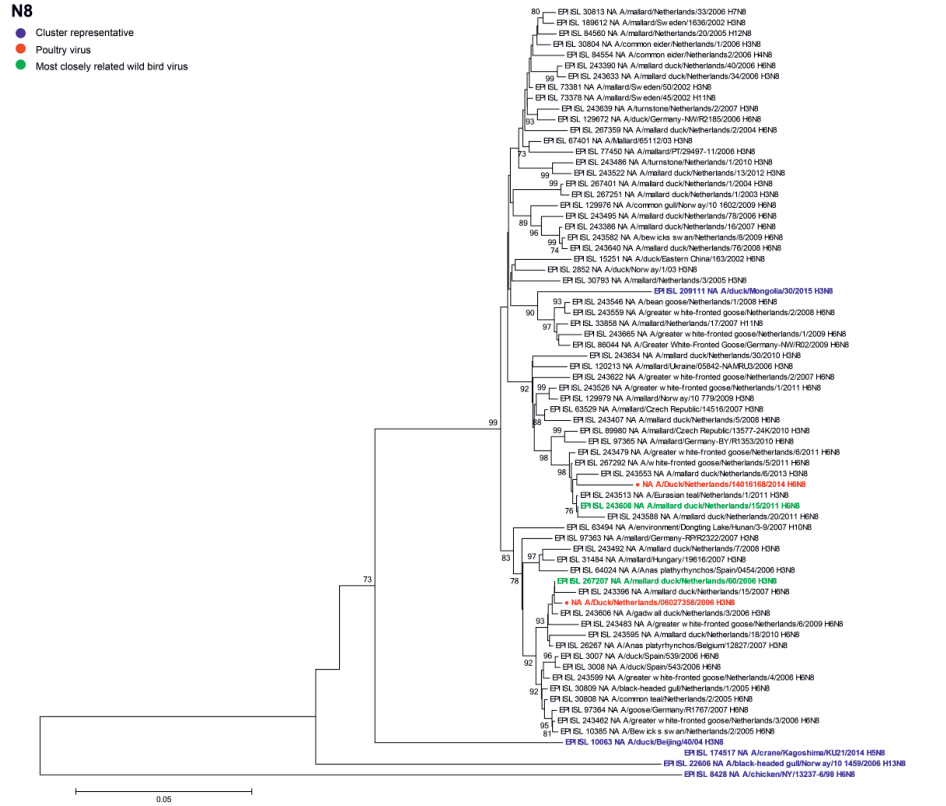
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EPI ISL 84560 NA A/mallard/Netherlands/20/2005 H12N8
EPI ISL 30804 NA A/common eider/Netherlands/1/2006 H3N8
EPI ISL 84554 NA A/common eider/Netherlands2/2006 H4N8
EPI ISL 243390 NA A/mallard duck/Netherlands/40/2006 H6N8
EPI ISL 243633 NA A/mallard duck/Netherlands/34/2006 H3N8
EPI ISL 73381 NA A/mallard/Sweden/50/2002 H3N8
EPI ISL 73378 NA A/mallard/Sweden/45/2002 H11N8
EPI ISL 243639 NA A/turnstone/Netherlands/2/2007 H3N8
EPI ISL 129672 NA A/duck/Germany-NW/R2185/2006 H6N8
EPI ISL 267359 NA A/mallard duck/Netherlands/2/2004 H6N8
EPI ISL 67401 NA A/Mallard/65112/03 H3N8
EPI ISL 77450 NA A/mallard/PT/29497-11/2006 H3N8
EPI ISL 243486 NA A/turnstone/Netherlands/1/2010 H3N8
EPI ISL 243522 NA A/mallard duck/Netherlands/13/2012 H3N8
EPI ISL 267401 NA A/mallard duck/Netherlands/1/2004 H3N8
EPI ISL 267251 NA A/mallard duck/Netherlands/1/2003 H3N8
EPI ISL 129976 NA A/common gull/Norway/10 1602/2009 H6N8
EPI ISL 243495 NA A/mallard duck/Netherlands/78/2006 H6N8
EPI ISL 243386 NA A/mallard duck/Netherlands/16/2007 H6N8
EPI ISL 243582 NA A/bewicks swan/Netherlands/8/2009 H6N8
EPI ISL 243640 NA A/mallard duck/Netherlands/76/2008 H6N8
EPI ISL 15251 NA A/duck/Eastern China/163/2002 H6N8
EPI ISL 2852 NA A/duck/Norway/1/03 H3N8
EPI ISL 30793 NA A/mallard/Netherlands/3/2005 H3N8
EPI ISL 209111 NA A/duck/Mongolia/30/2015 H3N8
EPI ISL 243546 NA A/bean goose/Netherlands/1/2008 H6N8
EPI ISL 243559 NA A/greater white-fronted goose/Netherlands/2/2008 H6N8
EPI ISL 33858 NA A/mallard/Netherlands/17/2007 H11N8
EPI ISL 243665 NA A/greater white-fronted goose/Netherlands/1/2009 H6N8
EPI ISL 86044 NA A/Greater White-Fronted Goose/Germany-NW/R02/2009 H6N8
EPI ISL 243634 NA A/mallard duck/Netherlands/30/2010 H3N8
EPI ISL 120213 NA A/mallard/Ukraine/05842-NAMRU3/2006 H3N8
EPI ISL 243622 NA A/greater white-fronted goose/Netherlands/2/2007 H6N8
EPI ISL 243526 NA A/greater white-fronted goose/Netherlands/1/2011 H6N8
EPI ISL 129979 NA A/mallard/Norway/10 779/2009 H3N8
EPI ISL 63529 NA A/mallard/Czech Republic/14516/2007 H3N8
EPI ISL 243407 NA A/mallard duck/Netherlands/5/2008 H6N8
EPI ISL 89980 NA A/mallard/Czech Republic/13577-24K/2010 H3N8
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EPI ISL 243479 NA A/greater white-fronted goose/Netherlands/6/2011 H6N8
EPI ISL 267292 NA A/white-fronted goose/Netherlands/5/2011 H6N8
EPI ISL 243553 NA A/mallard duck/Netherlands/6/2013 H3N8
NA A/Duck/Netherlands/14016168/2014 H6N8
EPI ISL 243513 NA A/Eurasian teal/Netherlands/1/2011 H3N8
EPI ISL 243608 NA A/mallard duck/Netherlands/15/2011 H6N8
EPI ISL 243588 NA A/mallard duck/Netherlands/20/2011 H6N8
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EPI ISL 243492 NA A/mallard duck/Netherlands/7/2008 H3N8
EPI ISL 31484 NA A/mallard/Hungary/19616/2007 H3N8
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NA A/Duck/Netherlands/06027358/2006 H3N8
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EPI ISL 243483 NA A/greater white-fronted goose/Netherlands/6/2009 H6N8
EPI ISL 243595 NA A/mallard duck/Netherlands/18/2010 H6N8
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EPI ISL 30808 NA A/common teal/Netherlands/2/2005 H6N8
EPI ISL 97364 NA A/goose/Germany/R1767/2007 H6N8
EPI ISL 243462 NA A/greater white-fronted goose/Netherlands/3/2006 H6N8
EPI ISL 10385 NA A/Bewicks swan/Netherlands/2/2005 H6N8
EPI ISL 10063 NA A/duck/Beijing/40/04 H3N8
EPI ISL 174517 NA A/crane/Kagoshima/KU21/2014 H5N8
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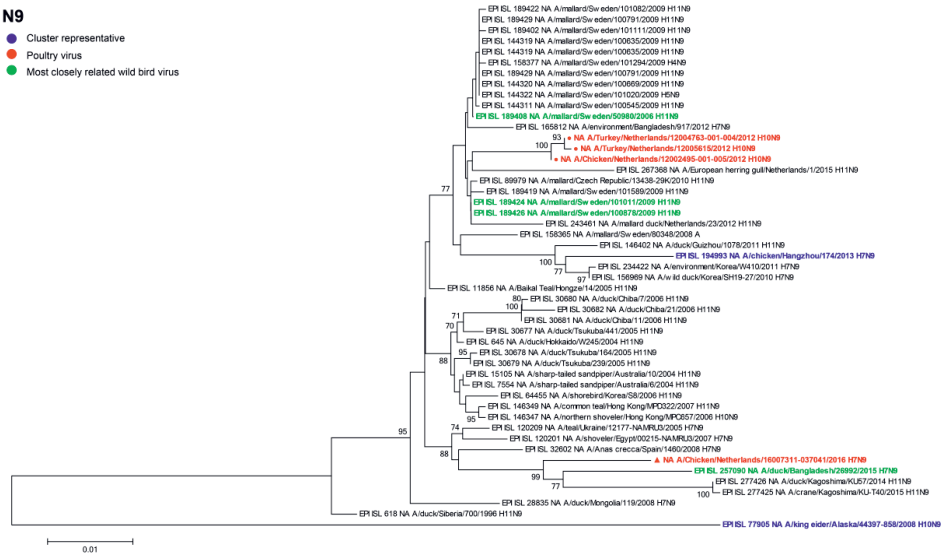
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Poultry virus
Most closely related wild bird virus
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EPI ISL 144319 NA A/mallard/Sweden/100635/2009 H11N9
EPI ISL 144319 NA A/mallard/Sweden/100635/2009 H11N9
EPI ISL 158377 NA A/mallard/Sweden/101294/2009 H4N9
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EPI ISL 144320 NA A/mallard/Sweden/100669/2009 H11N9
EPI ISL 144322 NA A/mallard/Sweden/101020/2009 H5N9
EPI ISL 144311 NA A/mallard/Sweden/100545/2009 H11N9
EPI ISL 189408 NA A/mallard/Sweden/50980/2006 H11N9
EPI ISL 165812 NA A/environment/Bangladesh/917/2012 H7N9
NA A/Turkey/Netherlands/12004763-001-004/2012 H10N9
NA A/Turkey/Netherlands/12005615/2012 H10N9
NA A/Chicken/Netherlands/12002495-001-005/2012 H10N9
EPI ISL 267368 NA A/European herring gull/Netherlands/1/2015 H11N9
EPI ISL 89979 NA A/mallard/Czech Republic/13438-29K/2010 H11N9
EPI ISL 189419 NA A/mallard/Sweden/101589/2009 H11N9
EPI ISL 189424 NA A/mallard/Sweden/101011/2009 H11N9
EPI ISL 189426 NA A/mallard/Sweden/100878/2009 H11N9
EPI ISL 243461 NA A/mallard duck/Netherlands/23/2012 H11N9
EPI ISL 158365 NA A/mallard/Sweden/80348/2008 A
EPI ISL 146402 NA A/duck/Guizhou/1078/2011 H11N9
EPI ISL 194993 NA A/chicken/Hangzhou/174/2013 H7N9
EPI ISL 234422 NA A/environment/Korea/W410/2011 H7N9
EPI ISL 156969 NA A/wild duck/Korea/SH19-27/2010 H7N9
EPI ISL 11856 NA A/Baikal Teal/Hongze/142005 H11N9
EPI ISL 30680 NA A/duck/Chiba/7/2006 H11N9
EPI ISL 30682 NA A/duck/Chiba/21/2006 H11N9
EPI ISL 30681 NA A/duck/Chiba/11/2006 H11N9
EPI ISL 30677 NA A/duck/Tsukuba/441/2005 H11N9
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EPI ISL 30678 NA A/duck/Tsukuba/164/2005 H11N9
EPI ISL 30679 NA A/duck/Tsukuba/239/2005 H11N9
EPI ISL 15105 NA A/sharp-tailed sandpiper/Australia/10/2004 H11N9
EPI ISL 7554 NA A/sharp-tailed sandpiper/Australia/6/2004 H11N9
EPI ISL 64455 NA A/shorebird/Korea/S8/2006 H11N9
EPI ISL 146349 NA A/common teal/Hong Kong/MPD322/2007 H11N9
EPI ISL 146347 NA A/northern shoveler/Hong Kong/MPD57/2006 H10N9
EPI ISL 120209 NA A/teal/Ukraine/12177-NAMRU3/2005 H7N9
EPI ISL 120201 NA A/shoveler/Egypt/00215-NAMRU3/2007 H7N9
EPI ISL 32602 NA A/Anas crecca/Spain/1460/2008 H7N9
NA A/Chicken/Netherlands/16007311-037041/2016 H7N9
EPI ISL 257090 NA A/duck/Bangladesh/26892/2015 H7N9
EPI ISL 277426 NA A/duck/Kagoshima/KU57/2014 H11N9
EPI ISL 277425 NA A/crane/Kagoshima/KU-T40/2015 H11N9
EPI ISL 28835 NA A/duck/Mongolia/119/2008 H7N9
EPI ISL 618 NA A/duck/Siberia/700/1996 H11N9
EPI ISL 77905 NA A/king eider/Alaska/44397-858/2008 H10N9
0.01

CHAPTER 3

Susceptibility of chickens to low pathogenic avian influenza (LPAI) viruses of wild bird- and poultry-associated subtypes

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Abstract

Analysis of low pathogenic avian influenza (LPAI) viruses circulating in the Netherlands in a previous study revealed associations of specific hemagglutinin (HA) and neuraminidase (NA) subtypes with wild bird- or poultry hosts. In this study, we identified putative host-associations in LPAI virus internal proteins. We show that LPAI viruses isolated from poultry more frequently carried the allele A variant of the nonstructural protein (NS) gene, compared to wild bird viruses. We determined the susceptibility of chickens to wild bird-associated subtypes H3N8 and H4N6 and poultry-associated subtypes H8N4 and H9N2, carrying either NS allele A or B, in an infection experiment. We observed variations in virus shedding and replication patterns, however, these did not correlate with the predicted wild bird- or poultry-associations of the viruses. The experiment demonstrated that LPAI viruses of wild bird-associated subtypes can replicate in chickens after experimental infection, despite their infrequent detection in poultry. Although the NS1 protein is known to play a role in immune modulation, no differences were detected in the limited innate immune response to LPAI virus infection. This study contributes to a better understanding of the infection dynamics of LPAI viruses in chickens.

Keywords: avian influenza virus; low pathogenic avian influenza; wild birds; poultry; chickens; shedding; innate immune response

Introduction

Avian influenza (AI) viruses are influenza A viruses that circulate among a broad range of wild bird species, particularly birds of the orders *Anseriformes* (ducks, geese, swans) and *Charadriiformes* (gulls, terns, waders) [1], and can also infect domestic poultry. AI viruses are divided into subtypes based on the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), which are involved in the attachment and release from host cells [2], respectively. Most viruses are low pathogenic avian influenza (LPAI) viruses that produce subclinical infections in poultry or, occasionally, cause mild respiratory disease, a reduction in egg production and low mortality [3]. LPAI viruses of HA subtypes H5 and H7 can mutate into highly pathogenic avian influenza (HPAI) variants that can cause severe progressive disease and high mortality in birds [4]. Outbreaks of HPAI viruses can have serious impact on animal health and economic consequences for the commercial poultry industries. In addition, some strains can be transmitted to humans [5], causing major concern for public health worldwide.

In birds, 16 HA subtypes (H1-H16) and 9 NA (N1-N9) subtypes have been found in numerous combinations [6,7]. Some of these subtypes are specific to certain avian species, of which H13 and H16 viruses in gulls is a prime example [7]. Our previous study on LPAI viruses circulating in wild birds and poultry in the Netherlands also revealed close host-dependent associations among other HA and NA subtypes [8]. During a ten-year surveillance period in the Netherlands, LPAI viruses of subtypes H3N8 and H4N6 were predominantly detected in wild ducks, in particular mallards, but rarely detected in poultry. In contrast, LPAI viruses of subtypes H8N4 and H9N2 were most frequently detected in Dutch poultry, but only sporadically detected in wild birds. These findings suggest that transmission to poultry is selective, and likely influenced by viral factors that determine host range.

Besides HA and NA, the viral genome encodes for internal proteins including polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), matrix proteins (M1 and M2), nucleoprotein (NP) and nonstructural proteins (NS1 and NS2). The NS proteins circulate in two lineages (NS allele A and B) that differ by around 30% of their amino acids [9]. The internal proteins are responsible for viral functions, such as genome replication and expression, and virus assembly [10]. The NS1 protein can modulate the host immune response and protein expression, and thereby acts as an important virulence factor [11]. Several of the internal proteins have been described to influence the infectivity, pathogenicity and transmissibility of AI viruses in a host-dependent manner [12], but their role in host-dependent transmission between avian species is largely unknown. Increased knowledge on the ability of different AI virus subtypes and genotypes to infect poultry would contribute to a better understanding of virus epidemiology. In addition, the identification of viral factors that influence host range can be used for predicting the risk of infection in poultry, and can help to design more efficient surveillance programs for the early detection of potentially dangerous strains.

In this study, we analysed the association of LPAI virus internal proteins with wild bird or poultry hosts. We show that LPAI viruses isolated from poultry more frequently contain NS allele A, compared to wild bird viruses. The susceptibility of chickens to wild duck-origin LPAI viruses of wild bird-associated subtypes H3N8 and H4N6 and poultry-associated subtypes H8N4 and H9N2, carrying either NS allele A or B, was studied in an infection experiment. We analysed the pattern and route of viral shedding, which not only provides information on

potential replication sites but also gives an indication of the ability of virus transmission to other birds. In addition, we analysed virus replication and immunological responses in the respiratory and intestinal organs, which are main target tissues of LPAI viruses. This study contributes to a better understanding of the infection dynamics of different LPAI virus strains in chickens, which can be used to improve current surveillance programs.

Material and Methods

ETHICAL STATEMENT

The animal experiment and associated procedures were in accordance with the national regulations on animal experimentation and the project license was approved by the Dutch Central Authority for Scientific Procedures on Animals (CCD) (permit number ADV401002015317; experiment number 2016.D-0057.001). The animal procedures were performed conform the guidelines from the European Union directive 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes [13].

VIRUS SELECTION

The subtypes tested in this study were selected based on their association with wild birds or poultry as observed in a previous surveillance study in the Netherlands [8]. To identify host-associated features in the internal proteins, a statistical comparative analysis was performed using the metadata-driven comparative analysis tool for sequences (meta-CATS) of the Influenza Research Database (IRD) (https://www.fludb.org) [14]. For this analysis, we used the amino acid (aa) sequences of the internal proteins (PB1, PB2, PA, M1, M2, NP, NS1 and NS2) of the same set of 162 wild bird viruses and 42 poultry viruses as analysed previously [8]. For each protein, a multiple sequence alignment was generated in MUSCLE version 3.8.31 [15]. Subsequently, the alignments were submitted to the automated meta-CATS pipeline in two groups according to their host source (i.e. wild bird or poultry). In the statistical tool, a chi-square test of independence was performed at each aa position to identify residues that significantly differed between the groups ($p<0.05$).

VIRUS ISOLATION AND PROPAGATION

The viruses used in this study were provided by Erasmus MC (Rotterdam, the Netherlands) (Table S1). The viruses were initially isolated from oropharyngeal (OP) or cloacal (CL) swabs collected from wild ducks as part of the national AI virus surveillance program in wild birds in the Netherlands. The whole genome sequences were generated in a previous study [8]. Virus stocks were generated by two passages in 9-11 day-old specific pathogen free (SPF) embryonated chicken eggs (ECEs). The virus stocks were titrated using standard methods to determine the median egg infectious dose (EID50) titres [16]. The median tissue culture infective dose (TCID50) titres were determined by end-point titration in Madin-Darby Canine Kidney (MDCK) cells, as described previously [17]. The virus stocks were diluted in sterile phosphate buffered saline (PBS) immediately prior to use in order to obtain 106 EID50/ml inoculum.

ANIMALS AND HOUSING

A total of 184 six-week-old SPF White Leghorn chickens (*Gallus gallus domesticus*) of both sexes were obtained from MSD Animal Health (Boxmeer, the Netherlands). White Leghorn chickens were chosen for this research as it represents the most common and economically important poultry type in the Netherlands [18]. The experiment was performed in biosafety level 2 (BSL 2) facilities at Wageningen Bioveterinary Research (WBVR, Lelystad, the Netherlands). The chickens were housed in temperature-controlled rooms under optimal light conditions and humidity, and feed and water were provided *ad libitum*. Each experimental group was housed separately in floor pens with solid livestock dividing panels, and personnel changed clothes to prevent cross-contamination between groups.

EXPERIMENTAL DESIGN

The chickens were randomly divided into eight experimental groups (virus-inoculated groups) of 20 chickens and one experimental group (control group) of 24 chickens (Figure 1). Individual birds of each experimental group were numbered randomly to select chickens for tissue collection at different time points. At day 0, prior to inoculation, OP and CL swabs were collected from all chickens to confirm the absence of current AI virus infection. In addition, blood samples were collected by heart puncture from the control group (n=4) under anaesthesia by intramuscular (IM) administration of 0.4 ml Xylazine and Ketamine mixture. After blood collection, the chickens were immediately euthanized by intravenous (IV) administration of 2.0 ml Euthasol 50% solution (AST Farma, Oudewater, the Netherlands), and tissue samples of various organs were collected, including the trachea, lung and ileum.

Inoculation was performed via intranasal (IN) and intratracheal (IT) administration of 0.1 ml of 10^6 EID_{50}/ml inoculum per route (inoculation dose of $10^{5.3}$ EID_{50} per bird). The inoculation dose was confirmed by back-titration on MDCK cells and conversion of the $TCID_{50}$ titres into equivalent EID_{50} titres. For each experimental group, the viral dose was within the range of 0.5 log10 EID_{50}/ml of the target titre. All birds were observed daily for clinical signs of disease. OP and CL swabs were taken for virus detection from live birds daily to 7 days post inoculation (dpi) to determine viral shedding. Four chickens from each experimental group were euthanized, using the same method as the control group, at 1, 2, 3, 5 and 7 dpi to collect blood and organs. The experiment was terminated at 7 dpi.

ANTIBODY DETECTION

Blood samples were left overnight at RT for serum separation [19], and serum samples were stored at -20°C until testing. For influenza virus-specific antibody detection, serum was tested by anti-NP ELISA (FlockChek AI MultiS-Screen Ab Test Kit (IDEXX Europe B.V., Hoofddorp, the Netherlands)), according to the manufacturers' instructions. Hemagglutination inhibition (HI) testing for HA subtype-specific antibodies was done using standardized homologous H3, H4, H8 and H9 antigens. HI test results were reported as log_2 HI titres, with titres of 3 log_2 or higher considered positive.

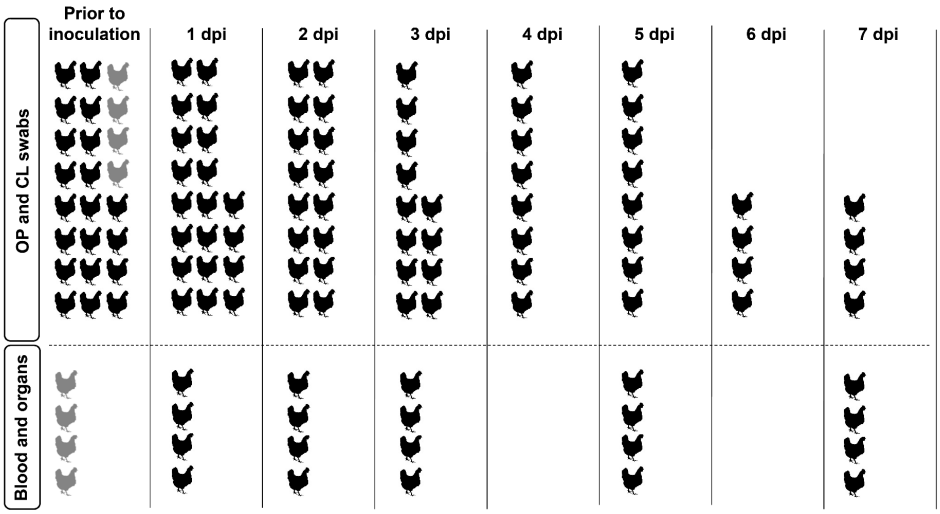

FIGURE 1. SAMPLE COLLECTION PER EXPERIMENTAL GROUP.
A total of 184 six-week-old specific pathogen free (SPF) White Leghorn chickens were divided into eight experimental groups of 20 chickens (virus-inoculated groups) and one experimental group of 24 chickens (control group). Prior to inoculation, oropharyngeal (OP) and cloacal (CL) swabs were collected from all chickens of each experimental group to confirm the absence of current AI virus infection. In addition, blood and organs were collected from four chickens in the control group (shown in grey). After inoculation, OP and CL swabs were taken from live birds daily to 7 days post inoculation (dpi) to determine viral shedding. Four chickens from each experimental group were euthanized at 1, 2, 3, 5 and 7 dpi to collect blood and organs. The experiment was terminated at 7 dpi.

VIRUS DETECTION IN SWABS

Swabs were placed in 2.0 ml Tryptose Phosphate Broth 2.95% containing gentamicin and stored at -80°C until testing. For virus detection, total RNA was extracted from the swab specimens using the MagNA Pure 96 system (Roche, Basel, Switzerland) with the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche). Influenza A virus was detected by a quantitative real-time reverse transcription polymerase chain reaction targeting the matrix gene (M-PCR), as described previously [20]. For each virus, a standard curve for virus quantification was taken along that consisted of ten-fold serial dilutions of the working stocks with a known EID_{50} titre. The standard curve was used to convert the Ct values into equivalent EID_{50} titres and determine PCR efficiency. Results were reported as mean equivalent log10 EID_{50}/ml titres and their standard deviation (SD) with a lower detection limit of $10^{1.7}$ EID_{50}/ml, and plotted using Graphpad PRISM 8. Chickens were considered positive for viral shedding if virus was detected in swab samples at any time during the experiment.

VIRUS DETECTION IN TISSUE

Tissues collected for virus detection were snap-frozen on dry ice and stored at -80°C until processing. The frozen tissue samples (100 mg) were homogenised in 1.0 ml Trizol (TRI Reagent (Thermo Fisher Scientific, Waltham, MA, USA)) [21]. Isolation of total RNA from the homogenized tissue was performed using the Direct-Zol RNA Microprep isolation kit (Zymo Research, Irvine, CA, USA) with DNAse treatment according to the manufacturers' protocol. A M-PCR was performed to quantitate viral RNA in the tissue samples, as described previously [20]. Results were reported as mean Ct values and their SD.

CYTOKINE EXPRESSION IN TISSUE

To measure cytokine expression, the quantity of the extracted RNA from tissue samples was assessed using the Nanodrop spectrophotometer (Thermo Fisher Scientific). RNA quality was inferred using the 2100 Bioanalyzer system (Aligent Technologies, Santa Clara, CA, USA). Samples meeting a RNA integrity number (RIN) score of 6.5 or higher have been classified as high-quality RNA samples obtained from frozen tissue [22], and were therefore included in this study. RNA (200 ng) was converted into cDNA using the Superscript IV First-Strand Synthesis System (Thermo Fisher Scientific). Quantitative real-time PCR was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) in the ABI 7500 Real-Time PCR system (Thermo Fisher Scientific) [21].

A panel of five candidate reference genes in chickens (HPRT1, RPLP0, HMBS, TBP, RPL13) was selected based on literature [23] and tested using the extracted RNA samples from tissues of seven birds from the virus-inoculated and control group. From this panel, two stably-expressed reference genes (RPLP0 and TBP) were selected for each organ separately using NormFinder [24]. Cytokine and TLR mRNA expression was measured by a PCR targeting IL-6, IL-1β, IFN-α, IFN-β and TLR7 mRNAs. The primer sets used were identical to those described previously [21], except that the IL-1β forward primer was replaced with 5′-CAGCAGCCTCAGCGAAGAG-3′. A standard curve of a plasmid containing the gene of interest (GenScript Biotech, Piscataway, NJ, USA) was used to determine PCR efficiency. Results were reported as fold change in mRNA expression relative to the control group and their SD calculated using the ΔΔCt method ($2^{-\Delta\Delta Ct}$) [25]. Comparison between virus-inoculated and control groups was performed using the unpaired, two-tailed Student's t-test, with significance defined as $p<0.05$. The statistical analyses were conducted using Graphpad PRISM 8.

CLUSTER ANALYSIS OF INTERNAL GENES

We performed genetic cluster analysis based on the internal genes (PB1, PB2, PA, NP, MP, and NS) to determine if genes similar to those of the inoculated viruses have previously been isolated from poultry. For this analysis, we used a dataset of publicly available sequences of around 20,000 avian-origin AI viruses obtained from GISAID's EpiFlu database (http://www.gisaid.org) [26] on 27 May 2019. For each internal gene, the sequences were aligned in MAFFT version 7.427 [27,28] and curated in Aliview version 1.26 [29]. Partial sequences and sequences containing multiple ambiguous bases (Ns) were excluded from the analysis. The

remaining sequences were clustered against the sequences of the inoculated viruses in CD-HIT-EST-2D [30,31]. A sequence identity threshold value of 1.5% was used to generate clusters of sequences that share nucleotide sequence identities of at least 98.5% with the inoculated virus. For each cluster, the total number of sequences (cluster size) and the number of sequences derived from poultry were determined.

Results

IDENTIFICATION OF HOST-ASSOCIATIONS IN THE INTERNAL PROTEINS

To identify amino acid (aa) residues in the internal proteins that significantly differ between viruses isolated from wild birds and poultry, we applied meta-CATS analysis using the sequences of 162 wild bird viruses and 42 poultry viruses isolated in the Netherlands. The chi-squared analysis identified 135 statistically significant aa residues that differed between the host groups ($p<0.05$). A total of 82 aa residues were located in the NS proteins due to the presence of two distinct NS alleles (NS allele A and B) (Table S2). Viruses isolated from poultry contained relatively more frequently NS allele A (83%) than viruses isolated from wild birds (62%). This skewed distribution may suggest that poultry is more prone to infection with LPAI virus strains carrying NS allele A.

We identified 53 statistically significant aa residues that differed between wild bird and poultry viruses, but were not linked to the NS alleles. These residues were located in PB1 (10 of 48), PB2 (11 of 53), PA (14 of 53), NP (11 of 53), M1 (1 of 53), M2 (1 of 53), NS1 (4 of 53) and NS2 (1 of 53). A total of 44 residues were present in more than 90% of the viruses in both host groups, and four residues were located at highly variable aa positions, as three or more aa variants were detected at these positions. The five aa residues for which larger differences in occurrence between wild bird and poultry viruses were found are listed in Table S2. These were mostly arginine (R) to lysine (K) substitutions, with unknown effect on protein function. Based on the suspected host-association of the NS protein, we selected strains of NS allele A and B for further evaluation in the animal experiment.

CLINICAL SIGNS AND SEROCONVERSION IN LPAI INFECTED CHICKENS

Six-week-old SPF chickens were inoculated with eight wild duck-origin LPAI viruses of wild bird-associated (H3N8 and H4N6) and poultry-associated subtypes (H8N4 and H9N2), with either NS allele A or B, to study their susceptibility to infection (Table S1). The chickens were examined for clinical signs, swabbed daily, and euthanized at selected time points to collect organs and blood (Figure 1).

During the 7-day experiment, no obvious clinical signs and no mortality were observed. There were no antibodies detected in serum of the control chickens. Influenza A virus NP-specific antibodies were detected by ELISA in chickens inoculated with H8N4 NS A virus (4 of 4 chickens) and H9N2 NS A virus (1 of 4 chickens) at 5 dpi (Table S3), indicating rapid initiation of antibody production in these chickens. At 7 dpi, seroconversion was observed in chickens from all experimental groups, with the exception of chickens inoculated with H8N4 NS B virus. HA subtype-specific antibodies were detected upon inoculation with H8N4 NS A virus at 5 dpi in 3 of 4 chickens (mean HI titre of 4.3 ± 0.6 $\log_2$) and at 7 dpi in 2 of

4 chickens (HI titres of 5 $\log_2$). In the groups inoculated with H3N8 NS A and B viruses, subtype-specific antibodies were detected at 7 dpi in 1 of 4 chickens (HI titres of 3 $\log_2$).

VIRAL SHEDDING OF LPAI INFECTED CHICKENS

Viral shedding upon inoculation of the LPAI viruses was measured daily by the detection of viral RNA in swab samples using influenza virus-specific M-PCR. The mean virus titres in oropharyngeal (OP) and cloacal (CL) swabs for each experimental group are plotted over time in Figure 2 and 3, respectively. The number of birds shedding virus and the mean shedding titres are provided in Table S4 and S5. No virus was detected in swab samples collected from the control birds. Viral shedding was observed in all virus-inoculated groups, except in chickens inoculated with H8N4 NS B virus.

Shedding through the oropharyngeal route was predominantly detected in chickens inoculated with H3N8 NS A virus (12 of 20 chickens), H3N8 NS B virus (17 of 20 chickens), H4N6 NS A virus (19 of 20 chickens) and H4N6 NS B virus (16 of 20 chickens). The mean onset of OP shedding was 1.4±1.1 dpi. In chickens inoculated with H3N8 NS A virus, a peak of shedding was observed at 1 and 5 dpi, with a mean shedding titre of $10^{3.8}$ EID_{50}. In this group, one chicken was tested positive for OP shedding at 7 dpi, although the virus titre was low ($10^{2.4}$ EID_{50}). No OP shedding was observed at 7 dpi in other experimental groups. In chickens inoculated with H3N8 NS B virus, a peak of shedding was observed at 1-2 dpi, with a mean shedding titre of $10^{4.7}$ EID_{50}, which declined and resolved by 5 dpi. H4N6 NS A virus-infected chickens showed a peak of shedding at 1 dpi (mean shedding titre of $10^{4.8}$ EID_{50}), followed by a fast decline to low virus titres between 3-7 dpi. Chickens inoculated with H4N6 NS B virus showed a peak of OP shedding at 1-2 dpi, with a mean shedding titre of $10^{4.8}$ EID_{50}, which declined and resolved by 7 dpi. However, limited shedding was detected in chickens inoculated with H8N4 NS A virus (7 of 20 chickens), H9N2 NS A virus (4 of 20 chickens) and H9N2 NS B virus (6 of 20 chickens), and no OP shedding was detected in chickens inoculated with H8N4 NS B virus (0 of 20 chickens).

In addition, limited shedding through the cloacal route was observed in chickens inoculated with H3N8 NS A virus (2 of 20 chickens), H3N8 NS B virus (7 of 20 chickens) and H8N4 NS A virus (3 of 20 chickens). The majority of these chickens (11 of 12 chickens) were also positive for OP shedding. The mean onset of CL shedding was 3.9±1.5 dpi. The mean virus titre was highest at 7 dpi in all three experimental groups, with mean shedding titres ranging between $10^{3.7\text{-}6.8}$ EID_{50}. No virus was detected in the CL swabs of chickens inoculated with both H4N6 viruses, H8N4 NS B virus and both H9N2 viruses. The results demonstrate variations in viral shedding patterns and routes between LPAI viruses of wild bird- and poultry-associated subtypes and NS alleles in chickens.

VIRAL REPLICATION IN THE RESPIRATORY AND INTESTINAL TRACT

Tissues collected from the trachea, lung and ileum were tested by M-PCR to determine viral replication in the respiratory and intestinal tract of four chickens from each experimental group (Table 1). No virus was detected in tissues collected from the control chickens At 1 dpi, virus was detected in trachea and lung tissues of chickens from all virus-inoculated groups,

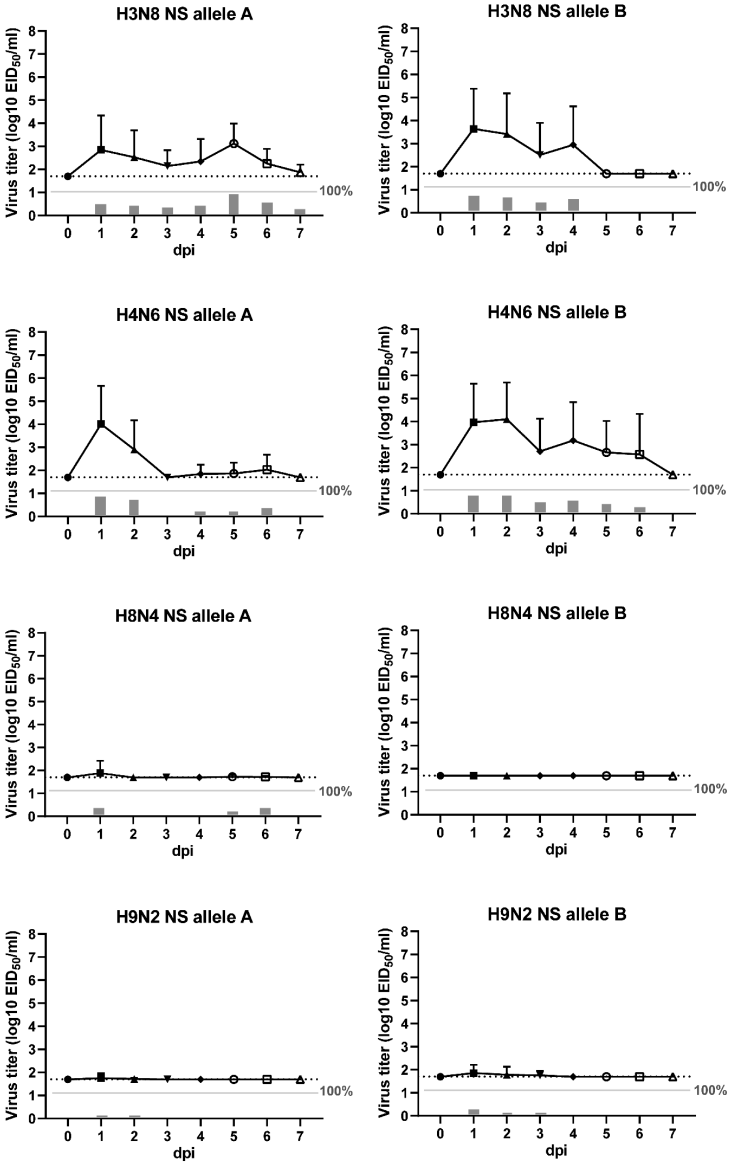

FIGURE 2. OROPHARYNGEAL SHEDDING.

Virus detected in oropharyngeal (OP) swabs collected from chickens inoculated by the intranasal (IN) and intratracheal (IT) route with eight strains of low pathogenic avian influenza (LPAI) viruses (105.3 median egg infectious dose (EID50) per bird). The swabs were taken daily from live birds to 7 days post inoculation (dpi) for virus detection by influenza virus-specific PCR (M-PCR). Viral shedding is expressed as the mean equivalent log10 EID_{50}/ml titre ± standard deviation (SD) with a lower detection limit of $10^{1.7}$ EID_{50}/ml (dashed line). The grey bars below the solid line indicate the percentage of positive swabs.

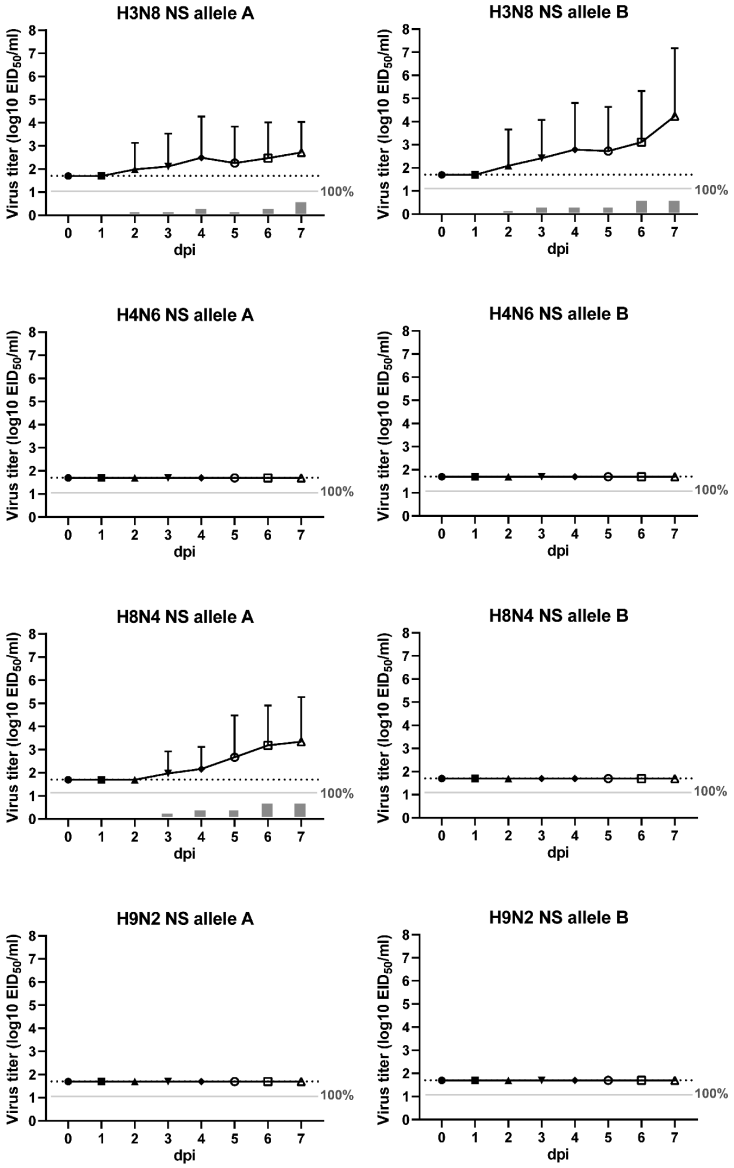

FIGURE 3. CLOACAL SHEDDING.

Virus detected in cloacal (CL) swabs collected from chickens inoculated by the intranasal (IN) and intratracheal (IT) route with eight strains of low pathogenic avian influenza (LPAI) viruses ($10^{5.3}$ median egg infectious dose (EID_{50}) per bird). The swabs were taken daily from live birds to 7 days post inoculation (dpi) for virus detection by influenza virus-specific PCR (M-PCR). Viral shedding is expressed as the mean equivalent log10 EID_{50}/ml titre ± standard deviation (SD) with a lower detection limit of $10^{1.7}$ EID_{50}/ml (dashed line). The grey bars below the solid line indicate the percentage of positive swabs.

TABLE 1. VIRAL REPLICATION IN THE TRACHEA, LUNG AND ILEUM.
The ratio of chickens virus-positive in tissue to the number of virus-inoculated chickens. The tissues were collected from four euthanized birds of each experimental group at 1, 3, 5 and 7 days post inoculation (dpi). Virus was detected by influenza virus-specific PCR (M-PCR). Viral titres are expressed as the mean Ct value ± standard deviation (SD).

	Trachea		Lung		Ileum		
Virus	**1 dpi**	**3 dpi**	**1 dpi**	**5 dpi**	**1 dpi**	**5 dpi**	**7 dpi**
H3N8 NS allele A	3/4 (32.6±1.8)	1/4 (34.3)	4/4 (31.6±3.4)	0/4	0/4	0/4	1/4 (26.3)
H3N8 NS allele B	1/4 (29.5)	0/4	4/4 (34.6±2.8)	0/4	0/4	1/4 (23.2)	2/4 (23.4±0.2)
H4N6 NS allele A	3/4 (27.5±0.8)	0/4	3/4 (26.9±2.9)	0/4	0/4	0/4	0/4
H4N6 NS allele B	1/4 (36.6)	0/4	3/4 (32.5±8.3)	0/4	0/4	0/4	0/4
H8N4 NS allele A	2/4 (32.3±0.2)	0/4	4/4 (30.2±0.5)	0/4	0/4	0/4	4/4 (34.2±4.9)
H8N4 NS allele B	0/4	0/4	1/4 (35.7)	1/4 (30.0)	0/4	0/4	0/4
H9N2 NS allele A	2/4 (30.9±0.9)	0/4	0/4	0/4	0/4	0/4	0/4
H9N2 NS allele B	2/4 (30.1±2.3)	0/4	2/4 (33.5±2.0)	0/4	0/4	0/4	0/4

dpi, days post inoculation; NS, nonstructural protein; n.d., not done.

indicative of local virus replication. Remarkably, virus was detected in trachea or lung of all chickens inoculated with H8N4 NS A virus and 3 of 4 chickens inoculated with H9N2 NS B virus, despite OP shedding was limited in these groups. At 3 and 5 dpi, most tissues examined were tested virus-negative, indicating a short persistence of LPAI virus in the chicken respiratory tract. At 7 dpi, virus was detected in the ileum collected from chickens inoculated with H3N8 NS A (1 of 4 chickens), H3N8 NS B (2 of 4 chickens) and H8N4 NS A (all 4 chickens) viruses, which were also positive for CL shedding. This shows that LPAI virus replication in the intestine and subsequent excretion via the cloaca is strain-dependent. Overall, virus was mainly detected in the respiratory tissues at 1 dpi and intestinal tissues at 7 dpi, which correlates with the pattern of viral shedding over time.

CYTOKINE mRNA EXPRESSION IN THE TRACHEA AND ILEUM

For analysis of the inflammatory cytokine response, we measured the levels of IL-6, IL-1β, IFN-α, IFN-β and TLR7 mRNA in tissues collected from the trachea and ileum by quantitative PCR (Figure 4). The measured expression levels were normalized using a selected panel of reference genes. We determined the mean fold change in mRNA expression in chickens infected with LPAI viruses of wild bird-associated (H3N8 and H4N6) and poultry-associated (H8N4 and H9N2) subtypes, carrying either NS allele A or B, relative to uninfected chickens in the control group. Different time points were selected for the trachea (1 and 3 dpi) and ileum (5 and 7 dpi) based on the detection of virus in swabs and organs. Levels of IL-6 mRNA were almost undetectable in all tissues examined, and therefore excluded for analysis.

In the trachea, no significant change in cytokine expression was observed at 1 dpi. At 3 dpi, levels of IFN-β mRNA were slightly induced in chickens inoculated with the NS allele A variants of wild bird-associated subtypes (3.2±1.1 mean fold induction) and poultry-associated subtypes (2.6±1.1 mean fold induction). In the ileum, slightly reduced levels of IFN-α mRNA were measured at 5 dpi in chickens inoculated with wild bird-associated subtypes of NS allele A and B (mean fold reduction of 2.3±0.2 and 2.1±0.1, respectively). At 7 dpi, we observed increased within-group variability in cytokine mRNA levels in the ileum of chickens inoculated with wild bird-associated subtypes of NS allele B. This suggests that these strains led to changes in cytokine expression in a subset of the infected chickens. However, overall, the results indicate that LPAI viruses elicit limited innate immune responses in chickens.

GENETIC CLUSTER ANALYSIS OF THE INOCULATED VIRUSES

The susceptibility of chickens to the LPAI viruses of specific subtypes or NS alleles was not consistent with their frequency of isolation in the field. We therefore performed genetic cluster analysis for the internal genes (PB1, PB2, PA, NP, MP, and NS) to determine if genes similar to those of the inoculated viruses have previously been isolated from poultry. This may reveal genetic links between the inoculated viruses and poultry hosts that can help to explain the unexpected results of the infection experiment. For this analysis, the full-length nucleotide sequences of the internal genes were compared with those deposited in online databases. Genes similar to those of the inoculated viruses were identified by genetic cluster analysis using a sequence identity threshold value of 1.5%. For each genetic cluster, the cluster size and the number of viruses isolated from poultry were determined (Table 2).

The genetic cluster analysis confirmed the association of NS allele A with poultry, as viruses carrying NS allele A clustered more often with poultry viruses compared to viruses carrying NS allele B. Interestingly, one or more gene segments encoding for the viral polymerase subunits (PB1, PB2, and PA) clustered with poultry viruses for both H3N8 viruses, both H4N6 viruses and H8N4 NS A virus, which could also replicate in chickens. In contrast, H8N4 NS B virus and both H9N2 viruses, which did not replicate in chickens, contained polymerase gene segments that were restricted to wild bird isolates. These findings suggest that the polymerase complex may play a role in infection of chickens, and may be involved in the transmissibility of LPAI viruses between avian hosts.

Discussion

In a previous study on LPAI viruses circulating in wild birds and poultry in the Netherlands, we found close host-dependent associations among HA and NA subtypes, suggesting selective virus transmission to poultry [8]. Viruses of subtypes H3N8 and H4N6 were found to be associated with wild birds, whereas H8N4 and H9N2 were found to be associated with poultry. In the present study, we examined these host-subtype associations *in vivo*. Chickens were inoculated with eight strains of wild duck-origin LPAI viruses of wild bird- and poultry-associated subtypes. No clinical signs were observed during the experiment, which is

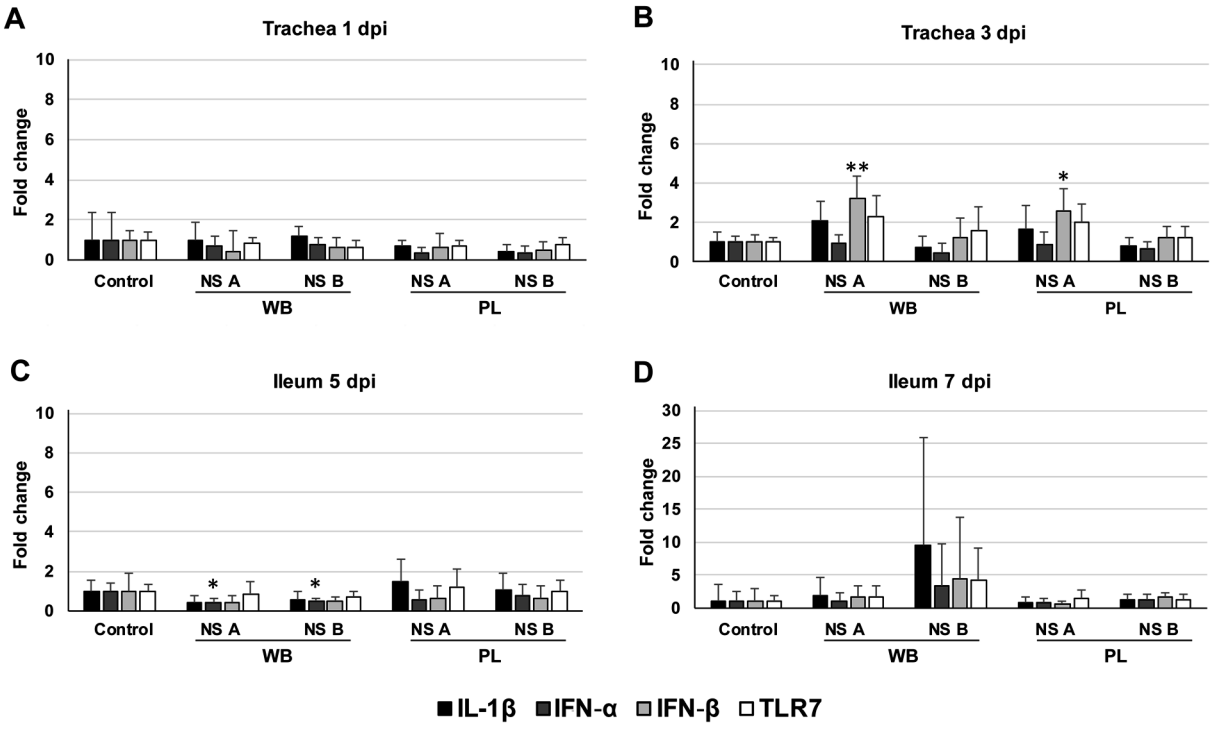

FIGURE 4. CYTOKINE MRNA EXPRESSION IN THE TRACHEA AND ILEUM.

Fold change in IL-1β, IFN-α, IFN-β, and TLR7 mRNA levels in trachea at (A) day 1 and (B) day 3, and in ileum at (C) day 5 and (D) day 7 post inoculation (dpi) of chickens with low pathogenic avian influenza (LPAI) viruses of wild bird-associated H3N8 and H4N6 (WB) and poultry-associated subtypes H8N4 and H9N2 (PL) carrying nonstructural protein (NS) allele A or B. Results of two experimental groups were pooled into a single representative group as follows: H3N8 and H4N6 viruses of NS allele A (WB/NS A), H3N8 and H4N6 viruses of NS allele B (WB/NS B), H8N4 and H9N2 viruses of NS allele A (PL/NS A), and H8N4 and H9N2 viruses of NS allele B (PL/NS B). mRNA expression is shown as the mean fold change relative to the control group ± standard deviation (SD). * $p<0.05$, ** $p<0.01$.

TABLE 2. GENETIC CLUSTER ANALYSIS OF THE INOCULATED VIRUSES.
Clusters of internal genes similar to those of the inoculated viruses, presented as the number of sequences that originate from poultry out of the total number of sequences within each cluster (cluster size). Genetic clusters were generated by clustering publicly available sequences of around 20,000 avian-origin AI viruses obtained from GISAID's EpiFlu database (http://www.gisaid.org) [26] on 27 May 2019 against the sequences of the inoculated viruses, using a sequence identity threshold value of 1.5%. We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database on which this research is based.

Virus	PB1	PB2	PA	NP	MP	NS
H3N8 NS allele A	3/15	0/91	0/23	2/37	0/49	0/67
H3N8 NS allele B	0/4	2/6	0/7	0/32	7/101	0/103
H4N6 NS allele A	4/17	0/59	1/5	1/27	6/304	13/557
H4N6 NS allele B	6/17	0/97	0/3	0/95	34/568	0/108
H8N4 NS allele A	0/160	2/33	0/21	0/92	38/698	33/886
H8N4 NS allele B	0/61	0/20	0/20	4/56	93/1034	0/79
H9N2 NS allele A	0/59	0/64	0/58	0/108	14/510	2/86
H9N2 NS allele B	0/15	0/16	0/56	0/1	0/149	8/35

PB2, polymerase basic protein 2; PB1, polymerase basic protein 1; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; MP, matrix protein; NS, nonstructural protein.

consistent with the low pathogenicity of the viruses. The absence of antibody responses in most virus-inoculated chickens may be due to the short duration of the experiment. Viral shedding was observed in 82 of 160 virus-inoculated chickens, predominantly via the OP route early after inoculation, which is consistent with previous studies [32-34]. Evidence of viral replication was found in all virus-inoculated groups, but strain-dependent variations in susceptibility and shedding patterns were observed between groups.

Contrary to our expectations based on field observations, LPAI viruses of wild bird-associated subtypes H3N8 and H4N6 replicated in chickens. Most chickens inoculated with the H3N8 viruses shed virus through both the OP and CL route. In contrast, the H4N6 viruses were exclusively shed through the OP route, implying that the H4N6 viruses used in this study replicate more efficiently in the respiratory tract than in the intestinal tract. Previous studies have shown that Asian H3N8 and H4N6 strains of wild bird-origin are able to infect chickens, although replication efficiency strongly differed between strains [35-37]. In Europe, recent outbreaks of H3N1 viruses in Belgium have also demonstrated the ability of H3 viruses to infect chickens [38]. Phylogenetically, the HA gene of the H3N1 viruses clusters with other Eurasian strains of different subtype combinations and internal gene compositions, but no more than 98.3% nucleotide sequence identity was found by BLAST (results not shown). The results in this study demonstrate that chickens are experimentally susceptible for H3N8 and H4N6 viruses that have been circulating in Europe, suggesting that these viruses can also be transmitted from anseriform to galliform hosts. Nevertheless, these subtypes were rarely detected in chickens during a ten-year surveillance period in the Netherlands [8]. Of the LPAI virus subtypes H8N4 and H9N2 that were frequently found in poultry, only one H8N4 strain replicated efficiently in chickens. The H8N4 virus carrying NS allele A was shed in high concentrations through the cloaca, replicated in the respiratory and intestinal tract, and

induced a more rapid seroconversion in chickens compared to the other inoculated viruses. Despite the frequent detection of H8N4 and H9N2 subtypes in poultry during the ten-year surveillance period, no to limited virus was detected in the swabs or tissues of chickens inoculated with the other H8N4 and H9N2 strains, indicating low susceptibility for these strains.

Several factors may have contributed to these unexpected results. One explanation could be that H3N8 and H4N6 viruses have been introduced into poultry, but have not been detected during surveillance. This is considered unlikely, as poultry flocks are screened at least once a year for the presence of antibodies against AI viruses, which are generally detectable up to several weeks or months after infection [39]. However, the antibody responses may differ in duration and intensity between virus subtypes and strains. H3N8 and H4N6 viruses may cause shorter antibody responses in chickens compared to other subtypes, resulting in a less frequent detection in poultry. Analysis of humoral responses to diverse LPAI viruses in chickens could provide more insight in variations in the duration and intensity of antibody responses between subtypes. Furthermore, the fact that the LPAI viruses used in this study originate from wild ducks, and may have been adapted to anseriform hosts, could have contributed to the inefficient replication of most H8N4 and H9N2 strains in chickens. The H9N2 viruses used in this study are not related to the poultry-adapted H9N2 viruses that have circulated enzootically in poultry in Asia, North Africa and the Middle East, with sporadic spillovers to humans (results not shown). Finally, discrepancies between experimental studies and field observations may be due to environmental conditions, such as the number and density of chickens, virus inoculation dose and route, and concurrent infections, which can influence virus infection dynamics.

Alternatively, poultry have not been exposed to the LPAI viruses that were detected in wild birds. The fact that H3N8 and H4N6 viruses have frequently been isolated from cloacal swabs of wild birds indicates the possibility of viral exposure of poultry via faecal droppings. Therefore, these LPAI viruses may circulate in wild bird species that have not been in contact with poultry. Wild bird surveillance is strongly biased towards mallards [8], while these may not be risk species for AI virus introduction into poultry. This is supported by previous genetic analysis that showed no direct relationship between wild bird and poultry viruses [8]. The biased surveillance in wild birds may also explain why H8N4 and H9N2 viruses were less often detected in wild birds. Possibly these viruses predominantly circulate among other understudied wild bird species. An important limitation is that wild bird surveillance is solely based on virological monitoring. In contrast to antibody detection, virus can only be detected during a relatively short time frame. A previous surveillance study in Sweden reported the detection of H8 and H9 viruses during late spring and early summer, outside the prevalence peak of AI viruses in wild birds [40]. In this period, the sampling frequency of wild birds in the Netherlands is low, which may explain the limited detection of H8N4 and H9N2 viruses.

In this study, we also examined host-associations in genes encoding for internal proteins. This analysis revealed that NS allele A – although predominant in both wild birds and poultry – was relatively more frequently detected in poultry compared to wild birds. A similar distribution was observed among online available sequences, in which viruses collected from galliform species carried more often NS allele A compared to viruses collected from anseriform species [41]. The skewed distribution suggests that NS A viruses may have an increased ability to infect poultry. Previous studies have shown that the NS1 protein can influence viral replication across hosts and determines viral fitness and pathogenicity in chickens by evading

the immune response through inhibition of IFN type I production [42-44]. The distinct NS alleles have also been associated with host-specific transmission of AI viruses based on *in vitro* studies using avian and mammalian cells. In mammalian cells, NS A viruses were able to suppress IFN type I production more efficiently compared to NS B viruses [45,46]. In avian cells, NS A viruses replicated more efficiently in chicken and turkey cells, whereas NS B viruses replicated more efficiently in duck cells [47], suggesting that the NS alleles can also influence virus transmission at the wild bird-poultry interface.

Our study shows that LPAI viruses of both NS alleles were able to induce viral shedding and replicate in respiratory and intestinal organs of chickens. This demonstrates that the host range of NS alleles between the avian species is not strict, which is consistent with previous studies showing infection and persistence of both NS alleles A and B viruses in poultry [41,48]. In addition, NS allele A and B showed comparable shedding patterns and routes for most subtypes, except H8N4, of which the NS A variant was shed more efficiently than the NS B variant. In tissue samples, the detection of virus was variable between strains, which appeared to be independent of NS allele. The results thus indicate variable replication efficiencies of NS A and B viruses in chickens.

To study the innate immune response to the different LPAI viruses, we measured changes in the production of inflammatory cytokines in the respiratory and intestinal tract of the virus-inoculated chickens. We measured the levels of mRNA encoding for interleukins IL-6 and IL-1β and interferons IFN-α and IFN-β, which can inhibit viral replication by interaction with viral components and modulation of the host cell metabolism [43], and TLR-7, which is an endosomal pattern recognition receptors that can detect viral RNA at the site of infection to induce an inflammatory response [49]. In the trachea, no stimulation of the innate immune response was observed at 1 dpi, despite virus was detected in all virus-inoculated groups. At 3 dpi, IFN-β mRNAs were induced to higher levels in response to NS A viruses of both wild bird-associated and poultry-associated subtypes. The upregulation of both IFN-β mRNAs expression has previously been observed in LPAI virus-infected chickens [21]. However, the results do not correspond with the expected inhibition of IFN type I production by NS A viruses. In the ileum, no induction of cytokine mRNA expression was observed at 5 dpi. The expression of IFN-α mRNA was even slightly reduced in some groups. At 7 dpi, the expression of IL-1β, IFN-α, IFN-β and TLR7 mRNAs was induced by LPAI viruses of wild bird-associated subtypes carrying NS allele B in the ileum of some, but not all, chickens, indicating that immune responses vary between strains. The induction of inflammatory cytokines in the respiratory and intestinal tract has been reported in previous studies using LPAI viruses of subtypes H7N1 [21] and H9N2 [50], but is limited compared to HPAI viruses [43]. Our study also indicates that LPAI viruses elicit limited innate immune responses in chickens. The cytokine response remained limited to undetectable when only virus-positive samples were included (results not shown). It should be mentioned that the analysis of the cytokine response was limited to few time points and tissues. In addition, the involvement of other (non-studied) cytokines in limiting LPAI virus infection cannot be excluded.

The susceptibility of chickens to the viruses used in this infection experiment did not correlate with the detection frequencies of the HA and NA subtypes or NS alleles in the field. None of the poultry-associated aa residues, as specified in Table S2, were present in the inoculated viruses, except the K391R substitution in the PA protein of H3N8 NS B virus and both H4N6 and H9N2 viruses. In an attempt to explain the unexpected results, we also analysed potential links between the internal genes of the inoculated viruses and previous

isolations of similar genes from poultry hosts. Although the number of poultry viruses in the genetic clusters was often limited, this analysis suggested a potential role for the PB1, PB2, and PA genes in determining infectivity of these virus strains in chickens. Host-restriction factors in the polymerase genes have been described previously in several studies on avian-human transmission of AI viruses [51,52]. Also, previous proteotype analysis has shown specific combinations of the viral proteins among AI viruses of human and avian origin [53], Our study also indicates that a set of proteins may be the major host range determinant. However, the role of the polymerase proteins in transmission between avian hosts is poorly understood. Additional studies should be conducted in order to investigate the contribution of the polymerase activities in determining host range of AI viruses among avian species.

Concluding, this study provides valuable information on the susceptibility of chickens to LPAI viruses of various subtypes and genotypes. It demonstrates that wild bird-associated LPAI viruses of subtypes H3N8 and H4N6 can readily replicate in experimentally infected chickens, despite their infrequent detection in Dutch poultry flocks. The variable susceptibility of chickens to poultry-associated subtypes and NS alleles could not be explained by differences in the innate immune response, which was limited in all chickens. The results in this study increase our understanding of LPAI virus infection dynamics in chickens and can be used to optimize surveillance strategies for LPAI viruses in wild birds and poultry.

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AUTHOR CONTRIBUTIONS

Conceptualization: S.B., R.B., R.F. and N.B.; Methodology: S.B., E.G., R.B., R.F. and N.B.; Validation: S.B., E.G., M.R., M.E. and R.H.; Formal Analysis: S.B., E.G., M.A., M.R., M.E. and R.H.; Investigation: S.B. and M.A.; Resources: R.F. and N.B.; Data Curation: S.B.; Visualization: S.B.; Supervision: N.B.; Project Administration: N.B.; Funding Acquisition: R.F. and N.B.; Writing – Original Draft Preparation: S.B.; Writing – Review & Editing: E.G., M.A., M.R., M.E., R.H., R.B., R.F. and N.B.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Supplementary information

SUPPLEMENTARY TABLES

S1 Table. Viruses used in the infection experiment.
S2 Table. Meta-CATS analysis for virus selection.
S3 Table. Influenza virus-specific antibody detection.
S4 Table. Oropharyngeal shedding.
S5 Table. Cloacal shedding.

S1 TABLE. VIRUSES USED IN THE INFECTION EXPERIMENT.
Low pathogenic avian influenza (LPAI) viruses used in this study, including the isolate identification numbers as listed in GISAID's EpiFlu database (http://www.gisaid.org). The viruses were collected from wild ducks as part of the national avian influenza (AI) virus surveillance program in wild birds in the Netherlands.

Virus subtype	NS allele	Isolate name	GISAID isolate ID
H3N8	A	A/Mallard/Netherlands/17/2011	EPI_ISL_243527
H3N8	B	A/Mallard/Netherlands/30/2010	EPI_ISL_243634
H4N6	A	A/Mallard/Netherlands/7/2011	EPI_ISL_243399
H4N6	B	A/Mallard/Netherlands/26/2010	EPI_ISL_243498
H8N4	A	A/Mallard/Netherlands/1/2006	EPI_ISL_33854
H8N4	B	A/Mallard/Netherlands/11/2006	EPI_ISL_267765
H9N2	A	A/Mallard/Netherlands/19/2005	EPI_ISL_267193
H9N2	B	A/Eurasian wigeon/Netherlands/3/2005	EPI_ISL_64643

S2 TABLE. META-CATS ANALYSIS FOR VIRUS SELECTION.
A statistical comparative analysis was performed on 162 wild bird viruses and 42 poultry viruses using the metadata-driven comparative analysis tool for sequences (meta-CATS) of the Influenza Research Database (IRD) (https://www.fludb.org). In the statistical tool, a chi-square test of independence was performed at each aa position to identify residues that significantly differed between the groups ($p<0.05$). The sequences of the low pathogenic avian influenza (LPAI) viruses used in this study were generated in a previous study (for details see Material and Methods section).

Protein	Total no. of significant aa positions ($p<0.05$)	No. of significant aa positions present in >90% viruses in both groups	No. of significant aa positions with variable substitutions (≥ 3 aa variants)	No. of other significant aa positions (genetic feature found in poultry viruses)
PB2	11	10		1 (V255I)
PB1	10	7	2	1 (K577R)
PA	14	12	1	1 (K391R)
NP	11	9	1	1 (K77R)
M1	1	1		
M2	1	0		1 (K18R)
NS1	67	4		63 (NS allele A)
NS2	20	1		19 (NS allele A)
Total	**135**	**44**	**4**	**5**

PB2, polymerase basic protein 2; PB1, polymerase basic protein 1; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M1, matrix protein 1; M2, matrix protein 2; NS1, nonstructural protein 1; NS2, nonstructural protein 2.

S3 TABLE. INFLUENZA VIRUS-SPECIFIC ANTIBODY DETECTION.

The ratio of antibody-positive chickens to the number of virus-inoculated chickens as determined by influenza-specific ELISA and subtype-specific hemagglutinin inhibition (HI) tests. Serum samples taken at 5 and 7 days post inoculation (dpi) are shown. The HI titres are expressed as the mean log_2 values ± standard deviation (SD).

	ELISA positives		HI test positives (titre) [a]	
Virus group	**5 dpi**	**7 dpi**	**5 dpi**	**7 dpi**
H3N8 NS allele A	0/4	2/4	0/4	1/4 (3)
H3N8 NS allele B	0/4	2/4	0/4	1/4 (3)
H4N6 NS allele A	0/4	1/4	0/4	0/4
H4N6 NS allele B	0/4	4/4	0/4	0/4
H8N4 NS allele A	4/4	3/4	3/4 (4.3±0.6)	2/4 (5.0±0.0)
H8N4 NS allele B	0/4	0/4	0/4	0/4
H9N2 NS allele A	1/4	3/4	0/4	0/4
H9N2 NS allele B	0/4	1/4	0/4	0/4

dpi, days post inoculation; HI, hemagglutination inhibition; NS, nonstructural protein

[a] HI titres of 3 log^2 or higher were considered positive.

S4 TABLE. OROPHARYNGEAL SHEDDING.

The ratio of chickens positive for viral shedding through the oropharyngeal (OP) route to the number of virus-inoculated chickens. The chickens inoculated by the intranasal (IN) and intratracheal (IT) route with eight strains of low pathogenic avian influenza (LPAI) viruses ($10^{5.3}$ median egg infectious dose (EID_{50}) per bird). The swabs were taken daily from live birds to 7 days post inoculation (dpi) for virus detection by influenza virus-specific PCR (M-PCR). Viral titres for positive samples are expressed as the mean equivalent log10 EID_{50}/ml titre ± standard deviation (SD). The onset of viral shedding is reported as mean dpi ± SD.

Virus group	0 dpi	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	Total	Onset of viral shedding (dpi)
H3N8 NS allele A	0/20	9/20 (4.0±1.2)	6/16 (3.7±0.8)	4/12 (2.7±0.4)	3/8 (3.1±0.8)	7/8 (3.0±0.8)	2/4 (2.5±0.1)	1/4 (2.3)	12/20 (3.6±0.9)	1.8±1.4
H3N8 NS allele B	0/20	13/20 (4.7±1.2)	9/16 (4.8±1.1)	4/12 (4.1±1.4)	4/8 (4.2±1.5)	0/8	0/4	0/4	17/20 (4.6±1.2)	1.2±0.5
H4N6 NS allele A	0/20	15/20 (4.8±1.1)	9/16 (3.8±0.9)	0/12	1/8 (2.8)	1/8 (3.0)	1/4 (3.0)	0/4	19/20 (4.3±1.1)	1.5±1.4
H4N6 NS allele B	0/20	15/20 (4.7±1.2)	12/16 (4.9±0.8)	5/12 (4.0±1.2)	4/8 (4.6±0.7)	3/8 (4.2±0.6)	1/4 (5.2)	0/4	16/20 (4.7±1.0)	1.1±0.3
H8N4 NS allele A	0/20	5/20 (2.4±0.9)	0/16	0/12	0/8	1/8 (1.9)	1/4 (1.8)	0/4	7/20 (2.5±0.9)	2.3±2.2
H8N4 NS allele B	0/20	0/20	0/16	0/12	0/8	0/8	0/4	0/4	0/20	n.a.
H9N2 NS allele A	0/20	1/20 (2.7)	1/16 (1.9)	0/12	0/8	0/8	0/4	0/4	4/20 (2.3±0.5)	1.3±0.5
H9N2 NS allele B	0/20	4/20 (2.5±0.4)	1/16 (3.1)	1/12 (2.4)	0/8	0/8	0/4	0/4	6/20 (2.6±0.4)	1.2±0.4

OP, oropharyngeal; dpi, days post inoculation; n.a., not applicable; NS, nonstructural protein

S5 TABLE. CLOACAL SHEDDING.

The ratio of chickens positive for viral shedding through the cloacal (CL) route to the number of virus-inoculated chickens. The chickens inoculated by the intranasal (IN) and intratracheal (IT) route with eight strains of low pathogenic avian influenza (LPAI) viruses ($10^{5.3}$ median egg infectious dose (EID_{50}) per bird). The swabs were taken daily from live birds to 7 days post inoculation (dpi) for virus detection by influenza virus-specific PCR (M-PCR). Viral titres for positive samples are expressed as the mean equivalent log10 EID_{50}/ml titre ± standard deviation (SD). The onset of viral shedding is reported as mean dpi ± SD.

Virus group	0 dpi	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	Total	Onset of viral shedding (dpi)
H3N8 NS allele A	0/20	0/20	1/16 (6.3)	1/12 (6.6)	2/8 (4.9±2.7)	1/8 (6.2)	1/4 (4.8)	2/4 (3.7±1.0)	2/20 (5.1±1.6)	3.0±1.4
H3N8 NS allele B	0/20	0/20	1/16 (7.9)	3/12 (4.7±2.4)	2/8 (6.1±0.3)	2/8 (5.9±0.6)	2/4 (4.7±2.3)	2/4 (6.8±0.4)	7/20 (6.0±1.3)	4.1±1.8
H4N6 NS allele A	0/20	0/20	0/16	0/12	0/8	0/8	0/4	0/4	0/20	n.a.
H4N6 NS allele B	0/20	0/20	0/16	0/12	0/8	0/8	0/4	0/4	0/20	n.a.
H8N4 NS allele A	0/20	0/20	0/16	1/12 (4.9)	2/8 (3.5±1.1)	2/8 (5.5±0.2)	2/4 (4.6±0.3)	2/4 (4.9±0.6)	3/20 (4.7±0.9)	4.0±1.0
H8N4 NS allele B	0/20	0/20	0/16	0/12	0/8	0/8	0/4	0/4	0/20	n.a.
H9N2 NS allele A	0/20	0/20	0/16	0/12	0/8	0/8	0/4	0/4	0/20	n.a.
H9N2 NS allele B	0/20	0/20	0/16	0/12	0/8	0/8	0/4	0/4	0/20	n.a.

CL, cloacal; dpi, days post inoculation; n.a., not applicable; NS, nonstructural protein

CHAPTER 4

Genetic analysis identifies potential transmission of low pathogenic avian influenza (LPAI) viruses between poultry farms

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Abstract

Poultry can become infected with low pathogenic avian influenza (LPAI) viruses via (in)direct contact with infected wild birds, or by transmission of the virus between farms. This study combines routinely collected surveillance data with genetic analysis to assess the contribution of between-farm transmission to the overall incidence of LPAI virus infections in poultry. Over a ten-year surveillance period, we identified 35 potential cases of between-farm transmission in the Netherlands, of which ten formed geographical clusters. A total of 21 LPAI viruses was isolated from nine potential between-farm transmission cases, which were further studied by genetic and epidemiological analysis. Whole genome sequence analysis identified close genetic links between infected farms in seven cases. The presence of identical deletions in the neuraminidase stalk region and minority variants provided additional indications of between-farm transmission. Spatiotemporal analysis demonstrated that genetically closely related viruses were detected within a median time interval of eight days, and the median distance between the infected farms was significantly shorter compared to farms infected with genetically distinct viruses (6.3 vs. 69.0 km; $p<0.05$). The results further suggest that between-farm transmission was not restricted to holdings of the same poultry type and not related to the housing system. Although separate introductions from the wild bird reservoir cannot be excluded, our study indicates that between-farm transmission occurred in seven of nine virologically analysed cases. Based on these findings, it is likely that between-farm transmission contributes considerably to the incidence of LPAI virus infections in poultry.

Keywords: avian influenza virus; low pathogenic avian influenza; poultry; between-farm transmission; genetic analysis

Introduction

Avian influenza (AI) is a highly contagious viral disease that affects birds. AI viruses are widespread in wild waterfowl, that form the natural reservoir of AI viruses [1], and can occasionally be transmitted to commercial poultry. The viruses carry two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which are used for virus classification [2]. In birds, 16 HA (H1-H16) and 9 NA (N1-N9) subtypes have been identified [3,4].

Most AI viruses are low pathogenic avian influenza (LPAI) virus strains that cause subclinical infections in poultry [5]. In some cases, mild respiratory disease, a reduction in egg production or low mortality is observed [6]. AI viruses of subtypes H5 and H7 pose the greatest threat to commercial poultry because of their potential to evolve into highly pathogenic avian influenza (HPAI) viruses. HPAI viruses typically cause severe illness and high mortality in poultry [5], and some subtypes have been shown to also infect humans [7,8]. Hence, surveillance programmes are implemented for the early detection of LPAI and HPAI viruses of subtypes H5 and H7, which are also known as notifiable AI strains. In the Netherlands, poultry farms are screened serologically for AI virus infections at least once a year [9,10]. In addition, virological testing is performed upon notification of AI suspicions based on clinical signs or the detection of antibodies against H5 or H7 subtyped viruses. Non-notifiable LPAI virus infections are often considered to be of lower risk. However, their circulation in poultry may promote the emergence of influenza virus strains that have the ability to be transmitted efficiently among poultry and even humans [11]. Reassortment of these viruses with more pathogenic strains may have serious consequences for both animal and public health.

Although wild birds are considered the primary source of AI virus infections in poultry, flocks may also become infected by subsequent spread between farms. Recent HPAI outbreaks have demonstrated that AI viruses can spread rapidly between farms [12-14], leading to huge economic losses in the poultry industry. Sustained between-farm transmission of LPAI viruses has also been observed in commercial poultry, e.g. during LPAI outbreaks of subtypes H7N2 (1996-1998 and 2001-2002) in the United States [15-18], H7N1 (1999 and 2000-2001), H7N3 (2002-2003 and 2004) and H5N2 (2010-2012) in poultry-dense areas in Italy [19-21], and recurrent outbreaks of H9N2 infections in Asia and the Middle East (late 1990s-present) [20,22].

Various routes of between-farm transmission have been suggested, such as direct contact between poultry or indirect via the movement of persons (e.g. visitors, farm personnel), contaminated materials (e.g. farm equipment, clothing), or vectors (e.g. rodents, insects) between farms [23-27]. Moreover, transmission over short distances may occur when the virus is dispersed into the environment via water, air or dust [28-31]. Geographical clustering of infected farms implies the occurrence of transmission between neighbouring farms or separate introductions from the same environmental source [32,33]. However, the exact route of introduction into poultry often remains untraced and mechanisms underlying between-farm spread of AI viruses are not clearly understood.

Genetic analysis has frequently been used to study the emergence, evolution and between-farm transmission dynamics of HPAI viruses [34-37]. Similar studies for LPAI are limited by the lack of genetic information, in particular for non-notifiable AI strains. LPAI virus infections may remain unnoticed or are not reported because the mild symptoms are thought to be caused by other poultry diseases [38]. Therefore, LPAI viruses are primarily detected during routine serological screening without confirmation by virus detection. For this reason, the

contribution of between-farm transmission to the occurrence of LPAI virus infections in poultry is largely unknown.

This study combines routinely collected surveillance data with genetic analysis to assess the contribution of between-farm transmission to the overall incidence of LPAI virus infections in poultry. We analysed 220 serological and virological detections of LPAI virus infections, that occurred in commercial poultry in the Netherlands between 2006 and 2016, to identify potential between-farm transmission cases. Spatial analysis was performed for each potential between-farm transmission case separately to determine if infected farms clustered geographically. Subsequently, whole genome sequence analysis was performed to determine the genetic relationship between viruses isolated from potential between-farm transmission cases. Genetic analysis was combined with information regarding time, distance and poultry type to identify epidemiological variables associated with between-farm transmission. Better understanding of LPAI virus transmission routes into poultry and between farms is important to control virus spread in an early stage.

Material and methods

ETHICAL STATEMENT

Poultry blood and swab samples were collected as part of the national AI surveillance program in the Netherlands, which is carried out for detecting LPAI virus infections of H5 and H7 subtypes in poultry. Samples were taken by authorized veterinarians and sent to the laboratory for routine diagnosis of AI virus infections. Sampling of poultry was carried out in accordance with Council Directive 2005/94/EC of 20 December 2005 on European Union measures for the control of AI [39] and regulation TRCJZ/2005/1411 of 7 June 2005 concerning the prevention, control and monitoring of infectious animal diseases, zoonoses and transmissible spongiform encephalopathies (TSEs). This study analyses the test results obtained in the surveillance program. No new samples were collected for this study specifically.

STUDY POPULATION

Samples were collected between January 2006 and September 2016. The study population involved 2,379 commercial poultry farms in the Netherlands, consisting of farms holding broiler chickens (46%), layer chickens (42%), chicken breeders (8%), turkeys (2%) and domestic ducks (2%), with 45,000 animals per farm on average, as registered in 2013 with moderate fluctuations over the study period.

SEROLOGICAL MONITORING

For serological monitoring, blood samples were collected from all commercial poultry farms in the Netherlands once a year, except outdoor layer chicken and turkey farms, which were sampled four times a year and each production cycle, respectively. Screening of serum for the presence of influenza-specific antibodies was performed by the Dutch Animal Health Service (GD) using the FlockChek AI MultiS-Screen Ab Test Kit (IDEXX). Samples identified as positive

for influenza-specific antibodies were subsequently tested by the national reference laboratory Wageningen Bioveterinary Research (WBVR) in a H5 and H7 subtype-specific hemagglutination inhibition (HI) test according to the OIE Manual of Standards for Diagnostic Tests and Vaccines [40]. If no antibodies against virus subtypes H5 or H7 were detected, the subtype-specificity of the antibodies was determined using an in-house protein microarray or a bead-based multiplexed immunoassay of HA and NA antigens. Results were confirmed using influenza subtype-specific HI tests, neuraminidase inhibition (NI) tests and NA-specific enzyme-linked immunosorbent assays (ELISAs) [40].

VIROLOGICAL MONITORING

Virological monitoring was performed to check for virus circulation upon detection of antibodies against H5 and H7 subtyped viruses or in case of clinical notification. Tracheal and cloacal swabs were collected by a specialist team of the Netherlands Food and Consumer Product Safety Authority (NVWA). These samples were analysed by WBVR using the real-time reverse transcription polymerase chain reaction method targeting the matrix gene (M-PCR) [41]. Influenza virus-positive samples were subsequently tested in a H5 and H7 subtype-specific PCR [42,43]. The sequence of the HA proteolytic cleavage site was analysed to determine the pathogenicity of the virus [44]. Amplified HA and NA gene fragments were analysed by Sanger sequencing to determine the virus subtype [44,45]. To isolate viruses, swab samples were inoculated into the allantoic cavity specific-pathogen-free (SPF) embryonated chicken eggs (ECEs) [40]. Allantoic fluids positive for hemagglutination were characterized in a HI test using in-house prepared antisera.

SEQUENCING

Whole genome sequences of LPAI viruses were generated by next-generation sequencing (NGS), as described previously [46]. In short, RNA was purified from swab specimen or allantoic fluid using the High Pure Viral RNA Kit (Roche), amplified using universal primers, and sequenced with a minimum sequence coverage of 1,000 reads using the paired-end 200 Illumina MiSeq platform. Consensus sequences were generated in CLC Genomics Workbench (Qiagen) using a reference-based method [46] and submitted to GISAID's EpiFlu database (https://www.gisaid.org) [47] (S1 Table). A recent study identified a limit of 0.5% for reliable detection of minority variants in the influenza virus genome, based on the error-rate of the NGS procedure [48]. In this study, we used a minimum frequency of 2.0% and a minimum coverage of 1,000 reads, to ensure reliable detection of minority variants.

DATA ANALYSIS

Potential between-farm transmission cases were defined as two or more poultry farms testing positive for LPAI virus infection of the same HA/NA subtype within a time interval between two consecutive detections of maximum six months. To identify statistically significant spatial clusters of infected farms, spatial cluster analysis was performed using the free software program SaTScan version 9.6 (http://www.satscan.org) [49] for each potential between-farm transmission case separately. Input data was represented by the background poultry farm

population (variable 0), infected farms (variable 1), and geographical locations of individual farms specified as Cartesian coordinates. The Bernoulli probability model was used to scan for areas with a higher rate of infected farms than would be expected by chance ($p<0.05$). Geographical maps were generated using the statistical software package R version 3.4.0 [50]. Comparison of time intervals between virus detections and distances between infected farms was performed by using the non-parametric Wilcoxon Rank Sum test, with significance defined as $p<0.05$. Genetic analysis was performed by aligning the nucleotide consensus sequences for each gene segment separately in CLC Genomics Workbench (Qiagen). These alignments were used to calculate the nucleotide sequence identities between viruses and identify minority variants at consensus-level variant sites.

Results

IDENTIFICATION OF POTENTIAL BETWEEN-FARM TRANSMISSION CASES

To identify potential between-farm transmission cases, we analysed 220 serological and virological detections of LPAI virus infections that occurred in commercial poultry in the Netherlands between 2006 and 2016, which included 162 seropositive and 58 viropositive farms. Of the virologically confirmed infections, the genome sequence of 42 LPAI viruses was obtained. Over the ten-year surveillance period, we identified 35 potential between-farm transmission cases involving 132 farms, including 111 seropositive and 21 viropositive farms (Fig 1; S2 Table). Potential between-farm transmission cases involved various subtypes, of which some were detected in multiple years, e.g. H7N7 (2006, 2011, 2013 and 2015), H8N4 (2009, 2011, 2012, 2013 and 2015), H6N2 (2013, 2014 and 2015), H6N8 (2011, 2012 and 2013), and H9N2 (2010 and 2015). A total of ten spatial clusters were identified (referred to as clusters A-J) (S3 Table). Cluster radii ranged from 0.1 to 5.9 km, with a median radius of 1.5 km. Seven clusters included 2-3 infected farms (clusters A, C, D, E, H, I and J) and three clusters included 5-7 infected farms (clusters B, F and G). Most clusters were found in poultry-dense areas in the southern (clusters B, C, G, and I) and central (clusters A, F and J) part of the Netherlands, whereas some clusters (clusters D, E and H) were found in areas with a low poultry density. Geographical clustering of infected farms indicates potential transmission of LPAI viruses between neighbouring farms or separate introductions from the same environmental source.

GENETIC ANALYSIS OF POTENTIAL BETWEEN-FARM TRANSMITTED VIRUSES

NGS was performed to analyse potential transmissions between farms genetically. The LPAI virus sequences were obtained from 21 viropositive farms involved in nine potential between-farm transmission cases (S1 Table). In five of these cases, two or more viropositive farms were located within the same spatial cluster (clusters B, C, D, E and G). The collection locations of the 21 LPAI viruses were plotted in a geographical map of the Netherlands (Fig 2A).

After the whole genome sequences were determined, genetic analysis was performed by aligning the nucleotide consensus sequences of potentially between-farm transmitted viruses for each gene segment separately (Fig 2B). Viruses isolated from potential cases H1N5-2007, H10N7-2009, H6N1-2010, H10N9-2012, H5N3-2013 and H6N2-2014 shared nucleotide sequence identities of 99.70-100.00% in all eight gene segments.

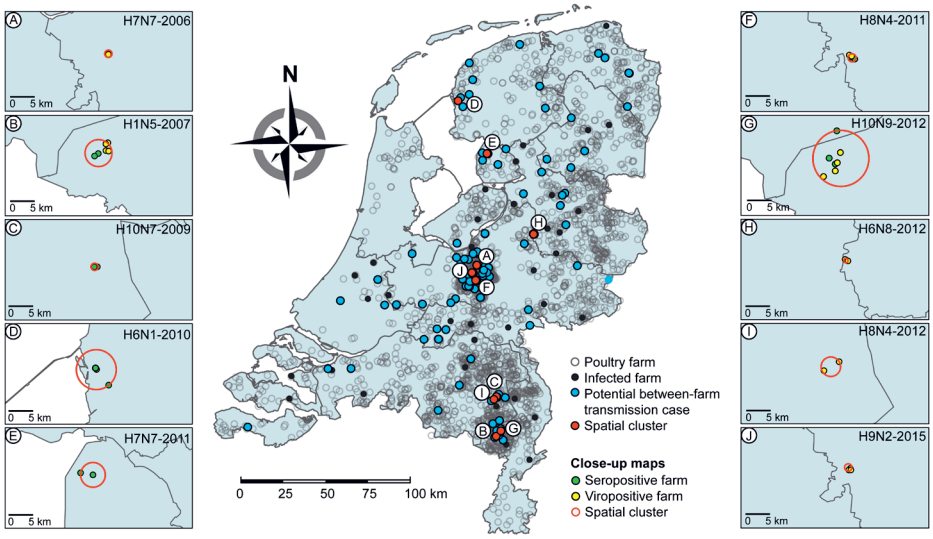

FIGURE 1. GEOGRAPHICAL MAP OF POTENTIAL BETWEEN-FARM TRANSMISSION CASES.
Map of the Netherlands showing the geographical distribution of commercial poultry farms (open dots) (n=2,379), farms infected with low pathogenic avian influenza (LPAI) virus (filled dots) (n=220), farms involved in potential between-farm transmission cases (blue) (n=132), and statistically significant spatial clusters (red) (n=10), including close-up maps of ten spatial clusters of seropositive farms (green) and viropositive farms (yellow) within potential between-farm transmission cases H7N7-2006 (A), H1N5-2007 (B), H10N7-2009 (C), H6N1-2010 (D), H7N7-2011 (E), H8N4-2011 (F), H10N9-2012 (G), H6N8-2012 (H), H8N4-2012 (I) and H9N2-2015 (J). Spatial cluster analysis was performed for each potential between-farm transmission case separately using the Bernoulli probability model ($p<0.05$). All samples were collected as part of the national avian influenza (AI) surveillance program in the Netherlands between January 2006 and September 2016.

Additionally, viruses isolated from potential cases H1N5-2007 and H10N7-2009 both contained a deletion in the stalk region of the NA protein of 18 and 21 amino acids, respectively. Viruses H7N7-2011-2 and H7N7-2011-3 also showed less than 0.3% nucleotide sequence divergence in each gene segment, whereas virus H7N7-2011-1 showed high sequence identities with viruses H7N7-2011-2 and H7N7-2011-3 in gene segments encoding polymerase basic protein 1 (PB1), polymerase acidic protein (PA), HA, NA, matrix protein (MP) and nonstructural protein (NS) (99.44-100.00%), but relatively low sequence identities in gene segments encoding polymerase basic protein 2 (PB2) and nucleoprotein (NP) (93.79-95.85%).

Low sequence identities were found between viruses isolated from potential cases H8N4-2011 and H7N7-2013. H8N4-2011 viruses showed high sequence identities in NP and MP (99.60%), but relatively low sequence identities in PB2, PB1, PA, HA, NA (93.81-98.35%). For NS only 53.84% sequence identity was observed, demonstrating that the viruses have distinct NS alleles. H7N7-2013 viruses shared relatively low sequence identities (<98.70%) in all gene segments, showing that the viruses were only distantly related. Altogether, whole genome sequence analysis identified highly similar viruses, sharing nucleotide sequence identities of 99.70-100.00% in all gene segments, in seven of nine potential cases involving 16

farms. As seven farms can be considered primary infected farms, these results suggest that nine of 21 viropositive farms may have become infected by between-farm virus transmission.

IDENTIFICATION OF MINORITY VARIANTS

NGS was applied to detect minority variants arising from biological variation in the virus population. Minority variants were analysed for all 16 genetically closely related viruses isolated from seven potential between-farm transmission cases using a minimum frequency of 2.0% and a minimum coverage of 1,000 reads (Table 1; S4 Table). The average coverage was 4,500 reads per nucleotide position. Shared minority variants were detected at two nucleotide positions in two potential cases, i.e. at nucleotide position 1017 in the HA gene of viruses H10N7-2009-1 and -2 (frequencies of 3%) and nucleotide position 1202 in the NP gene of viruses H10N9-2012-2 and -3 (frequencies of 7 and 10%, respectively).

More often, minority variants were identified at sites that differed between the consensus sequences of two aligned viruses, with frequencies ranging from 2 to 47%. In potential cases H1N5-2007 and H7N7-2011, minority variants were identified at all sites that

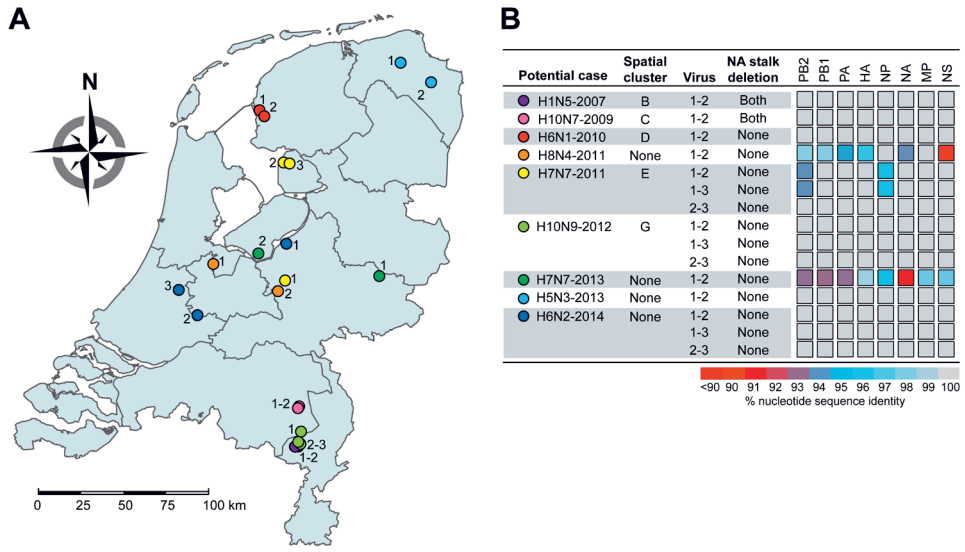

FIGURE 2. GENETIC ANALYSIS OF POTENTIAL BETWEEN-FARM TRANSMISSION CASES.
(A) Geographical map of the Netherlands showing the collection locations of low pathogenic avian influenza (LPAI) viruses isolated from potential between-farm transmission cases (n=21). (B) Genetic analysis of LPAI viruses isolated from potential between-farm transmission cases, showing the presence of deletions in the neuraminidase (NA) stalk region and the percentage of nucleotide sequence identity between viruses for each gene segment separately. All samples were collected as part of the national avian influenza (AI) surveillance program in the Netherlands between January 2006 and September 2016. Detailed information on the virus sequences is provided in S1 Table. PB2, polymerase basic protein 2; PB1, polymerase basic protein 1; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; MP, matrix protein; NS, nonstructural protein.

TABLE 1. Minority variant analysis of genetically closely related low pathogenic avian influenza (LPAI) viruses isolated from potential between-farm transmission cases.

Potential case	Virus alignment	No. of nucleotide differences	No. of shared minority variants	No. of minority variants at consensus-level variant sites
H1N5-2007	1-2	4	0	4
H10N7-2009	1-2	10	1	2
H6N1-2010	1-2	14	0	4
H7N7-2011	2-3	12	0	12
H10N9-2012	1-2	25	0	4
	1-3	25	0	12
	2-3	8	1	6
H5N3-2013	1-2	24	0	2
H6N2-2014	1-2	9	0	4
	1-3	11	0	4
	2-3	6	0	0

Note: The number of nucleotide differences, the number of shared minority variants (i.e. minority variants that are present in both viruses), and the number of minority variants at consensus-level variant sites (i.e. minority variants at sites that varied between viruses at consensus level) are shown. Minority variants were detected using a minimum frequency of 2.0% and a minimum coverage of 1,000 reads. All samples were collected as part of the national avian influenza (AI) surveillance program in the Netherlands between January 2006 and September 2016. Detailed information on the virus sequences is provided in S1 Table.

varied at consensus level, making a transmission event between these farms highly plausible. In addition, a relatively high number of nucleotide variants was found in the viral subpopulation of H10N9-2012-3, which were present in the consensus sequence of virus H10N9-2012-1. In potential case H6N2-2014, all nucleotide variants detected in the viral subpopulation of H6N2-2014-1 were fixed in the consensus sequence of viruses H6N2-2014-2 and -3. The detection of shared minority variants and minority variants at consensus-level variant sites provides additional indications that transmission of LPAI viruses between poultry farms occurred.

EPIDEMIOLOGICAL VARIABLES ASSOCIATED WITH BETWEEN-FARM TRANSMISSION

Genetic analysis was combined with information regarding time, distance and poultry type to identify epidemiological variables associated with between-farm transmission (Table 2). Genetically closely related viruses were isolated within a median time interval of 8 days (range 1-36 days), which was lower but not significantly different from that of the genetically distinct viruses (median time interval of 43 days; range 6-62 days) ($p=0.06$). The median distance between the collection locations of genetically closely related viruses was 6.3 km (range 0.6-68.9 km), which was significantly shorter compared to that of the genetically distinct viruses (median distance of 69.0 km; range 41.3-72.3 km) ($p<0.05$). Genetically closely related viruses isolated from potential cases H1N5-2007, H10N7-2009, H6N1-2010, H7N7-2011 and H10N9-

TABLE 2. Epidemiological information on low pathogenic avian influenza (LPAI) viruses isolated from potential between-farm transmission cases.

Potential between-farm case	Virus alignment	Genetic relationship [a]	Time interval (days)	Distance (km)	Spatial cluster (p<0.05) [b]	No. of infected farms within cluster	Cluster radius (km)	Poultry type
H1N5-2007	1-2	Close	22	0.9	B	6	3.0	Tu-Tu
H10N7-2009	1-2	Close	1	0.6	C	2	0.3	Tu-Oc
H6N1-2010	1-2	Close	28	4.5	D	3	4.4	Ic-Ic
H8N4-2011	1-2	Distant	62	41.3	None	n/a	n/a	Oc-Oc
H7N7-2011	1-2	Distant	42	69.2	E	2	2.7	Oc-Oc
	1-3	Distant	44	68.7				Oc-Oc
	2-3	Close	2	3.4				Oc-Tu
H10N9-2012	1-2	Close	28	6.3	G	7	5.9	Oc-Tu
	1-3	Close	36	6.9				Oc-Tu
	2-3	Close	8	1.3				Tu-Tu
H7N7-2013	1-2	Distant	6	72.3	None	n/a	n/a	Oc-Oc
H5N3-2013	1-2	Close	11	21.3	None	n/a	n/a	Oc-Oc
H6N2-2014	1-2	Close	5	18.5	None	n/a	n/a	Du-Oc
	1-3	Close	8	68.9				Du-Du
	2-3	Close	3	67.1				Oc-Du

Note: The time interval between virus detections, the distance between infected farms, the presence of statistically significant spatial clusters and poultry types are shown. All samples were collected as part of the national avian influenza (AI) surveillance program in the Netherlands between January 2006 and September 2016. Detailed information on the virus sequences is provided in Table S1.

Abbreviations: Du: domestic duck; Ic: indoor layer chicken; n/a: not applicable; Oc: outdoor layer chicken; Tu: turkey.

[a] The genetic relationship was considered 'close' if the viruses share nucleotide sequence identities of ≥ 99.70% in all gene segments. The genetic relationship was considered 'distant' if the viruses share a nucleotide sequence identity of <99.70% in at least one gene segment.

[b] The capital letters refer to close-up maps of spatial clusters presented in Figure 1.

2012 were collected within spatial clusters with distances between farms ranging from 0.6 to 6.9 km, suggesting local spread between farms or independent infections by the same local source. Additionally, seropositive farms were detected within the same spatial cluster in potential cases H1N5-2007, H6N1-2010 and H10N9-2012, indicating more farms were infected with the same virus. Interestingly, in potential cases H1N5-2007 and H10N9-2012, two infected farms within the same spatial cluster shared the same owner. Farms involved in potential cases H5N3-2013 and H6N2-2014 were located at respectively 21.3 and 18.5-68.9 km distance, indicating long-distance spread.

Finally, poultry types involved in potential between-farm transmission cases were examined. All genetically distinct viruses were isolated from outdoor chicken layer farms. The 16 genetically closely related viruses were isolated from six chicken layer farms with outdoor facilities (38%), and ten farms with an indoor housing system, including six turkey farms (38%), two duck farms (13%) and two chicken layer farms (13%). Potential spread within a poultry type was observed between farms infected with viruses H1N5-2007-1 and -2 (turkeys), H6N1-2010-1 and -2 (indoor chickens), H10N9-2012-2 and -3 (turkeys), H5N3-2013-1 and -2 (outdoor chickens), and H6N2-2014-1 and -3 (domestic ducks). Within the spatial clusters of potential cases H1N5-2007 and H6N1-2010, the seropositive farms were of the same poultry type, and no infections were detected in farms holding a different poultry type (S3 Table). In contrast, potential spread between farms holding different poultry types was observed in farms infected with viruses H10N7-2009-1 and -2 (turkeys-outdoor chickens), H7N7-2011-1 and -2 (outdoor chickens-turkeys), H10N9-2012-1 and the two other infected farms (outdoor chicken-turkeys), and H6N2-2014-2 and the two other infected farms (outdoor chickens-domestic ducks). The combined results suggest that between-farm transmission of LPAI viruses was not related to indoor or outdoor housing systems and not restricted to holdings of the same poultry type.

Discussion

This study evaluates the contribution of between-farm transmission to the overall incidence of LPAI virus infections in commercial poultry in the Netherlands. We analysed serological and virological detections of LPAI virus infections to identify potential between-farm transmission events. Subsequently, genetic analysis was combined with spatiotemporal and poultry type information to identify epidemiological variables associated with between-farm transmission. Over a ten-year surveillance period, we identified 35 potential between-farm transmission cases involving 132 of 220 infected poultry farms. We showed that in ten of these cases farms clustered geographically. The number of farms involved in each case was relatively small (2-7) infected farms), as compared to previous LPAI virus outbreaks, including those of subtypes H7N2 (1996-1998 and 2001-2002) in the United States, and H7N1 (1999 and 2000-2001), H7N3 (2002-2003 and 2004) and H5N2 (2010-2012) in Italy, that reported between 24-388 infected farms [20,21]. Some subtypes were detected in multiple years, which may be due to recurrent virus introductions from the wild bird population. However, no related wild bird viruses were detected in the same time frame between 2006 and 2011 [51], or in recent years. Also, none of the viruses were associated with HPAI outbreaks that were reported in the Netherlands in 2014, 2016 and 2017 [46,52,53].

Genetic analysis was performed using the whole genome sequences of 21 LPAI viruses isolated from nine potential cases. This analysis revealed that viruses isolated from potential cases H8N4-2011 and H7N7-2013 were only distantly related. Between-farm transmission could therefore be excluded in these two cases. In addition, virus H7N7-2011-1 showed low sequence identities with viruses H7N7-2011-2 and H7N7-2011-3 in two gene segments, which is presumably due to reassortment of gene segments between co-circulating viruses. Reassortment events are commonly observed in wild birds, the natural host of a vast diversity of AI viruses [54]. Therefore, reassortment likely occurred in the wild bird population and two distinct reassortment variants were subsequently introduced into the poultry facilities separately.

Genetically closely related viruses, showing less than 0.3% nucleotide sequence divergence in each gene segment, were identified in seven of nine virologically analysed cases involving 16 poultry farms. The close genetic relationship between the viruses suggests between-farm transmission or separate introductions from the same environmental source. NA stalk deletions were identified in potential cases H1N5-2007 and H10N7-2009. A deletion in the NA stalk region is considered a marker of virus adaptation to chickens, turkeys and other gallinaceous hosts [55,56], and is rarely detected in wild birds without a link to poultry. NA stalk deletions cause a change in tropism from the intestine to the respiratory tract in chickens [57,58], thereby increasing virus pathogenicity [59]. The length and position of NA stalk deletions is highly variable [56]. The fact that NA stalk deletions of identical length and position were identified strongly indicates that between-farm transmission occurred.

Moreover, shared minority variants or minority variants at consensus-level variant sites were identified in all seven potential between-farm transmission cases of genetically closely related viruses. These minority variants, although often present at low level, suggest that the viruses are genetically more closely related than predicted based on the consensus sequence. Interestingly, genetically closely related viruses isolated from potential cases H1N5-2007 and H7N7-2011 showed minority variants at all consensus-level variant sites. In these two cases, the virus on the secondary infected farm was likely isolated shortly after direct transmission from the primary infected farm or introduction from the same environmental source. In contrast, a relatively low number of minority variants together with a relatively high number of nucleotide differences in potential case H5N3-2013 suggests prolonged within-flock transmission before samples were collected. In some cases, the genetic relationship based on minority variants may be underestimated due to passaging of the virus strains in eggs.

Surprisingly, minority variant analysis indicated that virus H10N9-2012-1 was genetically more closely related to virus H10N9-2012-3 than to virus H10N9-2012-2, despite the larger time interval between the collection dates. At the same time, viruses H10N9-2012-1 and -2 shared two fixed nucleotide variants that were not present in virus H10N9-2012-3. We therefore hypothesize that the virus was transmitted from H10N9-2012-1 to H10N9-2012-2 and -3 via another (seropositive) farm within the same spatial cluster. This hypothesis is supported by the relatively high number of nucleotide differences between virus H10N9-2012-1 and the other two isolates. Minority variant analysis also revealed that viruses H6N2-2014-1 and -2 shared four fixed nucleotide variants that were not present in virus H6N2-2014-3, and viruses H6N2-2014-1 and –3 shared two fixed nucleotide variants that were not present in virus H6N2-2014-2. No minority variants were identified at sites that differed between H6N2-

2014-2 and -3. Based on these results, we assume that virus H6N2-2014-1 acted as a precursor virus for both viruses H6N2-2014-2 and -3.

Contact tracing to study the intensity of movements between farms could reveal potential modes of transmission, but is generally not performed for non-notifiable LPAI viruses. Here, we analysed other epidemiological links between farms, such as the time interval between virus detections, the distance between farms and poultry types, to identify variables that may be associated with between-farm transmission.

Temporal analysis demonstrated that genetically closely related viruses were detected within a median time interval of eight days (range 1-36 days). Previous studies have shown that viral shedding can already be observed from one day after experimental infection in chickens [60,61]. The mean infectious period of individual LPAI virus infected birds was estimated to range between 4-8 days [60,62,63]. However, the duration of the infectious period of an infected flock can take much longer, depending on within-flock transmission dynamics influenced by the virus and flock characteristics, such as poultry type, age of production and the presence of concomitant diseases [64]. At flock level, the infectious period is estimated to range between 1-2 months for chickens [65], and 2-11 months for turkeys [63]. This is much longer compared to HPAI, as most LPAI infections remain subclinical and control measures are not applied for subtypes other than H5 and H7. The time intervals between the potential cases fall within the estimated infectious period of LPAI virus infected flocks, and is therefore consistent with between-farm transmission.

Our study further suggests that both local and long-distance transmissions occurred, and that between-farm transmission was not restricted to holdings of the same poultry type. Additionally, no relation was found between indoor or outdoor housing systems and potential between-farm transmission. However, despite representing only 2% of the total poultry population in the Netherlands, turkeys were involved in a relatively high number of potential between-transmission cases. This may be explained by a higher susceptibility of this species to AI viruses [66]. Interestingly, all genetically distinct viruses were isolated from outdoor chickens, which may become infected more easily through direct or indirect contact with wild birds [67].

Potential local spread within a poultry type was observed between farms infected with viruses H1N5-2007-1 and -2, H6N1-2010-1 and -2, and H10N9-2012-2 and -3. During these events, transmission may have occurred via movement of persons or contaminated equipment between neighbouring farms, which is likely to occur between farms of the same poultry type because of a high probability of shared personnel, equipment and transport services [25-27,68]. This transmission route is supported by the fact that no influenza infections were detected in farms holding a different poultry type in potential cases H1N5-2007 and H6N1-2010, and two infected farms in potential cases H1N5-2007 and H10N9-2012 shared the same owner. Since AI viruses can persist for extended periods in the environment [31,69], transport of contaminated materials is also considered an important route of virus spread over long distances [33]. Between-farm transmission via human-mediated transport was therefore considered the most probable route of transmission for potential long-distance spread within a poultry type, which was observed between farms infected with viruses H5N3-2013-1 and -2, and H6N2-2014-1 and -3.

Alternatively, virus may have been transmitted between neighbouring farms by vectors [23,24], or via airborne transmission when virus particles or virus-contaminated dust

particles are being dispersed into the environment [28,30]. These transmission routes may explain potential local spread between farms holding different poultry types, which was observed between farms infected with viruses H10N7-2009-1 and -2, H7N7-2011-2 and -3, and from H10N9-2012-1 to the other infected farms. During the latter event, virus was detected in air samples up to 60 meters downwind of two infected turkey farms 2-9 days after infection was confirmed [28]. However, detection decreased rapidly with distance. Hence, the probability of between-flock transmission by air decreases with increasing distance [32,70], and will depend heavily on environmental conditions, such as wind [35]. In addition, most potential transmission events occurred between farms with indoor facilities, suggesting airborne transmission is less likely because of mechanical barriers.

In conclusion, our study indicates that between-farm transmission occurred in seven of nine virologically analysed cases. Based on these findings, transmission between poultry farms likely contributes considerably to the incidence of LPAI virus infections in poultry, although separate introductions from the wild bird reservoir cannot be excluded. In this study, genetic analysis was limited to few potential between-farm transmission cases for which virus was isolated. More frequent collection of samples for virological monitoring of non-notifiable LPAI viruses in poultry would be of great value to obtain more knowledge on LPAI virus transmission dynamics. This study highlights the value of genetic analysis to complement serological data and to improve epidemiological investigations on LPAI virus transmissions, which can be used to guide disease control strategies.

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CONFLICTS OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Supplementary information

SUPPLEMENTARY TABLES

S1 Table. Viruses isolated from potential between-farm transmission cases.
S2 Table. Identification of potential between-farm transmission cases.
S3 Table. Spatial clusters within potential between-farm transmission cases.
S4 Table. Identification of minority variants.

S1 TABLE. VIRUSES ISOLATED FROM POTENTIAL BETWEEN-FARM TRANSMISSION CASES.

Detailed information on low pathogenic avian influenza (LPAI) viruses isolated from potential between-farm transmission cases. All viruses were detected as part of the national avian influenza (AI) surveillance program in the Netherlands between January 2006 and September 2016. Whole genome consensus sequences were submitted to the GISAID's EpiFlu database (https://www.gisaid.org).

Potential case	Isolate number and name	Poultry type	No. of animals per farm	Farm culled	Passage details	Collection location (province)	Collection date	GISAID isolate ID
H1N5-2007	1. A/Turkey/Netherlands/07014290/2007	Turkey	n/a	No	E2	Limburg	2007-05-31	EPI_ISL_309820
	2. A/Turkey/Netherlands/07016245/2007	Turkey	19,000	No	E1	Limburg	2007-06-22	EPI_ISL_309821
H10N7-2009	1. A/Turkey/Netherlands/09006938/2009	Turkey	11,500	No	E1	Noord-Brabant	2009-04-14	EPI_ISL_309822
	2. A/Chicken/Netherlands/09006942/2009	Outdoor layer chicken	8,500	No	E1	Noord-Brabant	2009-04-15	EPI_ISL_309798
H6N1-2010	1. A/Chicken/Netherlands/10010413/2010	Indoor layer chicken	25,500	No	E1	Friesland	2010-06-21	EPI_ISL_309801
	2. A/Chicken/Netherlands/10012103/2010	Indoor layer chicken	43,000	No	E1	Friesland	2010-07-19	EPI_ISL_309802
H8N4-2011	1. A/Chicken/Netherlands/11004004/2011	Outdoor layer chicken	n/a	No	E2	Utrecht	2011-03-09	EPI_ISL_309804
	2. A/Chicken/Netherlands/11008325/2011	Outdoor layer chicken	n/a	No	Original	Gelderland	2011-05-10	EPI_ISL_309806
H7N7-2011	1. A/Chicken/Netherlands/11008327/2011	Outdoor layer chicken	9,000	Yes	E2	Gelderland	2011-05-12	EPI_ISL_309929
	2. A/Chicken/Netherlands/11011392/2011	Outdoor layer chicken	47,000	Yes	E2	Flevoland	2011-06-23	EPI_ISL_309808
	3. A/Turkey/Netherlands/11011530/2011	Turkey	7,000	Yes	E2	Flevoland	2011-06-25	EPI_ISL_309823
H10N9-2012	1. A/Chicken/Netherlands/12002495-001-005/2012	Outdoor layer chicken	22,000	No	E1	Noord-Brabant	2012-02-06	EPI_ISL_309809
	2. A/Turkey/Netherlands/12004763-001-004/2012	Turkey	4,000	No	E1	Limburg	2012-03-05	EPI_ISL_309825
	3. A/Turkey/Netherlands/12005615/2012	Turkey	33,000	No	E1	Limburg	2012-03-13	EPI_ISL_309826
H7N7-2013	1. A/Chicken/Netherlands/13003601/2013	Outdoor layer chicken	85,000	Yes	Original	Gelderland	2013-03-12	EPI_ISL_309811
	2. A/Chicken/Netherlands/13003983/2013	Outdoor layer chicken	24,000	Yes	Original	Flevoland	2013-03-18	EPI_ISL_309812
H5N3-2013	1. A/Chicken/Netherlands/13015884/2013	Outdoor layer chicken	10,000	Yes	Original	Groningen	2013-11-29	EPI_ISL_309813
	2. A/Chicken/Netherlands/13016263-031-035/2013	Outdoor layer chicken	12,000	Yes	Original	Groningen	2013-12-10	EPI_ISL_309814
H6N2-2014	1. A/Duck/Netherlands/14015610/2014	Duck	n/a	No	Original	Utrecht	2014-11-17	EPI_ISL_309833
	2. A/Chicken/Netherlands/14016059/2014	Outdoor layer chicken	n/a	No	Original	Zuid-Holland	2014-11-22	EPI_ISL_309930
	3. A/Duck/Netherlands/14016396/2014	Duck	12,000	No	Original	Gelderland	2014-11-25	EPI_ISL_309835

S2 TABLE. IDENTIFICATION OF POTENTIAL BETWEEN-FARM TRANSMISSION CASES.

Identification of potential between-farm transmission cases of low pathogenic avian influenza (LPAI) viruses based on serological and virological surveillance data. Potential between-farm transmission cases were defined as two or more poultry farms testing positive for LPAI virus infection of the same hemagglutinin (HA) and neuraminidase (NA) subtype combination within a time interval between two consecutive detections of maximum six months. All samples were collected as part of the national avian influenza (AI) surveillance program in the Netherlands between January 2006 and September 2016.

Potential case	Collection date first detection	Collection date last detection	Time span (days)	Total no. of infected farms	No. of infected chicken farms	No. of infected turkey farms	No. of infected duck farms	No. of seropositive farms	No. of vipositive farms
H7N7-2006	2006-07-26	2006-08-03	8	2	1			2	
H1N5-2007	2007-06-01	2007-08-21	81	6		6		4	2
H10N7-2009	2009-04-14	2009-04-15	1	2	1	1			2
H8N4-2009	2009-08-10	2010-07-06	330	6	6			6	
H6N1-2010	2010-05-25	2010-07-15	51	3	3			1	2
H7N4-2010	2010-05-14	2010-05-18	4	2	2			2	
H9N2-2010	2010-11-12	2013-03-12	851	12	11			12	
H6N8-2011	2011-01-19	2011-02-28	40	2	2			2	
H7N7-2011	2011-02-04	2011-06-26	142	4	2	2		1	3
H8N4-2011	2011-03-10	2011-10-06	210	9	7		2	7	2
H1N1-2011	2011-05-31	2011-07-12	42	2	1		1	2	
H10N9-2012	2012-02-07	2012-04-09	62	7	2	5		4	3
H2N3-2012	2012-02-20	2012-08-13	175	2	2			2	
H6N8-2012	2012-03-02	2012-05-28	87	4	4			4	
H2N9-2012	2012-04-03	2012-09-25	175	4	4			4	
H8N4-2012	2012-09-20	2013-01-19	121	2		2		2	
H11N9-2012	2012-12-20	2013-02-05	47	3	3			3	
H7N7-2013	2013-03-11	2013-06-13	94	3	3			1	2
H6N2-2013	2013-05-06	2013-06-20	45	2	2			2	
H7N1-2013	2013-05-31	2013-06-17	17	2	2			2	
H5N2-2013	2013-09-09	2014-03-31	203	3	2		1	3	
H8N4-2013	2013-09-23	2014-09-18	360	6	6			6	
H11N9-2013	2013-10-14	2013-10-14	0	2	2			2	
H5N3-2013	2013-11-28	2014-03-31	123	4	4			2	2

Potential case	Collection date first detection	Collection date last detection	Time span (days)	Total no. of infected farms	No. of infected chicken farms	No. of infected turkey farms	No. of infected duck farms	No. of seropositive farms	No. of viropositive farms
H5N3-2013	2013-11-28	2014-03-31	123	4	4			2	2
H6N8-2013	2013-11-29	2014-04-01	123	3	2	1		3	
H9N5-2013	2013-11-11	2014-01-03	53	3	3			3	
H2N7-2014	2014-02-27	2014-09-18	203	3	3			3	
H6N2-2014	2014-03-13	2015-02-23	347	7	4	1	2	4	3
H5N2-2014	2014-11-22	2015-04-02	131	2	2			2	
H2N5-2015	2015-01-05	2015-07-06	182	3	3			3	
H9N2-2015	2015-02-12	2016-03-07	389	8	7		1	8	
H6N2-2015	2015-11-12	2016-03-28	137	2	2			2	
H7N7-2015	2015-03-10	2015-06-22	104	3	3			3	
H8N4-2015	2015-06-23	2015-06-25	2	2	2			2	
H9N7-2016	2016-01-14	2016-03-07	53	2	1			2	
Total				**132**	**104**	**18**	**7**	**111**	**21**

S3 TABLE. SPATIAL CLUSTERS WITHIN POTENTIAL BETWEEN-FARM TRANSMISSION CASES.

Total number of farms and number of infected farms located within spatial clusters per poultry type. Spatial cluster analysis was performed for each potential between-farm transmission case separately using the Bernoulli probability model ($p<0.05$). All samples were collected as part of the national AI surveillance program in the Netherlands between January 2006 and September 2016.

Potential case	Spatial cluster [a]	Cluster radius (km)	Total no. of farms	Total no. of chicken farms	Total no. of turkey farms	Total no. of duck farms	No. of infected farms	No. of infected chicken farms	No. of infected turkey farms	No. of infected duck farms	No. of seropositive farms	No. of vaccinated farms
H7N7-2006	A	0.1	3	3			2	2			2	
H1N5-2007	B	3.0	25	16	9		6		6		4	2
H10N7-2009	C	0.3	4	3	1		2	1	1			2
H6N1-2010	D	4.4	5	4		1	3	3			1	2
H7N7-2011	E	2.7	3	2	1		2	1	1			2
H8N4-2011	F	0.9	10	9		1	5	4		1	4	1
H10N9-2012	G	5.9	90	75	14	1	7	2	5		4	3
H6N8-2012	H	0.5	2	2			2	2			2	
H8N4-2012	I	2.1	5	3	2		2		2		2	
H9N2-2015	J	0.8	8	8			3	3			3	
Total			**155**	**125**	**27**	**3**	**34**	**18**	**15**	**1**	**22**	**12**

[a] The capital letters refer to close-up maps of spatial clusters presented in Fig 1.

S4 TABLE. IDENTIFICATION OF MINORITY VARIANTS.

Minority variant analysis of genetically closely related low pathogenic avian influenza (LPAI) viruses isolated from potential between-farm transmission cases. All nucleotide positions that varied between viruses at consensus level are shown. Minority variants consist of shared minority variants (i.e. minority variants that are present in both viruses) and minority variants at consensus-level variant sites (i.e. minority variants at sites that varied between viruses at consensus level). Minority variants were detected using a minimum frequency of 2.0% and a minimum coverage of 1,000 reads. All viruses were detected as part of the national avian influenza (AI) surveillance program in the Netherlands between January 2006 and September 2016. Detailed information on the virus sequences is provided in S1 Table. PB2, polymerase basic protein 2; PB1, polymerase basic protein 1; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; MP, matrix protein; NS, nonstructural protein.

					Virus 1		Virus 2		Virus 3	
Potential case	**Gene segment**	**Nucleotide position**	**Var. A**	**Var. B**	**Var. A**	**Var. B**	**Var. A**	**Var. B**	**Var. A**	**Var. B**
H1N5-2007	PB2	2049	T	C	100%	0%	15%	85%		
	PA	885	A	C	67%	33%	0%	100%		
	HA	523	G	A	61%	39%	0%	100%		
	NS	521	C	T	65%	35%	0%	100%		
H10N7-2009	PB2	2061	A	G	100%	0%	26%	74%		
	PB2	2065	G	A	100%	0%	0%	100%		
	PB1	472	G	A	100%	0%	0%	100%		
	PB1	1930	G	A	100%	0%	0%	100%		
	PB1	2142	A	G	100%	0%	0%	100%		
	PA	2059	A	C	100%	0%	0%	100%		
	HA	766	C	T	100%	0%	11%	89%		
	HA	1017	T	A	97%	3%	97%	3%		
	NP	337	G	A	100%	0%	0%	100%		
	NS	30	G	A	100%	0%	0%	100%		
	NS	284	A	G	100%	0%	0%	100%		
H6N1-2010	PB2	646	C	A	100%	0%	0%	100%		
	PB2	2187	G	A	100%	0%	0%	100%		
	PB1	202	T	C	100%	0%	0%	100%		
	PA	73	A	G	100%	0%	0%	100%		
	PA	1053	T	C	100%	0%	0%	100%		
	PA	1071	A	T	100%	0%	0%	100%		
	PA	1133	T	G	100%	0%	0%	100%		
	PA	1892	G	T	100%	0%	0%	100%		
	NP	287	C	T	100%	0%	0%	100%		
	NA	284	C	T	100%	0%	2%	98%		
	NA	444	A	C	55%	45%	0%	100%		
	NA	710	G	A	57%	43%	0%	100%		
	NA	1297	G	A	100%	0%	0%	100%		
	NS	214	G	A	100%	0%	4%	96%		
H7N7-2011	PB2	232	A	C	100%	0%	16%	84%		
	PB2	513	C	T	100%	0%	16%	84%		
	PB2	1084	C	T	100%	0%	16%	84%		
	PB2	1359	C	T	100%	0%	16%	84%		
	PB1	1011	T	C	100%	0%	15%	85%		
	PA	497	T	C	73%	27%	10%	90%		
	PA	1842	G	A	100%	0%	15%	85%		

Potential case	Gene segment	Nucleotide position	Var. A	Var. B	Virus 1		Virus 2		Virus 3	
					Var. A	Var. B	Var. A	Var. B	Var. A	Var. B
	HA	445	G	A	100%	0%	32%	68%		
	HA	629	A	G	72%	28%	8%	92%		
	HA	1238	C	A	100%	0%	35%	65%		
	NA	631	T	C	100%	0%	12%	88%		
	NA	926	G	A	100%	0%	12%	88%		
H5N3-2013	PB2	260	G	A	100%	0%	0%	100%		
	PB2	702	A	G	100%	0%	0%	100%		
	PB2	741	A	C	100%	0%	0%	100%		
	PB1	213	C	T	100%	0%	0%	100%		
	PB1	456	A	G	100%	0%	0%	100%		
	PB1	578	A	G	100%	0%	0%	100%		
	PB1	1158	C	T	100%	0%	12%	88%		
	PB1	1611	C	T	100%	0%	0%	100%		
	PB1	1654	A	C	100%	0%	0%	100%		
	PB1	1869	T	C	100%	0%	0%	100%		
	PA	294	T	C	100%	0%	0%	100%		
	PA	1158	G	T	100%	0%	0%	100%		
	HA	460	T	-	100%	0%	0%	100%		
	HA	461	A	-	100%	0%	0%	100%		
	HA	462	T	-	100%	0%	0%	100%		
	HA	464	A	T	100%	0%	0%	100%		
	HA	606	A	G	100%	0%	0%	100%		
	HA	617	A	T	100%	0%	0%	100%		
	HA	859	G	A	100%	0%	0%	100%		
	HA	1077	C	T	100%	0%	0%	100%		
	NP	249	A	G	100%	0%	0%	100%		
	NP	260	A	G	100%	0%	0%	100%		
	NA	1143	A	G	100%	0%	0%	100%		
	MP	932	T	G	95%	5%	0%	100%		
H10N9-2012	PB2	590	A	C	100%	0%	32%	68%	100%	0%
	PB2	1048	C	T	100%	0%	32%	68%	100%	0%
	PB2	1960	G	A	100%	0%	0%	100%	3%	97%
	PB2	2300	T	G	100%	0%	0%	100%	0%	100%
	PB1	318	C	A	100%	0%	100%	0%	4%	96%
	PB1	1383	G	A	100%	0%	0%	100%	7%	93%
	PB1	1908	C	T	100%	0%	0%	100%	6%	94%
	PB1	2218	A	G	100%	0%	0%	100%	0%	100%
	PA	392	T	C	100%	0%	0%	100%	3%	97%
	PA	666	T	C	100%	0%	32%	68%	100%	0%
	PA	1284	A	G	100%	0%	0%	100%	6%	94%
	PA	1355	C	T	100%	0%	100%	0%	6%	94%
	PA	1524	C	T	100%	0%	0%	100%	6%	94%
	PA	1864	A	G	100%	0%	0%	100%	3%	97%
	HA	49	G	A	100%	0%	100%	0%	0%	100%
	HA	544	A	G	100%	0%	30%	70%	100%	0%
	HA	566	C	A	100%	0%	0%	100%	0%	100%
	HA	769	T	C	100%	0%	0%	100%	0%	100%
	HA	1379	T	C	100%	0%	0%	100%	0%	100%
	NP	681	G	A	100%	0%	0%	100%	3%	97%

Potential case	Gene segment	Nucleotide position	Var. A	Var. B	Virus 1		Virus 2		Virus 3	
					Var. A	Var. B	Var. A	Var. B	Var. A	Var. B
	NP	939	T	C	100%	0%	0%	100%	3%	97%
	NP	951	G	A	100%	0%	0%	100%	3%	97%
	NP	1090	A	G	100%	0%	0%	100%	0%	100%
	NP	1202	C	G	100%	0%	93%	7%	90%	10%
	NA	45	T	C	100%	0%	0%	100%	0%	100%
	NA	48	T	C	100%	0%	0%	100%	0%	100%
	NA	1054	G	A	100%	0%	100%	0%	0%	100%
	MP	421	A	G	100%	0%	0%	100%	0%	100%
	NS	209	A	G	100%	0%	0%	100%	0%	100%
	NS	338	G	A	100%	0%	0%	100%	0%	100%
H6N2-2014	PB2	1785	A	C	79%	21%	0%	100%	0%	100%
	PB1	239	G	A	64%	36%	0%	100%	0%	100%
	PB1	239	G	A	64%	36%	0%	100%	0%	100%
	PB1	354	T	C	100%	0%	0%	100%	0%	100%
	PB1	1833	G	A	78%	22%	0%	100%	0%	100%
	PB1	1836	G	A	100%	0%	0%	100%	0%	100%
	PA	318	A	G	100%	0%	100%	0%	0%	100%
	HA	236	A	G	100%	0%	100%	0%	0%	100%
	HA	376	G	T	100%	0%	0%	100%	100%	0%
	NP	46	A	G	53%	47%	0%	100%	0%	100%
	NA	45	T	C	100%	0%	100%	0%	0%	100%
	NA	558	A	G	100%	0%	0%	100%	100%	0%
	MP	604	A	G	100%	0%	0%	100%	0%	100%
	NS	430	A	G	100%	0%	100%	0%	0%	100%

CHAPTER 5

Spread of highly pathogenic avian influenza (HPAI) H5N5 viruses in Europe in 2016–2017 appears related to the timing of reassortment events

5

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Abstract

During the epizootic of highly pathogenic avian influenza (HPAI) H5N8 virus in Europe in 2016–2017, HPAI viruses of subtype H5N5 were also isolated. However, the detection of H5N5 viruses was limited compared to H5N8. In this study, we show that the genetic constellation of a newly isolated H5N5 virus is different from two genotypes previously identified in the Netherlands. The introduction and spread of the three H5N5 genotypes in Europe was studied using spatiotemporal and genetic analysis. This demonstrated that the genotypes were isolated in distinguishable phases of the epizootic, and suggested multiple introductions of H5N5 viruses into Europe followed by local spread. We estimated the timing of the reassortment events, which suggested that the genotypes emerged after the start of autumn migration. This may have prevented large-scale spread of the H5N5 viruses on wild bird breeding sites before introduction into Europe. Experiments in primary chicken and duck cells revealed only minor differences in cytopathogenicity and replication kinetics between H5N5 genotypes and H5N8. These results suggest that the limited spread of HPAI H5N5 viruses is related to the timing of the reassortment events rather than changes in virus pathogenicity or replication kinetics.

Keywords: avian influenza; highly pathogenic avian influenza; genetic analysis; reassortment

Introduction

Highly pathogenic avian influenza (HPAI) H5N1 viruses belonging to the A/Goose/Guangdong /1/1996 (GsGd) lineage were first detected in domestic geese in China in 1996 [1]. The virus caused outbreaks in poultry and humans in Hong Kong in 1997 [2], and was first detected in wild birds in 2002 [3]. The virus was subsequently disseminated intercontinentally from Asia to Europe, Africa and the Middle East by wild migratory birds in 2005 [4]. As descendants of the H5N1 GsGd lineage continued to circulate among poultry and wild birds, the hemagglutinin (HA) gene evolved into numerous phylogenetic clades. In addition, reassortment of H5N1 GsGd lineage viruses with co-circulating low pathogenic avian influenza (LPAI) viruses led to the emergence of new reassortant viruses of various gene constellations and subtype combinations. In 2014, HPAI H5 clade 2.3.4.4 viruses of subtype H5N8 emerged in Asia [5,6], which were divided into phylogenetic groups A and B. From 2014 onwards, several reassortant variants of HPAI H5 clade 2.3.4.4 viruses came to prominence, causing outbreaks of severe disease and high mortality among wild birds and commercial poultry worldwide.

In late 2014, HPAI H5N8 viruses belonging to clade 2.3.4.4 group A were introduced into Europe [7,8] and North America [9,10] by wild migratory birds. Intersubtype reassortment produced HPAI viruses of subtypes H5N1 and H5N2, the latter of which caused a large outbreak in commercial poultry in the United States in 2014-2015 [11]. In 2016, HPAI H5N8 viruses belonging to clade 2.3.4.4 group B re-emerged in Asia. The virus was first detected at the Qinghai Lake in China and the Ubsu-Nur Lake at the border between Russia and Mongolia, in May 2016 [12-14], and subsequently spread to Europe, Africa and the Middle East in the autumn and winter of 2016-2017 [15]. During this epizootic, over 2000 outbreaks in wild birds and poultry were reported in Europe [16]. In 2017, a reassortant virus of subtype H5N6 virus emerged from H5N8 clade 2.3.4.4 group B viruses in Asia [17], infecting wild birds and poultry in several European countries in the autumn and winter of 2017-2018 [18].

During the HPAI H5N8 clade 2.3.4.4 group B epizootic in 2016-2017, multiple reassortant variants of subtype H5N5 were detected in Europe (19-26). However, the number of birds detected with HPAI H5N5 virus was limited compared to H5N8 [19]. After the first detection of HPAI H5N5 virus in the Kamchatka region of Russia in October 2016 [20], a total of 24 outbreaks were reported in 11 European countries [19-26], mainly affecting wild birds. Infections of poultry and captive birds were reported during eight outbreaks [23-25]. Genetic analysis suggested that different H5N5 variants were introduced into Europe [21,23,26]. These H5N5 viruses contained the same HA cleavage site (PLREKRRKR/GLF) as was observed in the majority of the H5N8 isolates [20-22,24], and showed intravenous pathogenicity index (IVPI) scores in chickens comparable to H5N8 [22,24]. However, the limited number of infected birds may suggest that the H5N5 viruses exhibit characteristics different from H5N8, such as decreased infectivity, transmissibility or pathogenicity.

Here, we describe the genetic analysis of a newly isolated HPAI H5N5 virus that is genetically distinct from two H5N5 viruses previously isolated in the Netherlands. The Dutch H5N5 viruses were genetically compared with HPAI H5N5 viruses detected in other European countries, and the timing of reassortment was estimated. This study demonstrates that the three H5N5 genotypes were isolated in overlapping, but distinguishable outbreak phases. Results suggest multiple introductions of H5N5 viruses into Europe followed by local spread. We observed variations in the estimated timing of reassortment that led to the emergence of

the H5N5 genotypes. Experiments in primary chicken and duck cells showed only minor differences in cytopathogenicity and virus replication between H5N5 genotypes and H5N8. These findings suggests that the spread of H5N5 viruses in Europe is mainly driven by the timing of reassortment rather than changes in virus pathogenicity and replication kinetics.

Material and methods

VIRUS DETECTION AND SEQUENCING

Virus detection and sequencing were performed for one newly isolated HPAI H5N5 strain from a goose found dead in Utrecht, the Netherlands, on 22 May 2017 (A/Go/NL-Utrecht/17006881-001/2017; H5N5-19), as described previously [21]. In short, RNA was isolated from a tracheal swab sample using the MagNA Pure 96 system (Roche) with the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche). The sample was tested for the presence influenza A virus by using a real-time reverse transcription polymerase chain reaction targeting the matrix protein (MP) gene (M-PCR) [27], and subsequently tested in a H5 subtype-specific PCR as recommended by the European Union reference laboratory [28,29]. Sanger sequencing was performed to determine the pathogenicity and the neuraminidase (NA) subtype of the virus [30,31]. The whole genome sequence was generated by next-generation sequencing (NGS), as described previously [21]. Briefly, RNA was isolated from the swab sample using the High Pure Viral RNA Kit (Roche). Multi-segment amplification was performed using the SuperScript III One-Step RT-PCR System with the Platinum Taq DNA Polymerase High Fidelity kit (Thermo Fisher Scientific) and purified universal primers [32]. Purified amplicons were sequenced by using the Illumina Nextera DNA Sample Preparation kit and the paired-end 200 Illumina MiSeq platform with a minimum sequence coverage of 1,000 reads. The whole genome consensus sequence was generated by a reference-based method using the ViralProfiler-Workflow, an extension of the CLC Genomics Workbench version 11.0 (Qiagen) [21], and submitted to the GISAID's EpiFlu database (https://www.gisaid.org) [33] (EPI_ISL_288411). The most closely related viruses to the newly isolated H5N5 strain were identified by BLAST on 10 May 2019 (Table S1).

PHYLOGENETIC ANALYSIS

Phylogenetic trees were generated for each gene segment separately using the nucleotide sequences of the newly isolated HPAI H5N5 strain and other HPAI H5N5 viruses isolated during the epizootic in 2016-2017. Detailed information on the HPAI H5N5 virus sequences used in this study is provided in Table S2. We included the top 20 non-H5N5 sequence matches from the GISAID's EpiFlu Database for each H5N5 genotype to assess the origin of the gene segments. As a reference, HPAI H5N8 cluster representatives were included, representing clusters of H5N8 viruses isolated during the epizootic in 2016-2017. To select these cluster representatives, clustering of nucleotide sequences of around 675 HPAI H5N8 2016-2017 viruses available in the GISAID's EpiFlu Database on 10 May 2019 was performed using CD-HIT version 4.6.8 [34,35]. A nucleotide sequence identity threshold value of 1.5% was used to define clusters. The cluster representatives and the number of H5N8 viruses within each cluster are listed in Table S3. After selecting the best fit model of nucleotide substitution,

phylogenetic analysis was performed using the maximum likelihood (ML) method within the MEGA7 software package [36]. Trees were generated using the Tamura-Nei substitution model with a discrete gamma distribution (TN93+G) with 5 rate categories. Bootstrap support values above 70 (1,000 replicates) are shown at the branches.

NETWORK ANALYSIS

Network analysis was performed for viruses belonging to genotypes H5N5-GT2 and H5N5-GT3. For each virus, the full-length nucleotide sequences of the eight gene segments were concatenated and aligned in the software program DNA Alignment (Fluxus Technology) (http://www.fluxus-engineering.com). Sequence gaps were treated by complete deletion and ambiguous states were replaced by searching for the best replacement within the sequence having minimal distance. Phylogenetic networks were reconstructed using the median-joining method in the software program Network version 5 (Fluxus Technology) [37]. Networks were displayed in the software program Network Publisher version 2.1.1.2 (Fluxus Technology). The number of nucleotide substitutions between strains are shown as values near branches.

MOLECULAR CLOCK ANALYSIS

The time to the most recent common ancestor (TMRCA) was estimated for each gene segment of viruses belonging to genotypes H5N5-GT2 and H5N5-GT3. The sequences of HPAI H5N5 2016-2017 viruses were supplemented with HPAI H5N8 and H5N5 reference sequences and the top 100 most similar sequences of both genotypes obtained from the GISAID's EpiFlu Database on 24 January 2018. For gene segments PB1 and NP, we estimated the TMRCA for genotypes H5N5-GT2 and H5N5-GT3 using the top 100 most similar sequences of each genotype separately obtained from the GISAID's EpiFlu Database on 10 May 2019. Multiple sequence alignments were performed with MUSCLE version 3.8.31 [38] and curated in Aliview version 1.20 [39]. For each segment, ML trees were generated using MEGA7 [36] to select for the lineages of interest. Time scaled phylogenies were reconstructed using the Bayesian Markov chain Monte Carlo (MCMC) as implemented in BEAST version 1.8.4 (http://beast.community/beast), as described previously [21]. This analysis was conducted using the SRD06 substitution model [40], the Bayesian Skyline coalescent model, and an uncorrelated log-normal relaxed molecular clock. The Bayesian MCMC analysis was run for 100,000,000 states and the effective sample size (ESS) was checked in Tracer version 1.6 (http://beast.bio.ed.ac.uk/Tracer). Maximum clade credibility (MCC) tree files were summarized in Tree Annotator version 1.8 with a burn-in of 10%. The TMRCA values were obtained from the MCC trees visualized in FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

CELL CULTURES

Madin-Darby Canine Kidney (MDCK) cells were obtained from Philips-Duphar and maintained in complete Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific) supplemented with 5% fetal calf serum (FCS) (Harlan Bioproducts for Science) and 0.1% penicillin-streptomycin (Thermo Fisher Scientific). Primary chicken embryo fibroblast (CEF) and

duck embryo fibroblast (DEF) cells were prepared from 11-day-old specific-pathogen free (SPF) chicken embryos (*Gallus gallus domesticus*) and seronegative 14-day-old commercial Pekin duck embryos (*Anas platyrhynchos domesticus*), respectively, as described previously [41]. Briefly, embryo head, limbs and internal organs were removed and a single-cell suspension was prepared by trypsinization. The primary chicken and duck cells were seeded in growth medium containing 1.0x Medium 199 with Earle's salts (Thermo Fisher Scientific), 3.6% new born calf serum (NBCS) (Thermo Fisher Scientific), 0.12% sodium bicarbonate (Thermo Fisher Scientific), 2mM L-glutamine (Thermo Fisher Scientific), 0.1x MEM Vitamin solution (Thermo Fisher Scientific) and 0.1% gentamicin (Sigma-Aldrich). After one day, growth medium was replaced by maintenance medium containing 0.4x Medium 199 with Earle's salts (Thermo Fisher Scientific), 3.0% new born calf serum (NBCS) (Thermo Fisher Scientific), 0.05x Ham's F-10 Nutrient Mix, 0.11% sodium bicarbonate (Thermo Fisher Scientific), 1mM L-glutamine (Thermo Fisher Scientific), 0.1x M.E.M. Vitamins solutions (Thermo Fisher Scientific), 0.1% gentamicin (Sigma-Aldrich) and 0.12% tryptose phosphate broth. When confluence was reached after 2-3 days, cells were trypsinized and stored in liquid nitrogen for later use.

VIRUS PROPAGATION AND TITRATION

The viruses used for the *in vitro* experiments represent genotypes H5N5-GT1, H5N5-GT2, H5N5-GT3 and European H5N8 NL-Zeewolde-like virus, and are listed in Table S4. The viruses were isolated from swab samples using embryonated chicken eggs (ECEs), as described previously [42]. Virus stocks of second passage allantoic fluids were stored in aliquots at -80°C. The medium tissue culture infective dose (TCID50) titres of the virus stocks were determined by end-point titration in MDCK cells. In short, a total of $2.5x10^4$ MDCK cells/well was seeded in culture medium into each well of a 96-well tissue culture plate. The following day, infection medium was prepared by replacing FCS in culture medium by 0.3% bovine serum albumin (BSA). Cells were inoculated with ten-fold serial dilutions of the virus stocks in infection medium. After two days, an immunoperoxidase monolayer assay (IPMA) was performed using mouse anti-nucleoprotein (anti-NP) HB65 monoclonal antibodies and HRP-conjugated rabbit anti-mouse secondary antibodies (Dako) on monolayers fixed in 4% paraformaldehyde solution [43]. TCID50 titres were calculated using the Reed and Muench method [44].

VIRUS INFECTION OF PRIMARY CHICKEN AND DUCK CELLS

The cytopathogenic effect (CPE) of HPAI H5N5 and H5N8 viruses in primary CEF and DEF cells was measured using the real-time cell analysis (RTCA) system (xCelligence; Roche and ACEA Biosciences) [45]. For this real-time monitoring assay, a total of $3.5x10^5$ cells was seeded in growth medium into each well of the 8-well electronic tissue culture plate (E-plate) (ACEA Biosciences). After one day, cells were inoculated at a multiplicity of infection (MOI) of 0.001 in serum-free growth medium. Mock-infected cells were taken along as negative controls. The electrical impedance of the cell-covered electrodes, displayed as cell index (CI) value, was measured every 30 minutes. An increase in CI value indicates cell proliferation and adhesion, whereas a decrease in CI value indicates cell death. The CI values were normalized at 2 hpi to determine the time point when the CI value reaches half maximal (CI50) value. The experiment

was carried out three times in duplicate.

To generate growth curves of viral replication in CEF cells, a total of $3.5x10^5$ cells was seeded in growth medium into each well of a 24-well tissue culture plate. After one day, cells were inoculated with virus at a MOI of 0.001 in serum-free growth medium. Supernatants were collected at 4-hour intervals from 2 to 42 hpi and stored at -80°C until used for virus titration. TCID50 titres were determined by end-point titration in MDCK cells as described above. The experiment was carried out twice in triplicate. Results were expressed by the mean and its standard deviation (SD).

Results

GENETIC ANALYSIS OF HPAI H5N5 VIRUSES IN THE NETHERLANDS

We determined the whole genome sequence of a novel HPAI H5N5 virus that was isolated from a goose found dead in Utrecht, the Netherlands, in May 2017. To determine the gene constellation of this virus, the most closely related viruses were identified (Table S1), and phylogenetic trees were reconstructed for each gene segment separately (Figure S1). For phylogenetic analysis, we used the full-length nucleotide sequences of all HPAI H5N5 viruses isolated during the HPAI H5 2016–2017 epizootic (Table S2), and sequences of closely related viruses of other subtypes. In addition, HPAI H5N8 cluster representatives were included that represent the genetic diversity among H5N8 viruses during the epizootic (Table S3). The genetic analysis revealed that the newly isolated H5N5 strain was genetically distinct from two H5N5 viruses previously isolated in the Netherlands. This suggests that at least three different HPAI H5N5 reassortant variants circulated in the Netherlands, referred to as genotypes H5N5-GT1, H5N5-GT2 and H5N5-GT3.

The gene constellations of the genotypes are shown in Figure 1, where the gene segments are colored according to their phylogenetic cluster. Gene segments HA, MP and nonstructural protein (NS) of the three H5N5 genotypes clustered with the H5N8-China and Russia-Mongolia reference virus, whereas distinct clusters were identified for gene segments polymerase basic protein 1 (PB1), polymerase basic 2 (PB2), polymerase acidic (PA), NP and NA. The PB2 and NP genes of genotype H5N5-GT1 clustered with European H5N8 NL-Zeewolde-like viruses detected in the Netherlands (H5N8-PA I and H5N8-PA II), whereas the PA and NA genes are related to Eurasian LPAI viruses detected in previous years (Table S5). Genotype H5N5-GT2 contains reassorted PB2, PB1, NP and NA genes related to LPAI viruses detected in Eurasia in previous years. Genotype H5N5-GT3 is genetically highly similar to genotype H5N5-GT2, but contains reassorted PB1 and NP genes that were related to two LPAI viruses detected in the Netherlands in 2014.

INCIDENCE AND SPATIOTEMPORAL DISTRIBUTION OF HPAI H5N5 GENOTYPES IN EUROPE

The viruses that were detected in the Netherlands were genetically compared to HPAI H5N5 viruses detected in other European countries to reveal the incidence of the genotypes (Table 1; Figure S2). Results show that genotype H5N5-GT1 consisted of a unique gene constellation

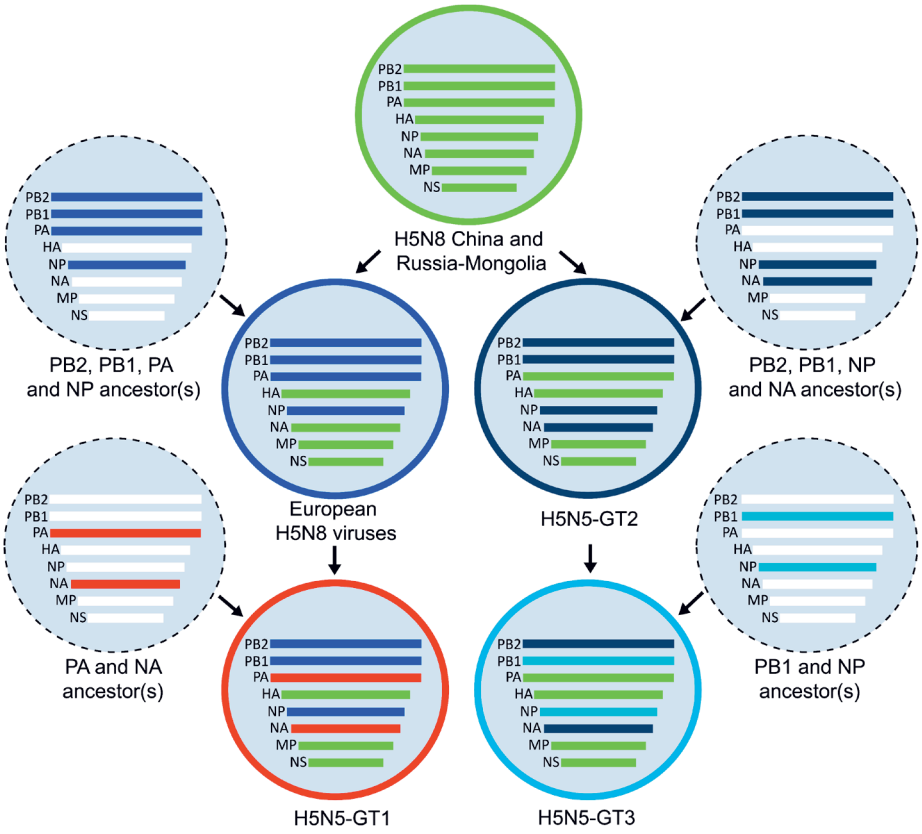

FIGURE 1. GENE CONSTELLATIONS OF HPAI H5N5 GENOTYPES.
Schematic representation of reassortment events that resulted in the emergence of three highly pathogenic avian influenza (HPAI) H5N5 genotypes detected during the HPAI H5 2016-2017 epidemic (H5N5-GT1, H5N5-GT2 and H5N5-GT3). Novel genes were obtained by reassortment of HPAI viruses with co-circulating low pathogenic avian influenza (LPAI) ancestor viruses. Gene segments are colored according to their phylogenetic cluster, as shown in Figure S1. PB2, polymerase basic protein 2; PB1, polymerase basic protein 1; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; MP, matrix protein; NS, nonstructural protein.

that was detected only once (H5N5-1). Genotype H5N5-GT2 viruses were most frequently detected, as 13 viruses were identified in Europe during the epizootic in 2016–2017 (H5N5-3 to -15). This genotype was first identified in Kamchatka, Russia, in October 2016 (H5N5-2). Four viruses (H5N5-16 to -19) were identified for genotype H5N5-GT3. For two H5N5 viruses (H5N5-20 and -21), the genotype could not be determined because of insufficient sequence data. Overall, this analysis demonstrates that the H5N5-GT2 was the most frequently isolated genotype of H5N5 viruses in Europe, followed by H5N5-GT3.

The collection locations of the European HPAI H5N5 viruses were plotted in a geographical map to elucidate the spatial distribution of the genotypes (Figure 2a). Genotype H5N5-GT1 was detected only once in the Netherlands. The collection locations of viruses

TABLE 1. Highly pathogenic avian influenza (HPAI) H5N5 viruses isolated during the HPAI H5 2016–2017 epidemic, ordered by genotype.

H5N5 genotype	H5N5 isolate number	Host	Collection date	Collection location
H5N5-GT1	1	Tufted duck	2016-11-14	Netherlands
H5N5-GT2	2	Environment	2016-10-01	Russia
	3	Swan	2016-12-13	Germany
	4	Mute swan	2016-12-13	Netherlands
	5 [a]	Barnacle goose	2016-12-22	Germany
	6	Greylag goose	2016-12-27	Germany
	7	Mute swan	2016-12-27	Croatia
	8	Eurasian wigeon	2016-12-29	Italy
	9	Gadwall	2017-01-10	Italy
	10	Mute swan	2017-01-20	Croatia
	11	Grey heron	2017-01-22	Germany
	12 [a]	Mute swan	2017-01-31	Poland
	13	Common buzzard	2017-02-06	Germany
	14	Mute swan	2017-02-14	Hungary
	15	Chicken	2017-03-07	Croatia
H5N5-GT3	16	Turkey	2017-01-22	Germany
	17	Cormorant	2017-01-30	Germany
	18	Egret	2017-02-14	Germany
	19	Goose	2017-05-22	Netherlands
Unknown	20 [a]	Mute swan	2017-02-09	Czech Republic
	21 [a]	Spot-billed pelican	2017-02-14	Czech Republic

Note: Detailed information on the virus sequences is provided in Table S2.

[a] Viruses with incomplete genome sequence were excluded for phylogenetic network analysis.

belonging to genotype H5N5-GT3 were restricted to areas in the northern part of Germany and the Netherlands. In contrast, H5N5-GT2 viruses were isolated from six European countries (The Netherlands, Germany, Poland, Italy, Croatia, and Hungary), demonstrating that H5N5-GT2 was geographically the most widespread genotype.

Analysis of the collection dates shows that HPAI H5N5 viruses were detected between October 2016 and May 2017 (Figure 2b). Genotype H5N5-GT1 was isolated in the early phase of the outbreak (in November 2016), H5N5-GT2 during the outbreak peak (between December 2016 and March 2017), and H5N5-GT3 during the outbreak peak and in the late phase of the outbreak (in January, February and May 2017). This shows that the H5N5 genotypes were isolated in overlapping, but distinguishable outbreak phases.

GENETIC RELATIONSHIPS BETWEEN HPAI H5N5 VIRUSES

Phylogenetic network analysis was performed to study genetic relationships between the HPAI H5N5 viruses of the same genotype. For viruses belonging to genotypes H5N5-GT2 and H5N5-GT3, the full-length nucleotide consensus sequences of all eight gene segments were concatenated and median-joining networks were reconstructed. This analysis was not

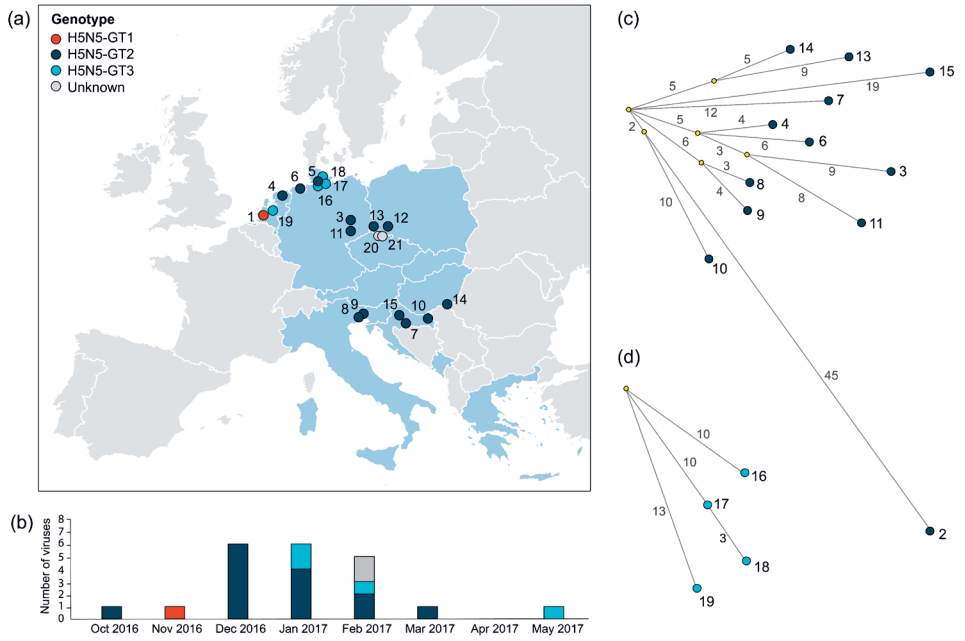

FIGURE 2. SPATIOTEMPORAL DISTRIBUTION AND PHYLOGENETIC NETWORK ANALYSIS OF HPAI H5N5 GENOTYPES. (a) Map of Europe showing the geographical distribution of highly pathogenic avian influenza (HPAI) H5N5 viruses isolated during the HPAI H5 2016–2017 epidemic, with countries reporting HPAI H5N5 virus infection (blue) and the collection locations of HPAI H5N5 viruses, colored by genotype. (b) Number of HPAI H5N5 viruses detected isolated during the HPAI H5 2016–2017 epidemic per month, colored per genotype. (c) Median-joining network analysis of viruses belonging to genotypes H5N5-GT2. (d) Median-joining network analysis of viruses belonging to genotype H5N5-GT3. The number of nucleotide substitutions between strains are shown as values near branches. Detailed information on the virus sequences is provided in Table S2.

performed for genotype H5N5-GT1, as only one virus of this genotype was isolated. In addition, viruses with incomplete genome sequences (H5N5-5, H5N5-12, H5N5-20 and H5N5-21) were excluded.

The median-joining network of genotype H5N5-GT2 viruses shows that the Russian Kamchatka virus (H5N5-2) was genetically relatively more distantly related to the European strains (Figure 2c). The Croatian virus H5N5-10 was genetically most closely related, showing 55 nt differences. The viruses shared two nucleotide variants that were not present in other European strains. The two other viruses isolated in Croatia (H5N5-7 and -15) were more distantly related to the Russian Kamchatka virus (H5N5-2) (59–66 nt differences). Moreover, the three Croatian viruses (H5N5-7, -10 and -15) were genetically relatively distantly related to each other, showing 24–27 nt differences. In contrast, the H5N5 viruses isolated in the Netherlands (H5N5-4) and in Germany (H5N5-3, -6 and -11) were genetically closely related (10–18 nt differences), as they share a predicted common ancestor. The network further revealed clustering of the Italian strains (H5N5-8 and -9), showing 7 nt differences, suggesting local virus circulation.

Phylogenetic network analysis of genotype H5N5-GT3 viruses identified the German isolate H5N5-17 as a direct precursor of virus H5N5-18 (3 nt differences) that was isolated in the same region (Figure 2d). In contrast, the German isolate H5N5-16 and the Dutch isolate H5N5-19 share a predicted common ancestor but are genetically more distantly related (20–23 nt differences). Phylogenetic network analysis shows that genetic relationships between the H5N5 viruses are largely consistent with geographical location, and indicates multiple virus introductions into Europe followed by local spread.

TIMING OF THE HPAI H5N5 REASSORTMENT EVENTS

To estimate the timing of the reassortment events that led to the emergence of the HPAI H5N5 genotypes, we performed molecular clock analysis. This analysis was not performed for genotype H5N5-GT1, which was detected only once. The median TMRCA for the individual gene segments of viruses belonging to genotypes H5N5-GT2 and H5N5-GT3 was estimated in the time-scaled phylogenetic trees (Table 1) (Figure S3). This molecular clock analysis showed that the European H5N5-GT2 viruses share a predicted common ancestor with the Russian Kamchatka virus, with a median TMRCA ranging from November 2015 (NA segment) to August 2016 (PA and MP segments) (nodes 1). These estimations of the TMRCA indicate that genotype H5N5-GT2 emerged in August 2016 (June–September 2016). The European H5N5-GT2 viruses share a predicted common ancestor with a median TMRCA ranging from April 2016 (NA segment) to November 2016 (MP and NS segments) (nodes 2). This analysis indicates that the European H5N5-GT2 viruses share a predicted common ancestor in November 2016 (November–December 2016).

Genotype H5N5-GT3 viruses share a predicted common ancestor with the Russian Kamchatka virus and the European H5N5-GT2 viruses for all gene segments except PB1 and NP, which were genetically most closely related to LPAI viruses detected in previous years. The median TMRCAs for the PB1 and NP gene segments of the H5N5-GT3 viruses were estimated in October 2016 (May 2016–January 2017) and September 2016 (March 2016–December 2016), respectively (nodes 3). These results suggest that the reassortment event that led to the emergence of genotype H5N5-GT3 occurred in the autumn of 2016.

The time-scaled phylogenetic trees further showed that although the HA, MP and NS genes of all three H5N5 genotypes fall in the same cluster of H5N8 China and Russia-Mongolia-like viruses, short phylogenetic distances within the clusters (so-called subclusters) were observed between genotype H5N5-GT1 and European H5N8 NL-Zeewolde-like viruses, and between genotypes H5N5-GT2 and H5N5-GT3. Interestingly, the MP gene of one H5N5-GT2 strain (H5N5-3) forms a phylogenetic subcluster with H5N8 viruses detected in Hungary, Germany and Poland in the same time period. These results suggest that this H5N5-GT2 virus may have obtained a novel MP gene by reassortment with H5N8 virus.

Altogether, molecular clock analysis shows variations in the timing of the reassortment events of the different H5N5 genotypes. The time at which the three genotypes emerged may have caused the differences in incidence and geographical distribution of the H5N5 viruses.

TABLE 2. TMRCA ESTIMATES OF HPAI H5N5 GENOTYPES. Estimated median time to the most recent common ancestor (TMRCA) for each gene segment of highly pathogenic avian influenza (HPAI) H5N5 viruses of genotype H5N5-GT2, the European H5N5-GT2 viruses and H5N5-GT3 viruses with 95% highest posterior density (HPD) intervals. The nodes for which the median TMRCAs estimates were determined are depicted in the time-scaled phylogenetic trees in Figure S3. PB2, polymerase basic protein 2; PB1, polymerase basic protein 1; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; MP, matrix protein; NS, nonstructural protein.

	H5N5-GT2 viruses (node 1)				European H5N5-GT2 viruses (node 2)				H5N5-GT3 viruses (node 3)			
Gene segment	**Median TMRCA**	**Lower 95% HPD**	**Upper 95% HPD**	**Posterior**	**Median TMRCA**	**Lower 95% HPD**	**Upper 95% HPD**	**Posterior**	**Median TMRCA**	**Lower 95% HPD**	**Upper 95% HPD**	**Posterior**
PB2	Mar-2016	Aug-2015	Aug-2016	0.9997	Sep-2016	May-2016	Nov-2016	0.9997	Oct-2016	Aug-2016	Nov-2016	0.4823
PB1	Dec-2015	Apr-2015	Jun-2016	0.9983	Jul-2016	Feb-2016	Oct-2016	0.9982	Oct-2016	May-2016	Jan-2017	0.9971
PA	Aug-2016	Jun-2016	Sep-2016	0.9982	Oct-2016	Sep-2016	Nov-2016	0.9995	Nov-2016	Oct-2016	Dec-2016	0.0942
HA	Jun-2016	Jan-2016	Aug-2016	0.9995	Oct-2016	Oct-2016	Jun-2016	0.9991	Dec-2016	Oct-2016	Jan-2017	0.9991
NP	Mar-2016	Aug-2015	Aug-2016	0.9988	Aug-2016	Apr-2016	Aug-2016	0.9988	Sep-2016	Mar-2016	Dec-2016	0.9991
NA	Nov-2015	Apr-2015	May-2016	0.9997	Apr-2016	Nov-2015	Aug-2016	0.9929	Oct-2016	Jun-2016	Jan-2017	0.9713
MP	Aug-2016	Jun-2016	Sep-2016	0.9929	Nov-2016	Nov-2016	Dec-2016	0.9928	Jan-2017	Dec-2016	Jan-2017	0.0325
NS	Jul-2016	Mar-2016	Sep-2016	0.9992	Nov-2016	Jun-2016	Aug-2016	0.9835	Dec-2016	Nov-2016	Dec-2016	0.0038

CYTOPATHOGENICITY AND REPLICATION OF HPAI H5N5 VIRUSES IN PRIMARY CHICKEN AND DUCK CELLS

We performed real-time cell analysis to determine the cytopathogenicity of HPAI H5N5 and H5N8 viruses in primary cultures of embryonic fibroblasts of chickens (CEF) and ducks (DEF). The cells were inoculated with viruses representing genotypes H5N5-GT1, H5N5-GT2, H5N5-GT3 and European H5N8 NL-Zeewolde-like virus (Table S4), and CPE was monitored by measuring the electrical impedance of the cell monolayer, which is expressed as the CI value. A decrease in CI value was observed between 10–12 hpi in inoculated CEF cells and between 12–14 hpi in inoculated DEF cells, marking the onset of virus-induced cytopathogenicity (Figure 3a and 3b). The CPE observed in the inoculated cultures increased over time, and resulted in complete cell death within 42 hours.

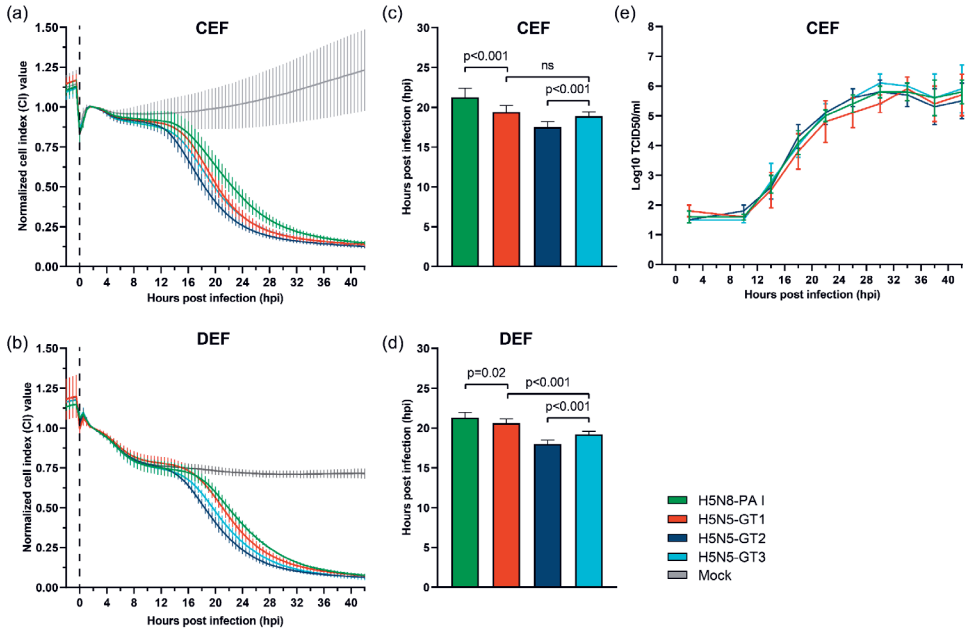

FIGURE 3. CYTOPATHOGENICITY AND REPLICATION OF HPAI H5N5 VIRUSES IN PRIMARY AVIAN CELLS. (a,b) Cytopathogenicity of highly pathogenic avian influenza (HPAI) H5N5 and H5N8 virus in primary chicken embryo fibroblast (CEF) and duck embryo fibroblast (DEF) cells measured by the real-time cell analysis (RTCA) system. The electrical impedance of the cell-covered electrodes was displayed as cell index (CI) value and normalized at two hours post infection (hpi). Virus was inoculated at a multiplicity of infection (MOI) of 0.001. Mock-infected cells were taken along as negative controls (grey). (c,d) The mean time at which the CI value decreased to 50% of the maximum (CI50) value after infection of primary CEF and DEF cells with HPAI H5N5 and H5N8 virus. The p-value was calculated using a two-tailed unpaired Student's t-test with $p<0.05$ considered statistically significant. (e) Growth curves of HPAI H5N5 and H5N8 virus in primary CEF cells. Virus was inoculated at a MOI of 0.001. Samples were taken at four hour intervals from 2 to 42 hpi and titrated to determine the medium tissue culture infective dose (TCID50) titres. Error bars indicate standard deviations (SD).

The time point at which half of the maximal CI value (CI50) was reached was determined to compare the cytopathogenicity of the different viruses. In CEF cells, the CI50 value was reached at 17.5 ± 0.7 hpi for H5N5-GT2 virus, at 18.9 ± 0.5 hpi for H5N5-GT3, and at 19.4 ± 0.8 hpi for H5N5-GT1 (Figure 3c). In DEF cells, cytopathogenicity for H5N5-GT2 was also highest (18.0 ± 0.5 hpi), followed by H5N5-GT3 (19.2 ± 0.4 hpi) and H5N5-GT1 (20.6 ± 0.5 hpi) (Figure 3d). Although the differences in cytopathogenicity between the viruses are small, these results suggest that genotype H5N5-GT2 is significantly more pathogenic to both primary chicken and duck cells compared to the other H5N5 genotypes ($p<0.001$). In addition, H5N5-GT3 appeared more pathogenic to DEF cells than H5N5-GT-1 ($p<0.001$). In contrast, upon inoculation with H5N8 virus, the CI50 value was reached at 21.3 ± 1.1 hpi in CEF cells and 21.3 ± 0.6 hpi in DEF cells, which was significantly slower compared to the H5N5 viruses ($p<0.001$ and $p<0.05$, respectively). This suggests that H5N8 is less pathogenic to primary chicken and duck cells compared to H5N5, although the measured differences are small.

In addition, we studied virus replication in CEF cells to investigate the relationship between cytopathogenicity and replication kinetics. Growth curves were generated by the collection of supernatants at four hour intervals (2–42 hpi), which were titrated to determine the infectious titres. Results show that H5N5 genotypes and H5N8 virus replicate to comparable virus titres in CEF cells (Figure 3e). The results suggest that the reassortment events may have resulted in minor changes in cytopathogenicity, whereas no changes in replication kinetics between H5N5 genotypes and H5N8 virus were observed in primary chicken cells.

Discussion

In 2016-2017, HPAI H5N8 clade 2.3.4.4 group B viruses caused a large-scale epizootic among wild birds and poultry in Europe. Concurrently, related HPAI viruses of subtype H5N5 were detected, although the number of birds detected with H5N5 infection was limited compared to H5N8. Genetic analysis demonstrates that three different genotypes of H5N5 were introduced into Europe. In this study, we analysed the emergence, spread and *in vitro* characteristics of these genotypes.

Viruses of genotype H5N5-GT2 were most frequently isolated and geographically the most widespread in Europe. Phylogenetic analysis demonstrated that the European H5N5-GT2 viruses share a predicted common ancestor with the H5N5 virus isolated in the Kamchatka region of Russia at the beginning of October 2016. Most European H5N5-GT2 viruses were isolated in December 2016 and January 2017. Phylogenetic network analysis demonstrated genetic relationships between H5N5-GT2 viruses largely corresponding with collection locations, as the Russian Kamchatka virus was genetically distinct from the viruses isolated in Europe, and the H5N5 viruses detected in the Netherlands and Germany were genetically distinct from viruses isolated in south-eastern European countries (Poland, Czech Republic, Hungary, Croatia and Italy). The phylogenetic network further revealed a close genetic relationship between the Italian strains. The results therefore indicate multiple introductions of H5N5-GT2 viruses into Europe followed by local spread, similar to H5N8 viruses [46].

Most H5N5-GT2 viruses were detected during the peak of the HPAI H5N8 outbreak in Europe, when high mortality rates among wild birds and multiple outbreaks in commercial poultry were reported [46,47]. Molecular clock analysis indicated that the European H5N5-GT2

viruses share a predicted common ancestor in November 2016, which is in accordance with molecular clock analysis that was performed for Italian strains [22]. The European H5N5-GT2 viruses share a common ancestor with the Russian Kamchatka virus in August 2016, after European H5N8 viruses emerged between May-August [21,22,26]. Similar results were obtained in a previous study on the emergence of H5N5-GT2 [26]. These results suggest that genotype H5N5-GT2 presumably emerged in the summer of 2016 on the breeding grounds of migratory wild birds in the northern part of Russia. The emergence of the virus at the end of the breeding season, just before or after the start of autumn migration, may resulted in limited spread compared to H5N8. The Kamchatka region is located in the Russian Far East, at a large distance from the known breeding sites for migratory wild birds in Russia. Therefore, the virus may have been dispersed from the common breeding areas to both Europe and the Kamchatka region during migration via separate flyways.

Phylogenetic analysis further indicated that, although belonging to genotype H5N5-GT2, one German virus (H5N5-3) obtained a novel MP gene by reassortment with H5N8 virus. This reassortment event resulted in a larger distance to other German H5N5-GT2 viruses (H5N5-6 and -11) and the Dutch H5N5-GT2 virus (H5N5-4). In the phylogenetic network, 6 out of 9 nucleotide differences between H5N5-3 and the predicted common ancestor of H5N5-3 and H5N5-11 were present in the MP gene.

The second most frequently detected HPAI H5N5 genotype is H5N5-GT3. Genetic analysis of the newly isolated H5N5 virus in the Netherlands revealed that this virus also belongs to genotype H5N5-GT3. The virus was isolated in May 2017, after a two-month gap of H5N5 detections in Europe. Phylogenetic analysis with other European H5N5 viruses showed that the virus shares a predicted common ancestor with viruses detected in Germany. Viruses with this gene constellation were solely detected in the northern part of Germany and in the Netherlands, indicating local virus circulation. The H5N5-GT3 viruses show a close genetic relationship with viruses of genotype H5N5-GT2, but contain reassorted PB1 and NP genes. These reassorted genes were genetically most closely related to LPAI viruses detected in the Netherlands in 2014, which may be explained by the intense wild bird surveillance activities in the Netherlands and the lack of recent sequence data. Molecular clock analysis estimated a common ancestor for the reassorted genes in September and October 2016. Possibly, an ancestor virus containing both gene segments has been circulating between 2014-2016, and was involved in the emergence of genotype H5N5-GT3 by reassortment with H5N5-GT2 virus during a single reassortment event. The timing of the reassortment event suggests that this occurred after the start of autumn migration, locally in Europe. No viruses of genotype H5N5-GT3 were detected on the breeding grounds, suggesting that relatively small amounts of wild birds became infected resulting in limited spread in Europe.

Genotype H5N5-GT1 was detected once, and this was the first detection of HPAI H5N5 virus in Europe. This detection was made in the Netherlands in November 2016, concurrently with the first cases of H5N8 in the Netherlands [21], and other European countries [16,48]. As previously reported, this genotype clustered phylogenetically with H5N8 viruses found in the Netherlands [21]. Therefore, H5N5-GT1 virus likely derived from reassortment of HPAI H5N8 and co-circulating LPAI viruses in the PA and NA genes [21,23,24]. Because no viruses with the same gene constellation were detected and recent sequence data on genetically related LPAI viruses was missing, molecular clock analysis could not be used to estimate the timing of the reassortment event giving rise to this genotype. However, the single detection of H5N5-GT1 may suggest that the reassortment event occurred after wild birds migrated from their

breeding grounds, which may have prevented the virus to spread among large populations of birds.

However, changes in virus characteristics, such as infectivity, transmissibility or pathogenicity may have also contributed to the limited spread of HPAI H5H5 viruses compared to H5N8 viruses. In this study, we infected primary chicken and duck cells to examine the cytopathogenicity and replication kinetics of the three H5N5 genotypes and H5N8 virus. For all tested viruses, infection of primary chicken and duck cells resulted in complete cell death within two days, demonstrating high cytopathogenicity. A comparison of the cytopathogenic effects revealed small differences between the three H5N5 genotypes and H5N8 virus in both primary chicken and duck cells. The H5N5 viruses appeared more cytopathogenic than the H5N8 virus, and cytopathogenicity of H5N5-GT2 was somewhat enhanced compared to H5N5-GT1 and H5N5-GT3. However, no changes in replication kinetics between the viruses were observed. The high *in vitro* cytopathogenicity and fast kinetics of virus replication are in accordance with the high IVPI scores that were reported for H5N5 viruses of genotypes H5N5-GT1 (3.00) ([21], unpublished results) and H5N5-GT2 (2.87-3.00) [22,24], which were comparable to H5N8 viruses (2.85-3.00) [13,22,23]. The H5N5 and H5N8 viruses carry the same HA, MP and NS gene segments, suggesting that the viral genetic factors associated with the high pathogenicity are likely present in these genes. The viruses contain the same HA cleavage site, which is the major determinant of the highly pathogenic phenotype. However, other genomic features may also contribute to the pathogenicity of the virus. A previous study identified truncations of the C-terminal of NS1 and the PB1-F2 protein, which are virulence factors associated with host adaptation [26]. These results indicate that the differences in the incidence and distribution between the viruses are no direct result from changes in pathogenicity or replication efficiency.

An important limitation of this study is that we used an *in vitro* system to investigate differences in pathogenicity and replication that will not fully represent the *in vivo* situation. The primary duck cells were obtained from Pekin ducks, a domestic duck breed derived from the mallard, while the viruses studied were isolated from a variety of wild bird species. In addition, pre-existing immunity in the wild bird population due to previous infections with related LPAI viruses may have protected wild birds against HPAI H5N5 infection, thereby influencing the spread of HPAI H5N5 viruses in Europe. In recent years, H5N2 virus descending from H5N8 group A viruses in 2014-2015 in North America [11], and H5N6 virus descending from H5N8 group B viruses in 2017-2018 in Europe [18], have dominated and even replaced co-circulating HPAI strains. However, the emergence of H5N5 virus from H5N8 group B viruses in 2016-2017 resulted in only limited infections. H5N2 viruses isolated during the outbreaks in North America in 2015 exhibited an unusual long pre-clinical period, long mean death time, and high level of viral shedding in turkeys, which may have contributed to the widespread distribution of H5N2 viruses [49]. Although H5N8 and H5N5 viruses both affected various poultry types, the high number of H5N8 outbreaks in poultry compared to H5N5 may have contributed to the increased dissemination of H5N8 viruses in Europe. Further experimental animal studies comparing H5N5 and H5N8 viruses should be performed to obtain insight in the infection dynamics of these viruses.

In conclusion, this study suggests that the limited spread and the differences in geographical distribution of HPAI H5N5 viruses are related to the timing of the reassortment events and introduction into Europe rather than changes in virus pathogenicity or replication kinetics.

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CONFLICTS OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Supplementary information

SUPPLEMENTARY TABLES

S1 Table. Viruses most closely related to the newly isolated H5N5 strain.
S2 Table. Detailed information on H5N5 virus sequences.
S3 Table. List of H5N8 cluster representatives.
S4 Table. Viruses used for in vitro experiments.
S5 Table. LPAI ancestor viruses of reassortant genes.

SUPPLEMENTARY FIGURES

S1 Figure. Phylogenetic analysis of H5N5 viruses.
S2 Figure. Gene constellations of H5N5 viruses.
S3 Figure. Maximum clade credibility trees.

S1 TABLE. VIRUSES MOST CLOSELY RELATED TO THE NEWLY ISOLATED H5N5 STRAIN.
Genetically most closely related viruses to the newly isolated highly pathogenic avian influenza (HPAI) H5N5-GT3 virus strain (A/Go/NL-Utrecht/17006881-001/2017; H5N5-19) as identified for each gene segment by BLAST in the GISAID's EpiFlu database (http://www.gisaid.org) on 10 May 2019. PB2, polymerase basic protein 2; PB1, polymerase basic protein 1; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; MP, matrix protein; NS, nonstructural protein.

Gene segment	Most closely related virus	Subtyp	GISAID isolate ID	Nucleotide sequence identity
PB2	A/M_Swan/NL-Groningen/16015826-001/2016	H5N5	EPI_ISL_287565	2311/2316 (99.8%)
PB1	A/egret/Germany-SH/R1459/2017	H5N5	EPI_ISL_259525	2281/2286 (99.8%)
PA	A/M_Swan/NL-Groningen/16015826-001/2016	H5N5	EPI_ISL_287565	2203/2208 (99.8%)
	A/Mute_swan/Hungary/5879/2017	H5N5	EPI_ISL_256462	
HA	A/M_Swan/NL-Groningen/16015826-001/2016	H5N5	EPI_ISL_287565	1746/1751 (99.7%)
NP	A/cormorant/Germany-SH/R896/2017	H5N5	EPI_ISL_260059	1507/1507 (100.0%)
	A/egret/Germany-SH/R1459/2017	H5N5	EPI_ISL_259525	
NA	A/M_Swan/NL-Groningen/16015826-001/2016	H5N5	EPI_ISL_287565	1434/1441 (99.5%)
	A/Mute_swan/Hungary/5879/2017	H5N5	EPI_ISL_256462	
MP	A/Mute_swan/Hungary/5879/2017	H5N5	EPI_ISL_256462	1002/1002 (100.0%)
NS	A/cormorant/Germany-SH/R896/2017	H5N5	EPI_ISL_260059	864/865 (99.9%)
	A/egret/Germany-SH/R1459/2017	H5N5	EPI_ISL_259525	
	A/Mute_swan/Hungary/5879/2017	H5N5	EPI_ISL_256462	

S2 TABLE. DETAILED INFORMATION ON H5N5 VIRUS SEQUENCES.

Sequences of highly pathogenic avian influenza (HPAI) H5N5 viruses used in this study as listed in the GISAID's EpiFlu database. We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu database on which this research is based. All submitters of data may be contacted directly via the GISAID website (http://www.gisaid.org).

Short isolate name	Full isolate name	Bird species	Collection date	Collection location	Submitting laboratory	GISAID isolate ID
H5N5-1	A/T_Dk/NL-Werkendam/16014159-001/2016	Tufted duck	2016-11-14	Netherlands	Wageningen Bioveterinary Research	EPI_ISL_287564
H5N5-2	A/Environment/Kamchatka/18/2016	Environment	2016-10-01	Russia	State Research Center of Virology and Biotechnology VECTOR	EPI_ISL_256301
H5N5-3	A/Swan/Germany-SN/R10645/2016	Swan	2016-12-13	Germany	Friedrich-Loeffler-Institut	EPI_ISL_240893
H5N5-4	A/M_Swan/NL-Groningen/16015826-001/2016	Mute swan	2016-12-13	Netherlands	Wageningen Bioveterinary Research	EPI_ISL_287565
H5N5-5	A/Barnacle goose/Germany-SH/R11505/2016	Barnacle goose	2016-12-22	Germany	Friedrich-Loeffler-Institut	EPI_ISL_243050
H5N5-6	A/Greylag goose/Germany-NIAR11353-L02142/2016	Greylag goose	2016-12-27	Germany	Friedrich-Loeffler-Institut	EPI_ISL_262056
H5N5-7	A/Mute swan/Croatia/102/2016	Mute swan	2016-12-27	Croatia	Croatian Veterinary Institute	EPI_ISL_240101
H5N5-8	A/Wigeon/Italy/16VIR9616-3/2016	Wigeon	2016-12-29	Italy	Istituto Zooprofilattico Sperimentale Delle Venezie	EPI_ISL_243085
H5N5-9	A/Gadwall/Italy/17VIR133-2/2017	Gadwall	2017-01-10	Italy	Istituto Zooprofilattico Sperimentale Delle Venezie	EPI_ISL_255189
H5N5-10	A/Mute swan/Croatia/42/2017	Mute swan	2017-01-20	Croatia	Croatian Veterinary Institute	EPI_ISL_243698
H5N5-11	A/Grey heron/Germany-SN/R572/2017	Grey heron	2017-01-22	Germany	Friedrich-Loeffler-Institut	EPI_ISL_260058
H5N5-12	A/Mute swan/Poland/64/2017	Mute swan	2017-01-31	Poland	National Veterinary Research Institut Poland	EPI_ISL_255917
H5N5-13	A/Common buzzard/Germany-SN/R1117/2017	Common buzzard	2017-02-06	Germany	Friedrich-Loeffler-Institut	EPI_ISL_259074
H5N5-14	A/Mute swan/Hungary/5879/2017	Mute swan	2017-02-14	Hungary	Central Agricultural Office Veterinary Diagnostic Directorate	EPI_ISL_256462
H5N5-15	A/Chicken/Croatia/104/2017	Chicken	2017-03-07	Croatia	Croatian Veterinary Institute	EPI_ISL_261332
H5N5-16	A/Turkey/Germany-SH/R425/2017	Turkey	2017-01-22	Germany	Friedrich-Loeffler-Institut	EPI_ISL_243049
H5N5-17	A/Cormorant/Germany-SH/R896/2017	Cormorant	2017-01-30	Germany	Friedrich-Loeffler-Institut	EPI_ISL_260059
H5N5-18	A/Egret/Germany-SH/R1459/2017	Egret	2017-02-14	Germany	Friedrich-Loeffler-Institut	EPI_ISL_259525
H5N5-19	A/Go/NL-Utrecht/17006881-001/2017	Goose	2017-05-22	Netherlands	Wageningen Bioveterinary Research	EPI_ISL_288411
H5N5-20	A/Mute swan/Czech Republic/2031-17/2017	Mute swan	2017-02-09	Czech Republic	State Veterinary Institute Prague	EPI_ISL_250920
H5N5-21	A/Spot-billed pelican/Czech Republic/2270-17/2017	Spot-billed pelican	2017-02-14	Czech Republic	State Veterinary Institute Prague	EPI_ISL_268981

S3 TABLE. LIST OF H5N8 CLUSTER REPRESENTATIVES.

Sequences of highly pathogenic avian influenza (HPAI) H5N8 viruses used as cluster representatives in this study as listed in the GISAID's EpiFlu™ Database. The cluster representatives were selected by clustering the nucleotide sequences of around 675 HPAI H5N8 2016-2017 viruses available in the GISAID's EpiFlu Database on 10 May 2019. A nucleotide sequence identity threshold value of 1.5% was used to define clusters. We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu™ Database on which this research is based. All submitters of data may be contacted directly via the GISAID website (http://www.gisaid.org). PB2, polymerase basic protein 2; PB1, polymerase basic protein 1; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleoprotein; MP, matrix protein; NS, nonstructural protein.

Gene segment	Cluster representative	GISAID isolate ID	No. sequences in cluster
PB1	A/Brown-headed_Gull/Qinghai/ZTO1-B/2016	EPI_ISL_224742	436
	A/Goose/Hungary/65817/2016	EPI_ISL_255174	18
PB2	A/Brown-headed_Gull/Qinghai/ZTO1-B/2016	EPI_ISL_224742	97
	A/T_Dk/NL-Zeewolde/16013976-001/2016	EPI_ISL_268674	261
	A/mute_swan/Hungary/51049/2016	EPI_ISL_237730	103
PA	A/Brown-headed_Gull/Qinghai/ZTO1-B/2016	EPI_ISL_224742	174
	A/T_Dk/NL-Monnickendam/16013865-006-008/2016	EPI_ISL_268669	15
	A/T_Dk/NL-Zeewolde/16013976-001/2016	EPI_ISL_268674	241
	A/domestic_duck/Siberia/50K/2016	EPI_ISL_240678	62
	A/chicken/Taiwan/x37/2016	EPI_ISL_256398	2
NP	A/Brown-headed_Gull/Qinghai/ZTO1-B/2016	EPI_ISL_224742	62
	A/T_Dk/NL-Zeewolde/16013976-001/2016	EPI_ISL_268674	260
	A/duck/India/10CA01/2016	EPI_ISL_237553	1
	A/domestic_duck/Siberia/50K/2016	EPI_ISL_240678	69
	A/mute_swan/Hungary/51049/2016	EPI_ISL_237730	101
	A/chicken/Astrakhan/3131/2016	EPI_ISL_240110	1
HA	A/Brown-headed_Gull/Qinghai/ZTO1-B/2016	EPI_ISL_224742	675
MP	A/Brown-headed_Gull/Qinghai/ZTO1-B/2016	EPI_ISL_224742	490
NS	A/Brown-headed_Gull/Qinghai/ZTO1-B/2016	EPI_ISL_224742	480

S4 TABLE. VIRUSES USED FOR IN VITRO EXPERIMENTS.

List of highly pathogenic avian influenza (HPAI) H5N8 and H5N5 viruses used for *in vitro* experiments, representing genotypes H5N5-GT1, H5N5-GT2, H5N5-GT3 and European H5N8 NL-Zeewolde-like virus. The GISAID isolate ID numbers refer to the whole genome sequences that were submitted to the GISAID's EpiFlu database (http://www.gisaid.org)

Genotype	Isolate name	Bird species	Collection date	Collection location	GISAID isolate ID
H5N8	A/Dk/NL-Biddinghuizen/16014829-011-015/2016	Domestic duck	2016-11-25	Biddinghuizen, The Netherlands	EPI_ISL_268629
H5N5-GT1	A/T_Dk/NL-Werkendam/16014159-001/2016	Tufted duck	2016-11-14	Werkendam, The Netherlands	EPI_ISL_287564
H5N5-GT2	A/M_Swan/NL-Groningen/16015826-001/2016	Mute swan	2016-12-13	Groningen, The Netherlands	EPI_ISL_287565
H5N5-GT3	A/Go/NL-Utrecht/17006881-001/2017	Goose	2017-05-22	Utrecht, The Netherlands	EPI_ISL_288411

S5 TABLE. LPAI ANCESTOR VIRUSES OF REASSORTANT GENES.

Novel genes were obtained by reassortment of the (HPAI) H5N5 genotypes detected during the HPAI H5 2016-2017 epizootic (H5N5-GT1, H5N5-GT2 and H5N5-GT3) with co-circulating low pathogenic avian influenza (LPAI) ancestor viruses. The most closely related LPAI ancestor viruses were identified by BLAST in the GISAID's EpiFlu database (http://www.gisaid.org) on 10 May 2019. PB2, polymerase basic protein 2; PB1, polymerase basic protein 1; NP, nucleoprotein; NA, neuraminidase.

Geno-type	Gene	Most closely related virus	Sub-type	GISAID isolate ID	Nucleotide sequence identity
H5N5-GT1	PA	A/duck/Mongolia/17/2011	H4N3	EPI_ISL_294001	2174/2209 (98%)
	NA	A/Mallard duck/Sweden/139899/2012	H15N5	EPI_ISL_267231	1410/1442 (97%)
H5N5-GT2	PB2	A/Barnacle goose/Netherlands/2/2014	H3N6	EPI_ISL_267243	2294/2316 (99%)
	PB1	A/Duck/Mongolia/709/2015	H10N7	EPI_ISL_206696	2288/2316 (98%)
	NP	A/Mallard duck/Netherlands/7/2014	H6N2	EPI_ISL_243620	1519/1540 (98%)
	NA	A/duck/Kyoto/261007/2014	H6N5	EPI_ISL_237150	1418/1439 (98%)
H5N5-GT3	PB1	A/mallard duck/Netherlands/9/2014	H6N2	EPI_ISL_267352	2279/2316 (98%)
	NP	/Barnacle goose/Netherlands/2/2014	H3N6	EPI_ISL_267243	1525/1540 (99%)

S1 FIGURE. PHYLOGENETIC ANALYSIS OF H5N5 VIRUSES.

Phylogenetic trees of individual gene segments of highly pathogenic avian influenza (HPAI) H5N5 viruses isolated during the HPAI H5 2016-2017 epizootic, including the top 20 sequence matches of other subtypes identified by BLAST in the GISAID's EpiFlu database (http://www.gisaid.org) on 10 May 2019. The H5N5 virus sequences are coloured according to their genotype (H5N5-GT1, red; H5N5-GT2, dark blue; H5N5-GT3, light blue). As a reference, HPAI H5N8 cluster representatives were included (green). Detailed information on the HPAI H5N5 and H5N8 virus sequences is provided in Table S2 and Table S3, respectively. The most closely related low pathogenic avian influenza (LPAI) ancestor viruses of the reassortant genes, as listed in Table S5, are depicted in bold. Phylogenetic analysis was performed using the maximum likelihood (ML) method within the MEGA7 software package. PB2, polymerase basic 2; PB1, polymerase basic 1; PA, polymerase acidic; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; MP, matrix protein; NS, nonstructural protein.


PB2
H5N5-GT1
H5N5-GT2
H5N5-GT3
H5N8 cluster representative
A/wigeon/Italy/16VIR9616-3/2016/EPI_ISL_243085/PB2/H5N5/2016-12-29
A/gadwall/Italy/17VIR133-2/2017/EPI_ISL_255189/PB2/H5N5/2017-01-10
A/Go/NL-Utrecht/17006881-001/2017/EPI_ISL_288411/PB2/H5N5/2017-05-22
A/grey_heron/Germany-SN/R572/2017/EPI_ISL_260058/PB2/H5N5/2017-01-22
A/M_Swan/NL-Groningen/16015826-001/2016/EPI_ISL_287565/PB2/H5N5/2016-12-13
A/egret/Germany-SH/R1459/2017/EPI_ISL_259525/PB2/H5N5/2017-02-14
A/greylag_goose/Germany-NI/AR11353-L02142/2016/EPI_ISL_262056/PB2/H5N5/2016-12-27
A/chicken/Croatia/104/2017/EPI_ISL_261332/PB2/H5N5/2017-03-07
A/mute_swan/Croatia/102/2016/EPI_ISL_240101/PB2/H5N5/2016-12-27
A/mute_swan/Poland/64/2017/EPI_ISL_255917/PB2/H5N5/2017-01-31
A/Mute_swan/Hungary/5879/2017/EPI_ISL_256462/PB2/H5N5/2017-02-14
A/common_buzzard/Germany-SN/R1117/2017/EPI_ISL_259074/PB2/H5N5/2017-02-06
A/cormorant/Germany-SH/R896/2017/EPI_ISL_260059/PB2/H5N5/2017-01-30
A/swan/Germany-SN/R10645/2016/EPI_ISL_240893/PB2/H5N5/2016-12-13
A/mute_swan/Croatia/42/2017/EPI_ISL_243698/PB2/H5N5/2017-01-20
A/environment/Kamchatka/18/2016/EPI_ISL_256301/PB2/H5N5/2016-10-01
A/barnacle_goose/Netherlands/2/2014/EPI_ISL_267243/PB2/H3N6/2014-12-15
A/spot-billed_duck/Korea/WA260/2017/EPI_ISL_309221/PB2/H7N7/2017-02-22
A/mallard/Korea/WA245/2017/EPI_ISL_309220/PB2/H7N7/2017-02-22
A/mandarin_duck/Korea/H987-3/2017/EPI_ISL_309222/PB2/H7N7/2017-03-07
A/mallard/Korea/H872-1/2017/EPI_ISL_309212/PB2/H7N7/2017-01-17
A/spot-billed_duck/Korea/H905-2/2017/EPI_ISL_309216/PB2/H7N7/2017-02-08
A/mandarin_duck/Korea/H890-3/2017/EPI_ISL_309214/PB2/H7N7/2017-01-24
A/mallard/Korea/H901-1/2017/EPI_ISL_309215/PB2/H7N7/2017-01-24
A/mallard/Korea/H673-4/2016/EPI_ISL_309209/PB2/H7N7/2016-12-28
A/mallard/Korea/H840-2/2017/EPI_ISL_309210/PB2/H7N7/2017-01-10
A/mandarin_duck/Korea/H539-3/2016/EPI_ISL_309211/PB2/H7N7/2016-12-20
A/mallard/Korea/H906-3/2017/EPI_ISL_309225/PB2/H7N7/2017-02-08
A/mallard/Korea/H1055-2/2017/EPI_ISL_309226/PB2/H7N7/2017-03-15
A/mallard/Korea/H915/2017/EPI_ISL_309217/PB2/H7N7/2017-02-14
A/green-winged_teal/Korea/H873-5/2017/EPI_ISL_309213/PB2/H7N7/2017-01-17
A/common_teal/Korea/H937-5/2017/EPI_ISL_309219/PB2/H7N7/2017-02-23
A/gadwall/Chany/893/2018/EPI_ISL_333615/PB2/H3N8/2018-10-19
A/mallard_duck/Netherlands/11/2015/EPI_ISL_328964/PB2/A/2015-06-05
A/common_teal/Netherlands/1/2015/EPI_ISL_328977/PB2/H11N1/2015-09-29
A/duck/Mongolia/62/2013/EPI_ISL_298543/PB2/H3N1/2013-09-01
A/mute_swan/Hungary/51049/2016/EPI_ISL_237730/PB2/A_/_H5N8/2016-10-19
A/Brown-headed_Gull/Qinghai/ZTO1-B/2016/EPI_ISL_224742/PB2/A_/_H5N8/2016-05-01
A/T_Dk/NL-Zeewolde/16013976-001/2016/EPI_ISL_268674/PB2/A_/_H5N8/2016-11-09
A/T_Dk/NL-Zeewolde/16013976-005/2016/EPI_ISL_268678/PB2/H5N8/2016-11-09
A/T_Dk/NL-Zeewolde/16013976-004-006/2016/EPI_ISL_268677/PB2/H5N8/2016-11-09
A/T_Dk/NL-Roggebotsluis/16014462-015/2016/EPI_ISL_268670/PB2/H5N8/2016-11-17
A/T_Dk/NL-Monnickendam/16013865-006-008/2016/EPI_ISL_268669/PB2/H5N8/2016-11-08
A/P falcon/NL-Vrouwenpolder (Zeeland)/16015510-001/2016/EPI ISL 268666/PB2/H5N8/2016-12-07
A/Mal/NL-Mastenbroek/16015378-002/2016/EPI_ISL_268665/PB2/H5N8/2016-12-05
A/Magpie/NL-Volendam/16014331-002/2016/EPI_ISL_268663/PB2/H5N8/2016-11-16
A/L-bl-ba-gull/NL-Sovon/16014324-014/2016/EPI_ISL_268661/PB2/H5N8/2016-11-16
A/Gull10/NL-Marker_Wadden/16014466-014/2016/EPI_ISL_268660/PB2/H5N8/2016-11-17
A/Gull/NL-Marker_Wadden/16014466-020/2016/EPI_ISL_268658/PB2/H5N8/2016-11-17
A/Gr_bk_bd_gull/NL-Slootdorp/16014102-005/2016/EPI_ISL_268656/PB2/H5N8/2016-11-11
A/Dk/NL-Biddinghuizen/16015145-021-025/2016/EPI_ISL_268631/PB2/H5N8/2016-12-01
A/Dk/NL-Biddinghuizen/16015083-016-020/2016/EPI_ISL_268630/PB2/H5N8/2016-11-30
A/Dk/NL-Biddinghuizen/16014829-011-015/2016/EPI_ISL_268629/PB2/H5N8/2016-11-25
A/Ch/NL-Den_Oever/16014231-001/2016/EPI_ISL_268624/PB2/H5N8/2016-11-15
A/C_Gull/NL-Slootdorp/16014102-003/2016/EPI_ISL_268621/PB2/H5N8/2016-11-11
A/Buzzard/NL-Durgerdam/16015100-004/2016/EPI_ISL_268620/PB2/H5N8/2016-11-30
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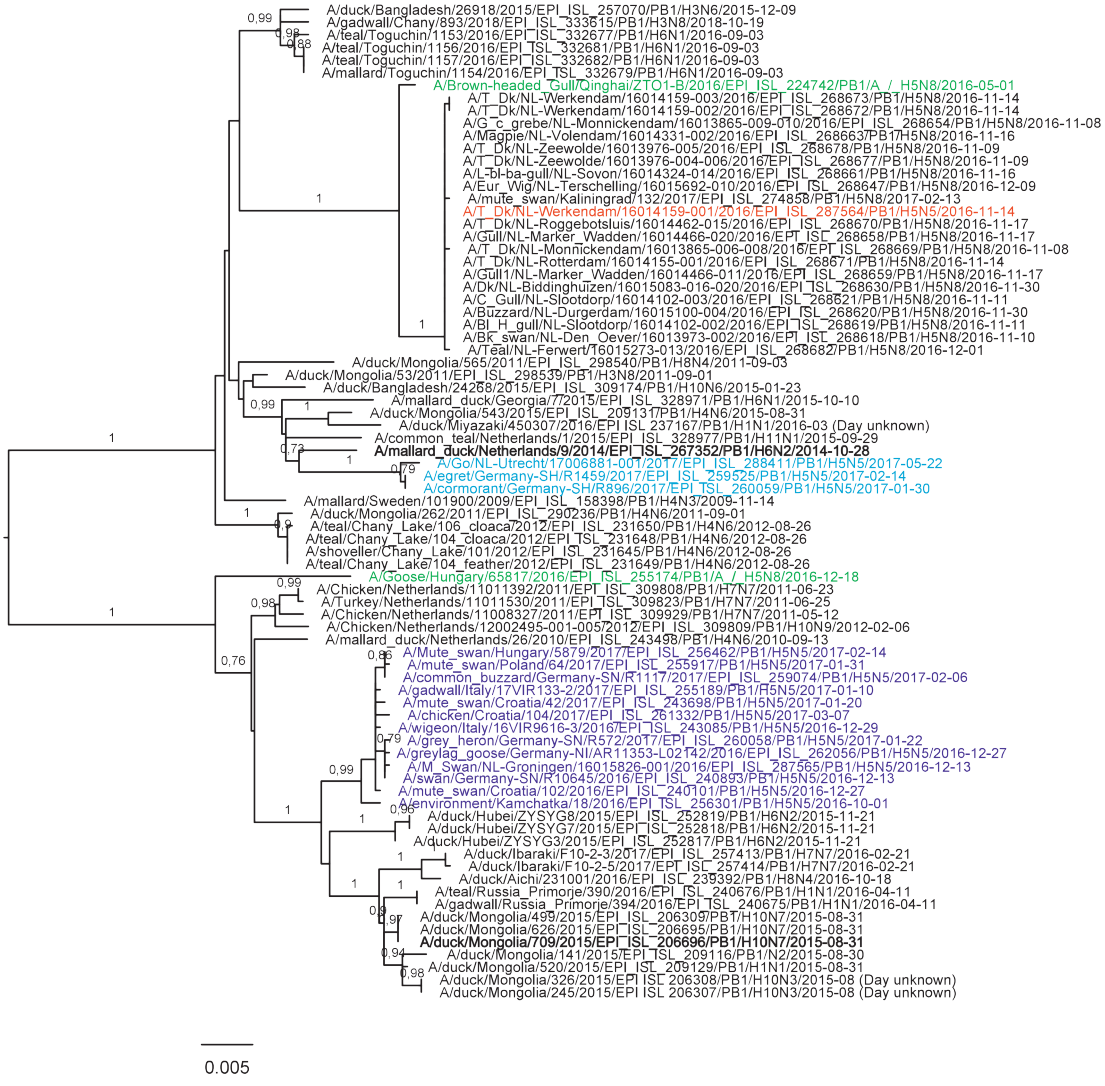
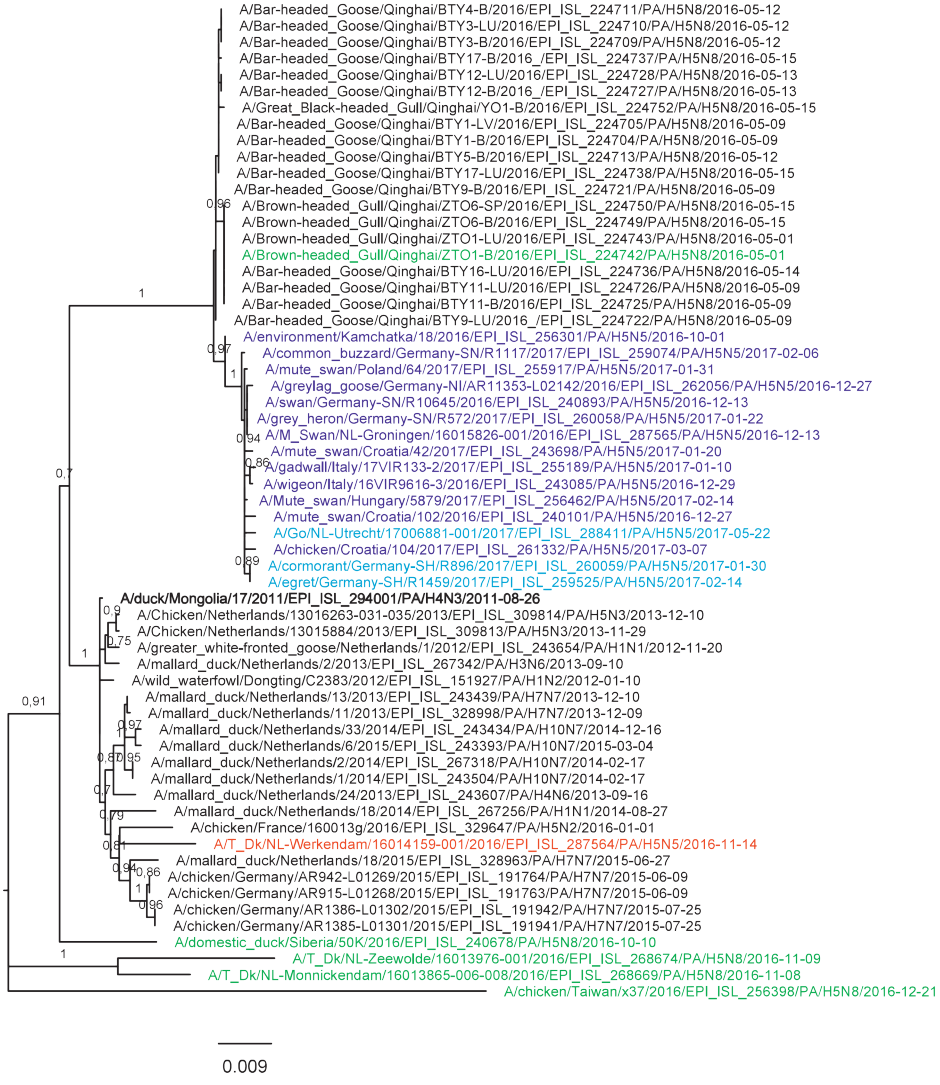
0.009

PB1

- H5N5-GT1
- H5N5-GT2
- H5N5-GT3
- H5N8 cluster representative




PA
H5N5-GT1
H5N5-GT2
H5N5-GT3
H5N8 cluster representative
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A/mute_swan/Poland/64/2017/EPI_ISL_255917/PA/H5N5/2017-01-31
A/greylag_goose/Germany-NI/AR11353-L02142/2016/EPI_ISL_262056/PA/H5N5/2016-12-27
A/swan/Germany-SN/R10645/2016/EPI_ISL_240893/PA/H5N5/2016-12-13
A/grey_heron/Germany-SN/R572/2017/EPI_ISL_260058/PA/H5N5/2017-01-22
A/M_Swan/NL-Groningen/16015826-001/2016/EPI_ISL_287565/PA/H5N5/2016-12-13
A/mute_swan/Croatia/42/2017/EPI_ISL_243698/PA/H5N5/2017-01-20
A/gadwall/Italy/17VIR133-2/2017/EPI_ISL_255189/PA/H5N5/2017-01-10
A/wigeon/Italy/16VIR9616-3/2016/EPI_ISL_243085/PA/H5N5/2016-12-29
A/Mute_swan/Hungary/5879/2017/EPI_ISL_256462/PA/H5N5/2017-02-14
A/mute_swan/Croatia/102/2016/EPI_ISL_240101/PA/H5N5/2016-12-27
A/Go/NL-Utrecht/17006881-001/2017/EPI_ISL_288411/PA/H5N5/2017-05-22
A/chicken/Croatia/104/2017/EPI_ISL_261332/PA/H5N5/2017-03-07
A/cormorant/Germany-SH/R896/2017/EPI_ISL_260059/PA/H5N5/2017-01-30
A/egret/Germany-SH/R1459/2017/EPI_ISL_259525/PA/H5N5/2017-02-14
A/duck/Mongolia/17/2011/EPI_ISL_294001/PA/H4N3/2011-08-26
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A/wild_waterfowl/Dongting/C2383/2012/EPI_ISL_151927/PA/H1N2/2012-01-10
A/mallard_duck/Netherlands/13/2013/EPI_ISL_243439/PA/H7N7/2013-12-10
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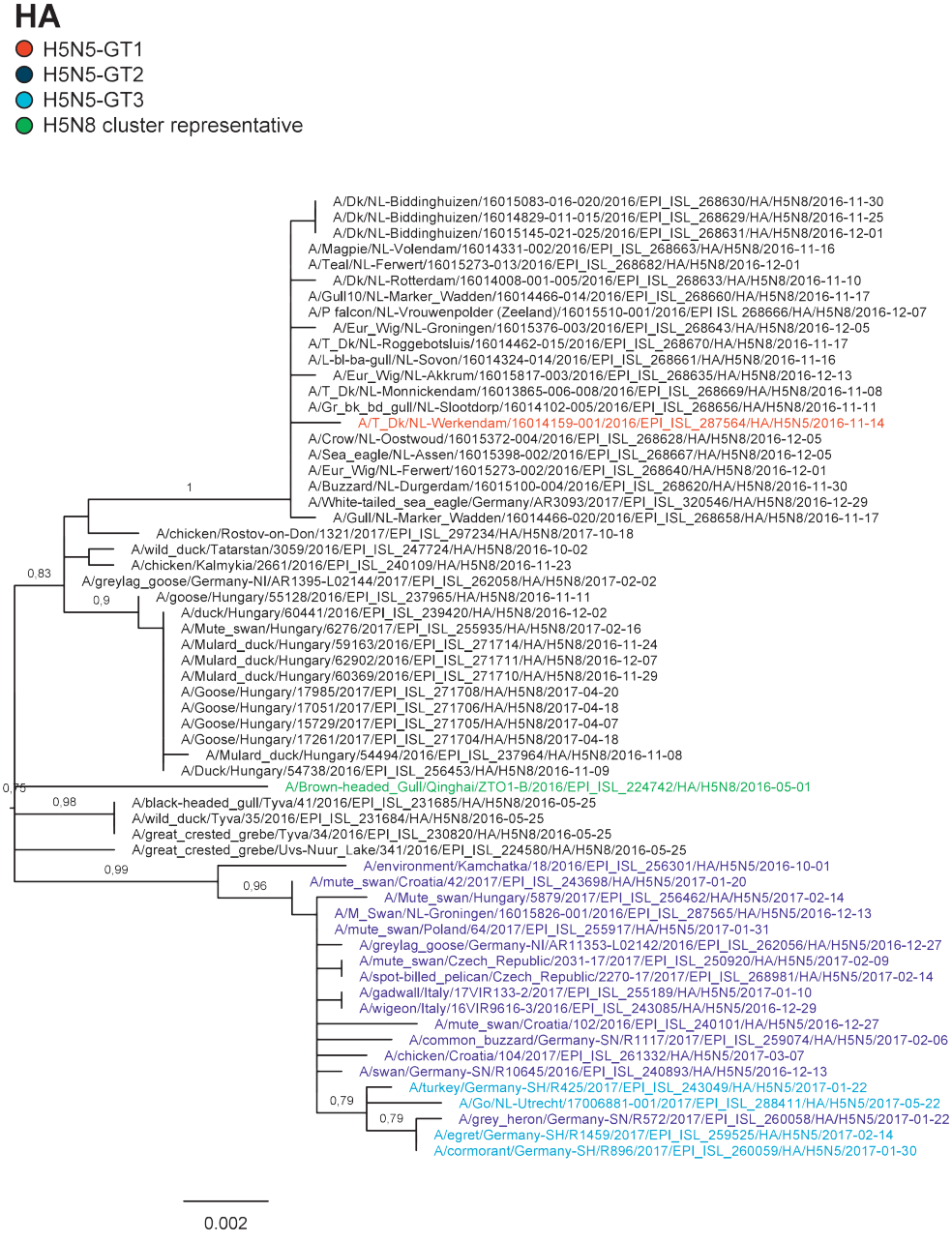
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H5N5-GT2
H5N5-GT3
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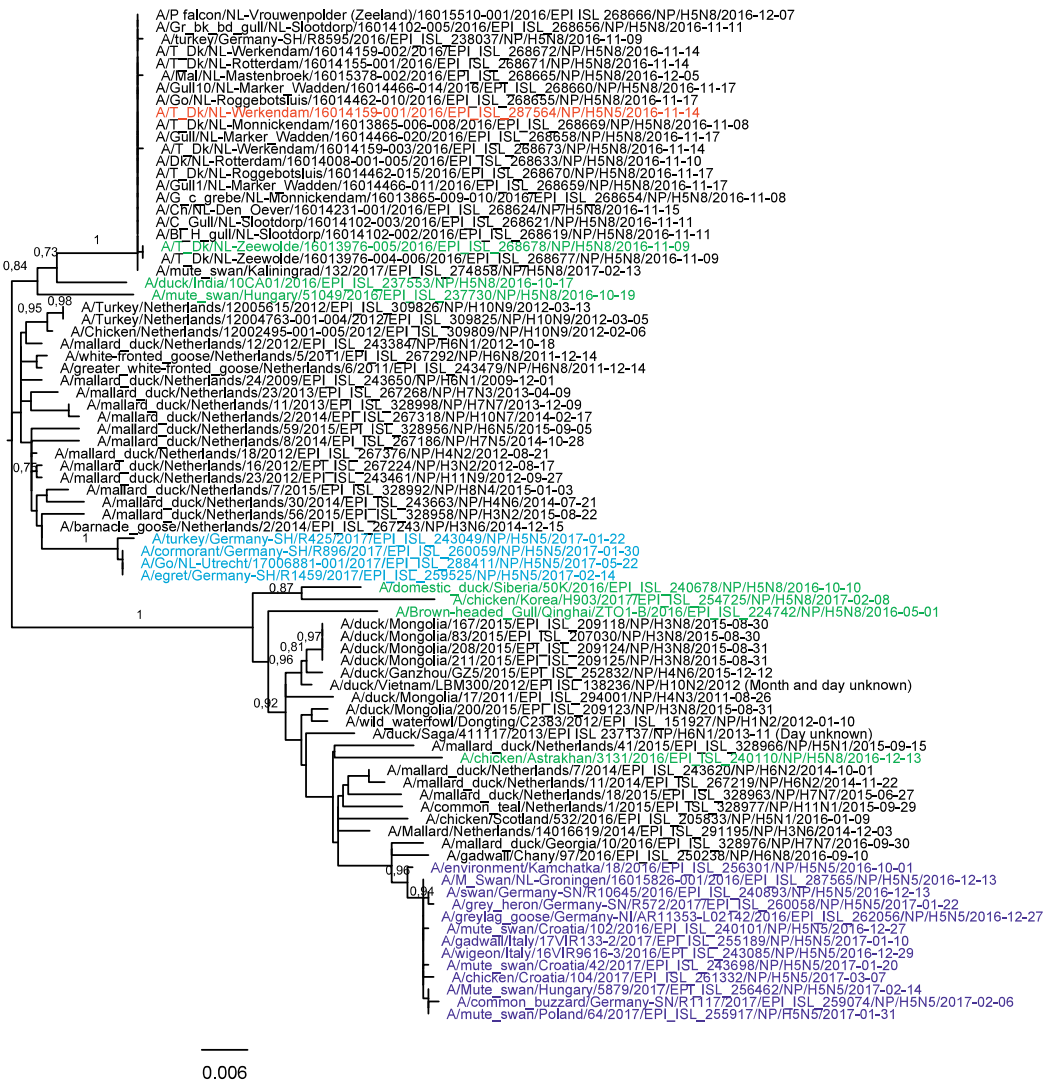
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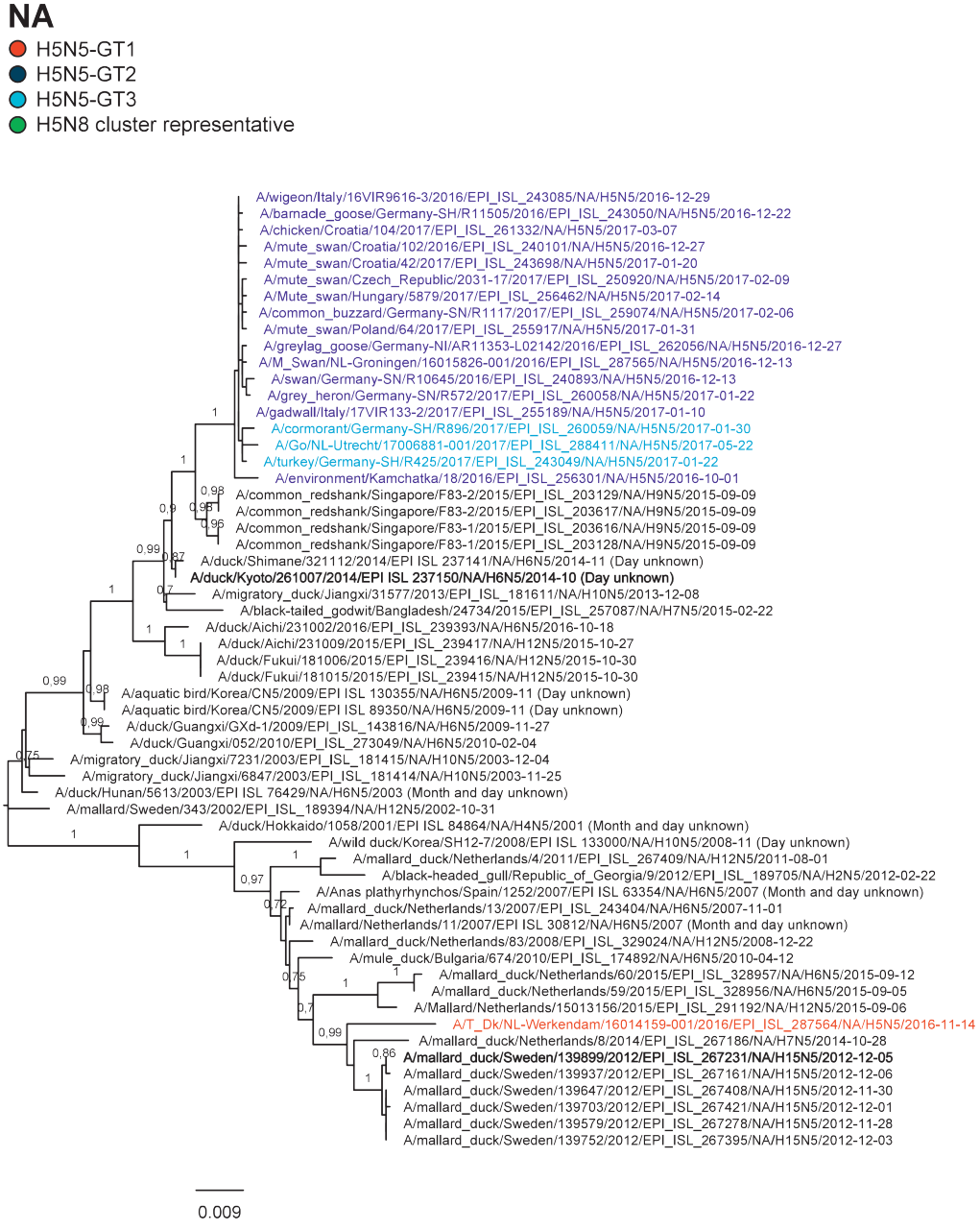
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A/migratory_duck/Jiangxi/31577/2013/EPI_ISL_181611/NA/H10N5/2013-12-08
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A/duck/Aichi/231002/2016/EPI_ISL_239393/NA/H6N5/2016-10-18
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A/wild duck/Korea/SH12-7/2008/EPI ISL 133000/NA/H10N5/2008-11 (Day unknown)
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H5N5-GT2
H5N5-GT3
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A/turkey/Italy/17VIR1574-1/2017/EPI_ISL_256306/MP/H5N8/2017-02-21
A/chicken/Poland/79A/2016/EPI_ISL_240104/MP/H5N8/2016-12-17
A/goose/Czech_Republic/136-17_1/2017/EPI_ISL_268930/MP/H5N8/2017-01-04
A/tawny_owl/Germany-SN/R1186/2017/EPI_ISL_258527/MP/H5N8/2017-02-08
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A/Goose/Hungary/17580/2017/EPI_ISL_271707/MP/H5N8/2017-04-19
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A/Goose/Hungary/59763/2016/EPI_ISL_271709/MP/H5N8/2016-11-27
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A/P falcon/NL-Vrouwenpolder (Zeeland)/16015510-001/2016/EPI ISL 268666/MP/H5N8/2016-12-07
A/Magpie/NL-Volendam/16014331-002/2016/EPI_ISL_268663/MP/H5N8/2016-11-16
A/L-bl-ba-gull/NL-Sovon/16014324-014/2016/EPI_ISL_268661/MP/H5N8/2016-11-16
A/Go/NL-RoggebotsIuis/16014462-010/2016/EPI_ISL_268655/MP/H5N8/2016-11-17
A/Eur_Wig/NL-Groningen/16015376-003/2016/EPI_ISL_268643/MP/H5N8/2016-12-05
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A/BI_H_gull/NL-Slootdorp/16014102-002/2016/EPI_ISL_268619/MP/H5N8/2016-11-11
A/Bk_swan/NL-Den_Oever/16013973-002/2016/EPI_ISL_268618/MP/H5N8/2016-11-10
A/gadwall/Kurgan/2442/2016/EPI_ISL_256298/MP/H5N8/2016-08-27
A/chicken/Kalmykia/2643/2016/EPI_ISL_247725/MP/H5N8/2016-11-21
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0,88
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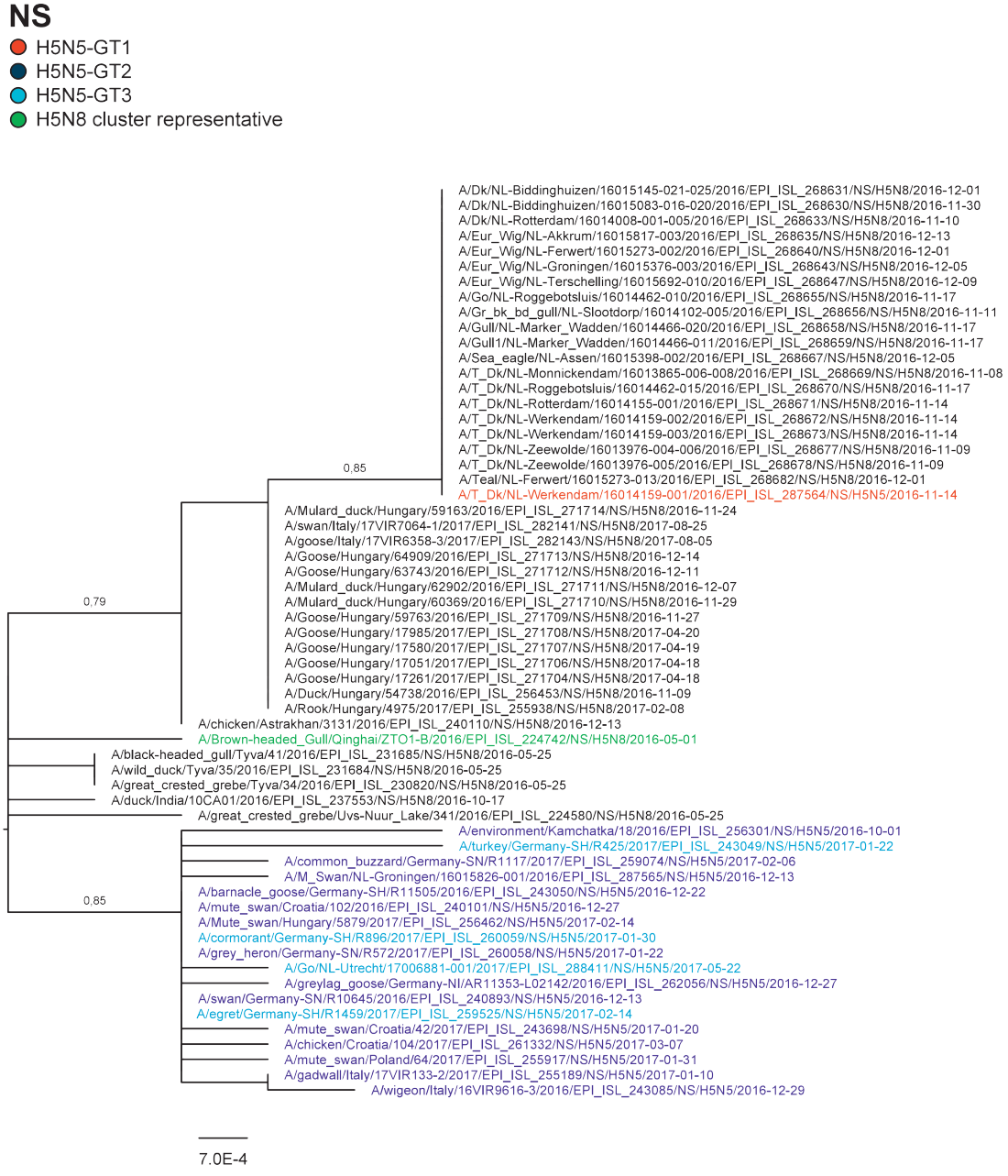
7.0E-4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H5N5-GT1
H5N5-GT2
H5N5-GT3
H5N8 cluster representative
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0,85
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A/Goose/Hungary/64909/2016/EPI_ISL_271713/NS/H5N8/2016-12-14
A/Goose/Hungary/63743/2016/EPI_ISL_271712/NS/H5N8/2016-12-11
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0,79
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A/Goose/Hungary/17580/2017/EPI_ISL_271707/NS/H5N8/2017-04-19
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A/Duck/Hungary/54738/2016/EPI_ISL_256453/NS/H5N8/2016-11-09
A/Rook/Hungary/4975/2017/EPI_ISL_255938/NS/H5N8/2017-02-08
A/chicken/Astrakhan/3131/2016/EPI_ISL_240110/NS/H5N8/2016-12-13
A/Brown-headed_Gull/Qinghai/ZTO1-B/2016/EPI_ISL_224742/NS/H5N8/2016-05-01
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0,85
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A/swan/Germany-SN/R10645/2016/EPI_ISL_240893/NS/H5N5/2016-12-13
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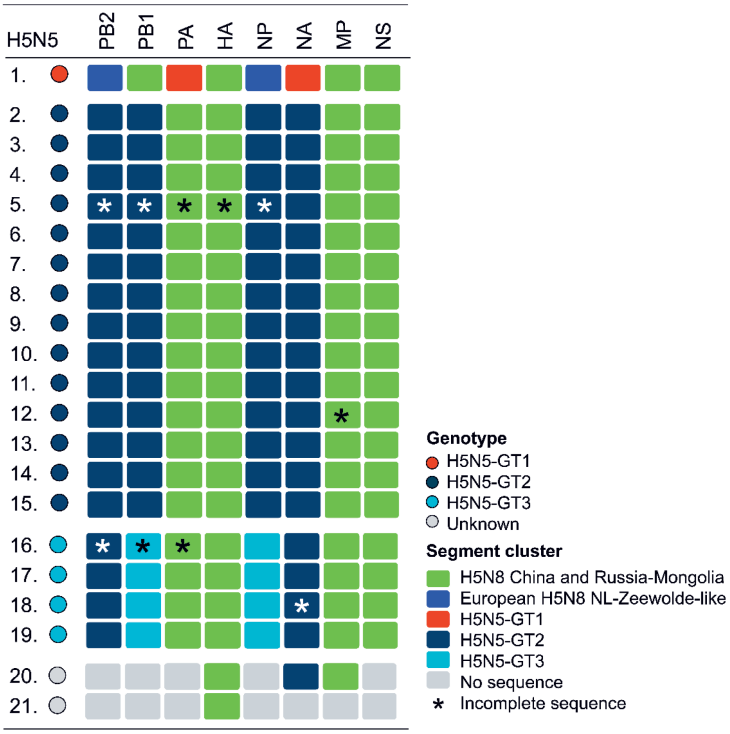
7.0E-4



S2 FIGURE. GENE CONSTELLATIONS OF H5N5 VIRUSES.
Highly pathogenic avian influenza (HPAI) H5N5 viruses isolated during the HPAI H5 2016-2017 epizootic, ordered by genotype. Gene segments are coloured according to their phylogenetic cluster. Detailed information on the virus sequences is provided in Table S2. PB2, polymerase basic protein 2; PB1, polymerase basic protein 1; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; MP, matrix protein; NS, nonstructural protein.

S3 FIGURE. MAXIMUM CLADE CREDIBILITY TREES.

Maximum clade credibility (MCC) trees were generated to estimate the median time to the most recent common ancestor (TMRCA) for each gene segment of highly pathogenic avian influenza (HPAI) H5N5 viruses isolated during the HPAI H5 2016-2017 epizootic. The H5N5 virus sequences are coloured according to their genotype (H5N5-GT1, red; H5N5-GT2, dark blue; H5N5-GT3, light blue). The median TMRCAs estimates were determined for H5N5-GT2 viruses (node 1), European H5N5-GT2 viruses (node 2), and H5N5-GT3 viruses (node 3). The estimated TMRCAs, the 95% highest posterior density (HPD) intervals and posterior values for each of the numbered nodes are listed in Table 2. PB2, polymerase basic 2; PB1, polymerase basic 1; PA, polymerase acidic; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; MP, matrix protein; NS, nonstructural protein.


PB2
H5N5-GT1
H5N5-GT2
H5N5-GT3
A/duck/Liangxi/33775/2013/EPI_ISL_181683/PB2/H10N3/2013-12-15
A/duck/Liangxi/33695/2013/EPI_ISL_181674/PB2/H10N3/2013-12-15
A/duck/Liangxi/33753/2013/EPI_ISL_181677/PB2/H10N3/2013-12-15
A/duck/Liangxi/33713/2013/EPI_ISL_181676/PB2/H10N3/2013-12-15
A/duck/Liangxi/33757/2013/EPI_ISL_181678/PB2/H10N3/2013-12-15
A/duck/Liangxi/33663/2013/EPI_ISL_181672/PB2/H10N3/2013-12-15
A/duck/Liangxi/33701/2013/EPI_ISL_181457/PB2/H10N3/2013-12-15
A/duck/Liangxi/33759/2013/EPI_ISL_181679/PB2/H10N3/2013-12-15
A/duck/Liangxi/33699/2013/EPI_ISL_181675/PB2/H10N3/2013-12-15
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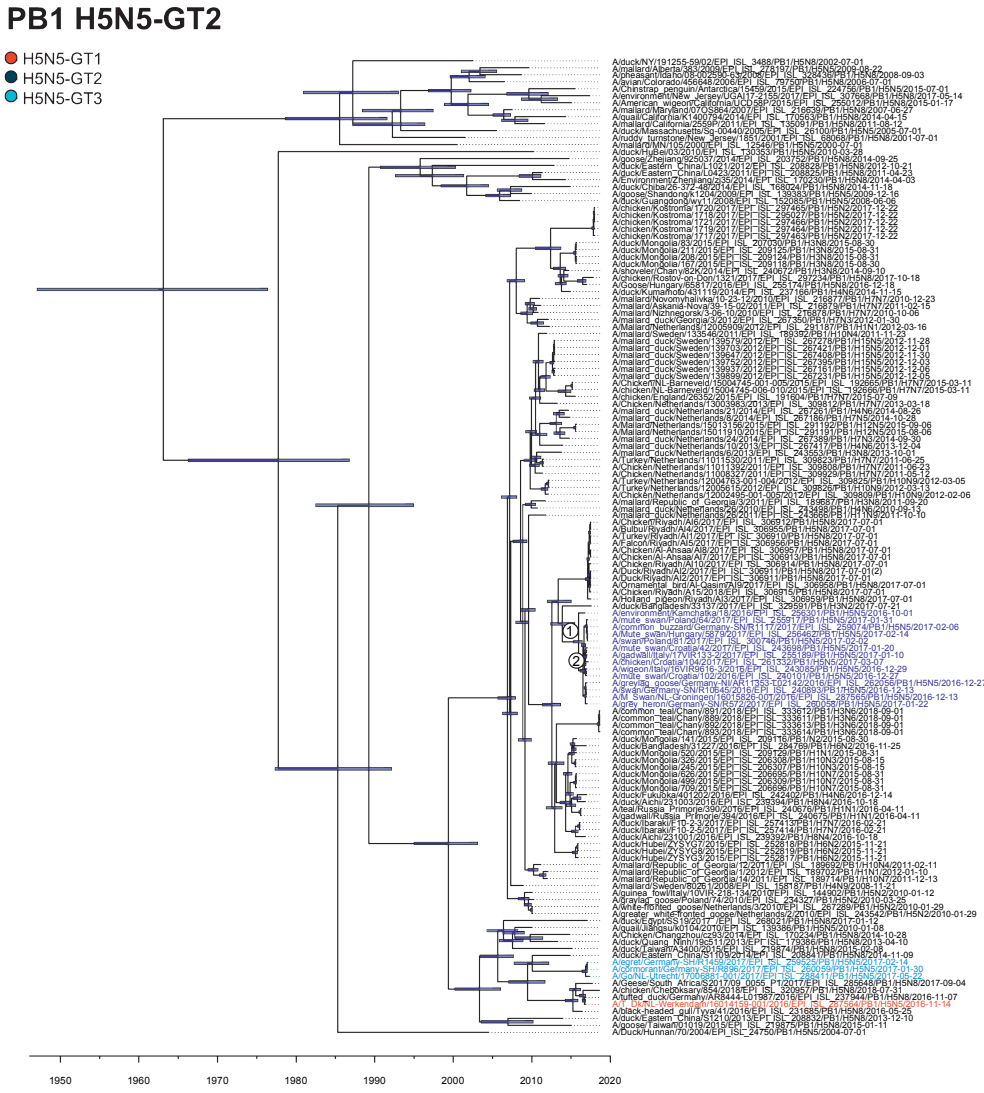
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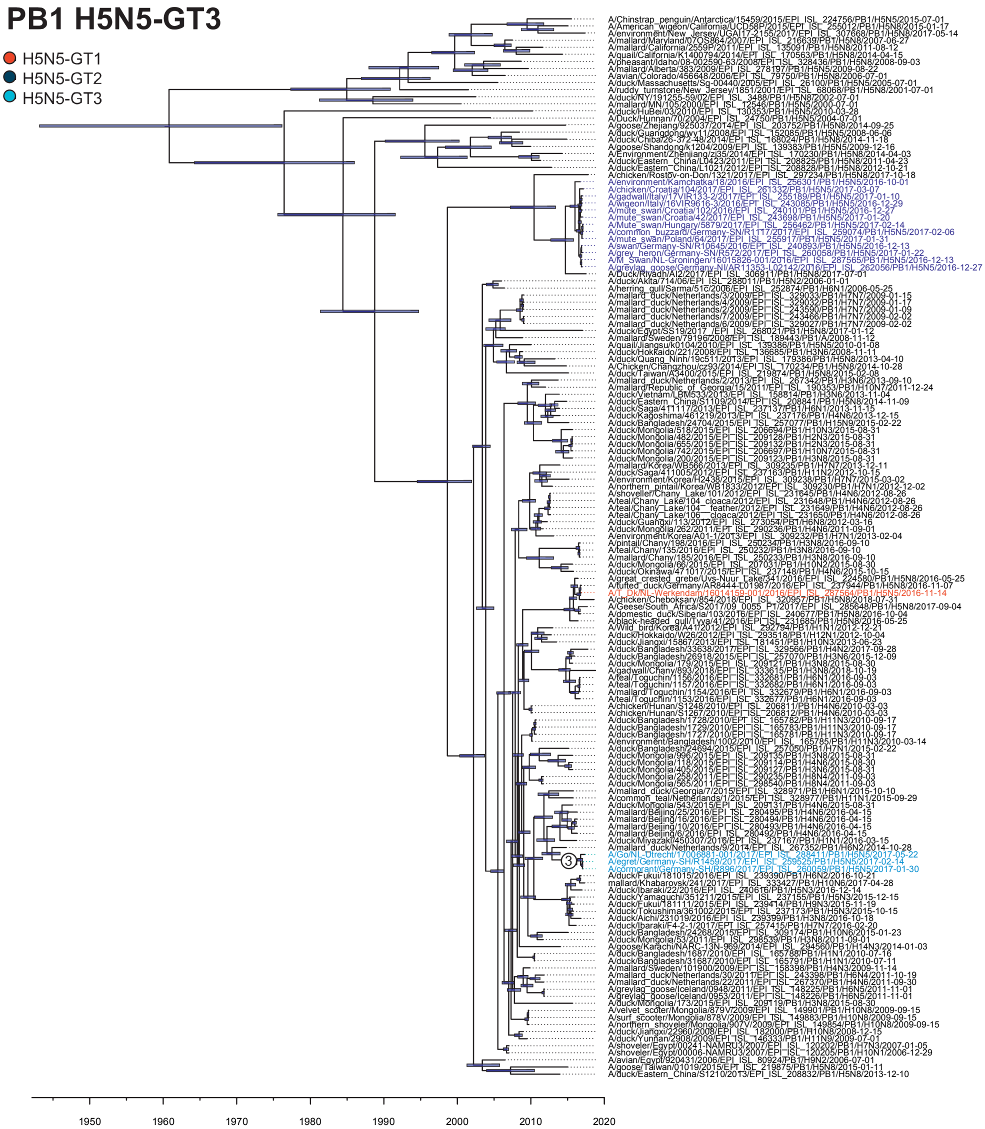
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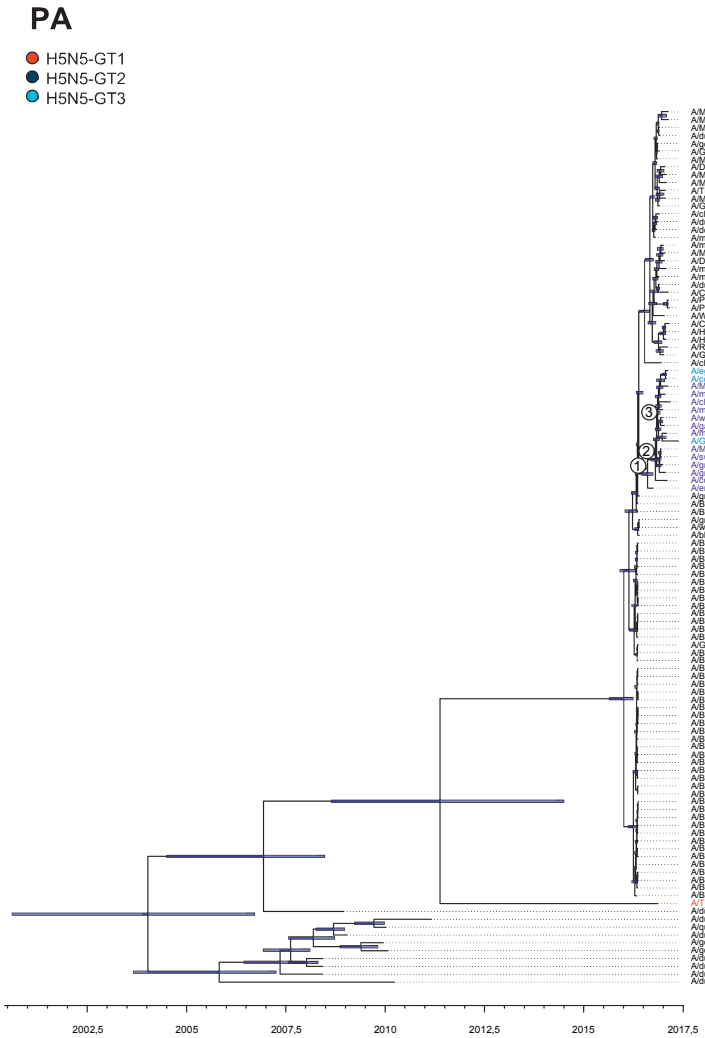
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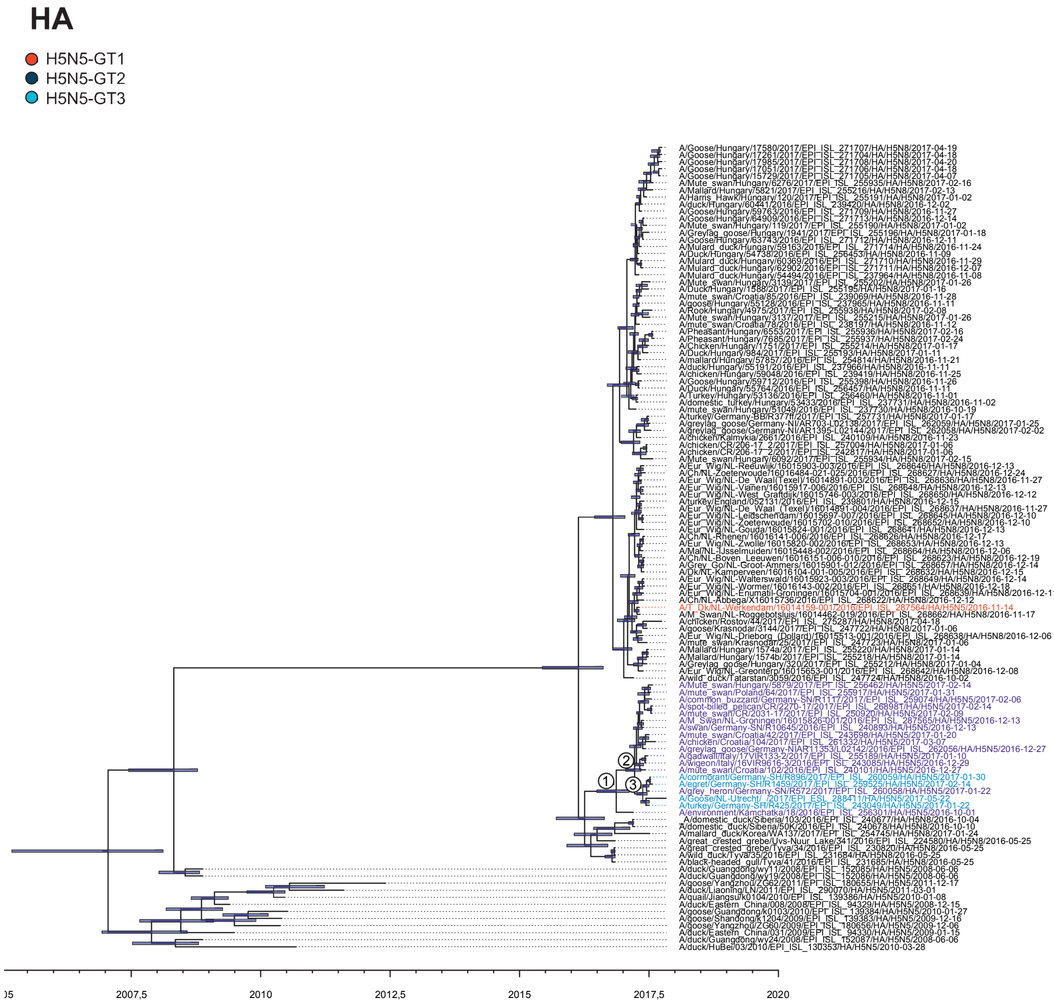
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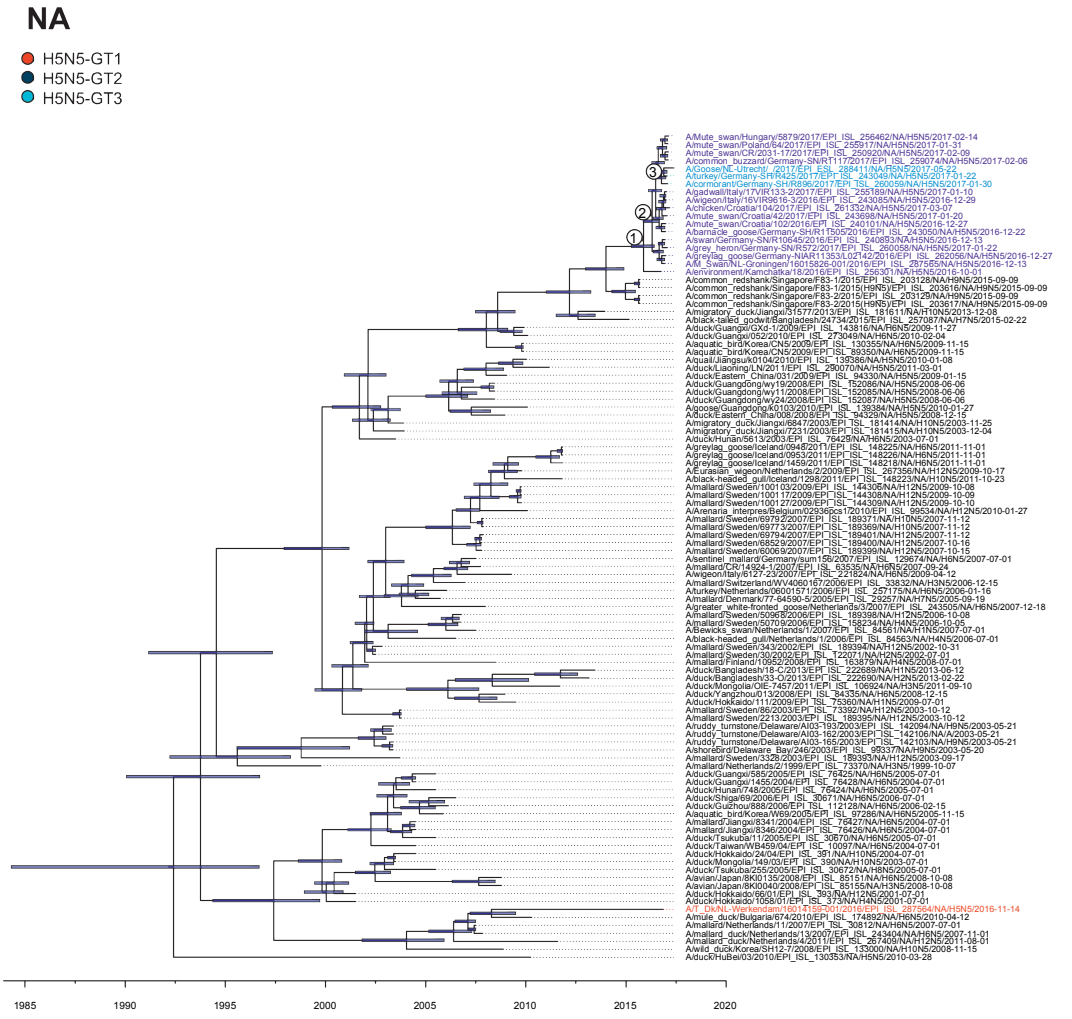
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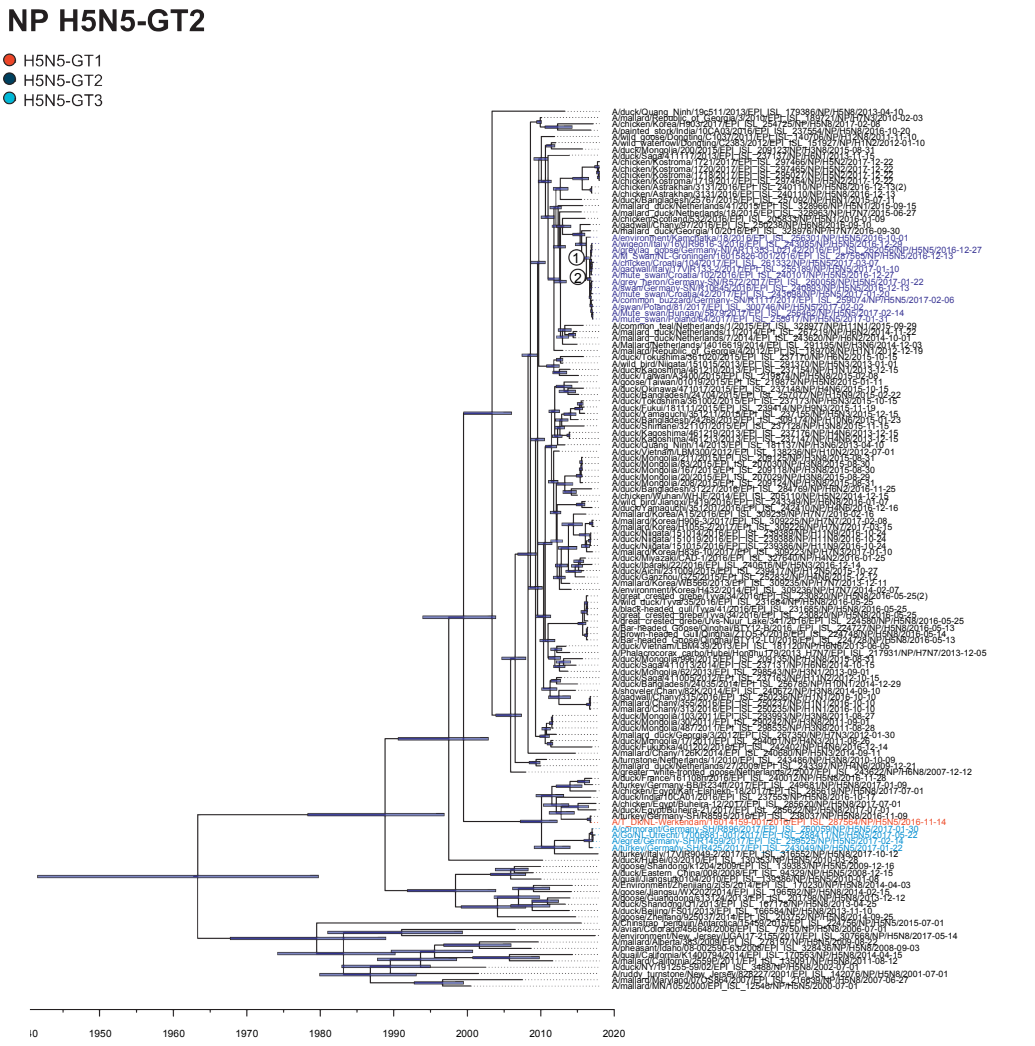
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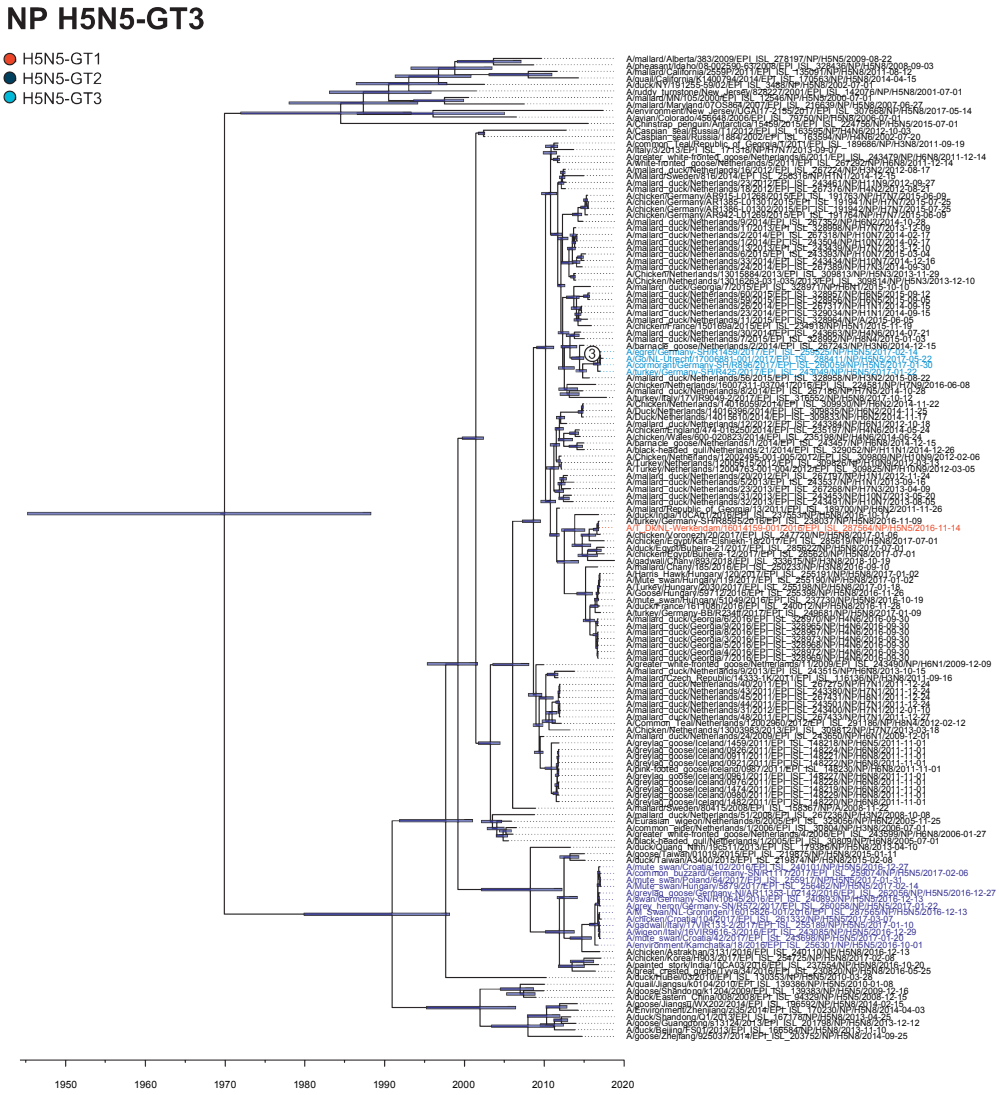
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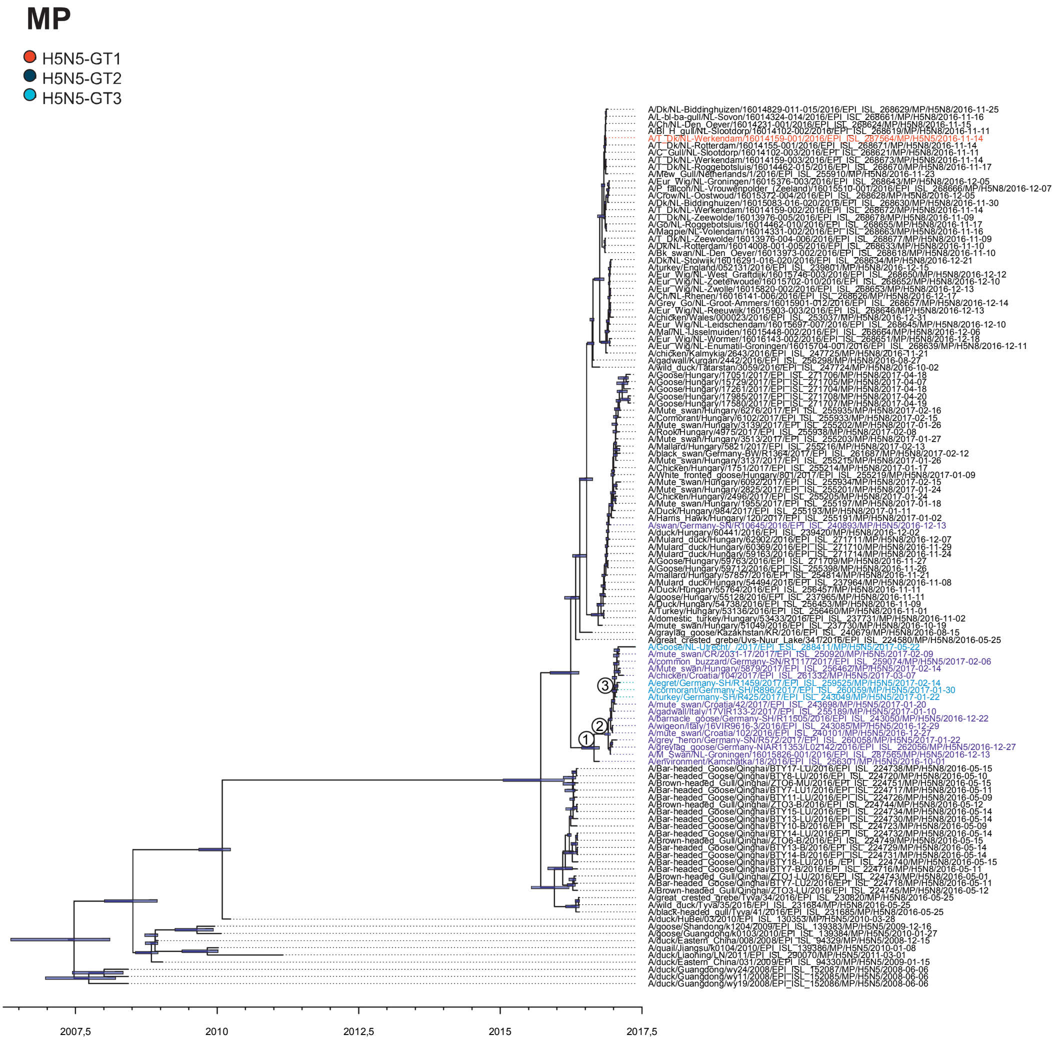
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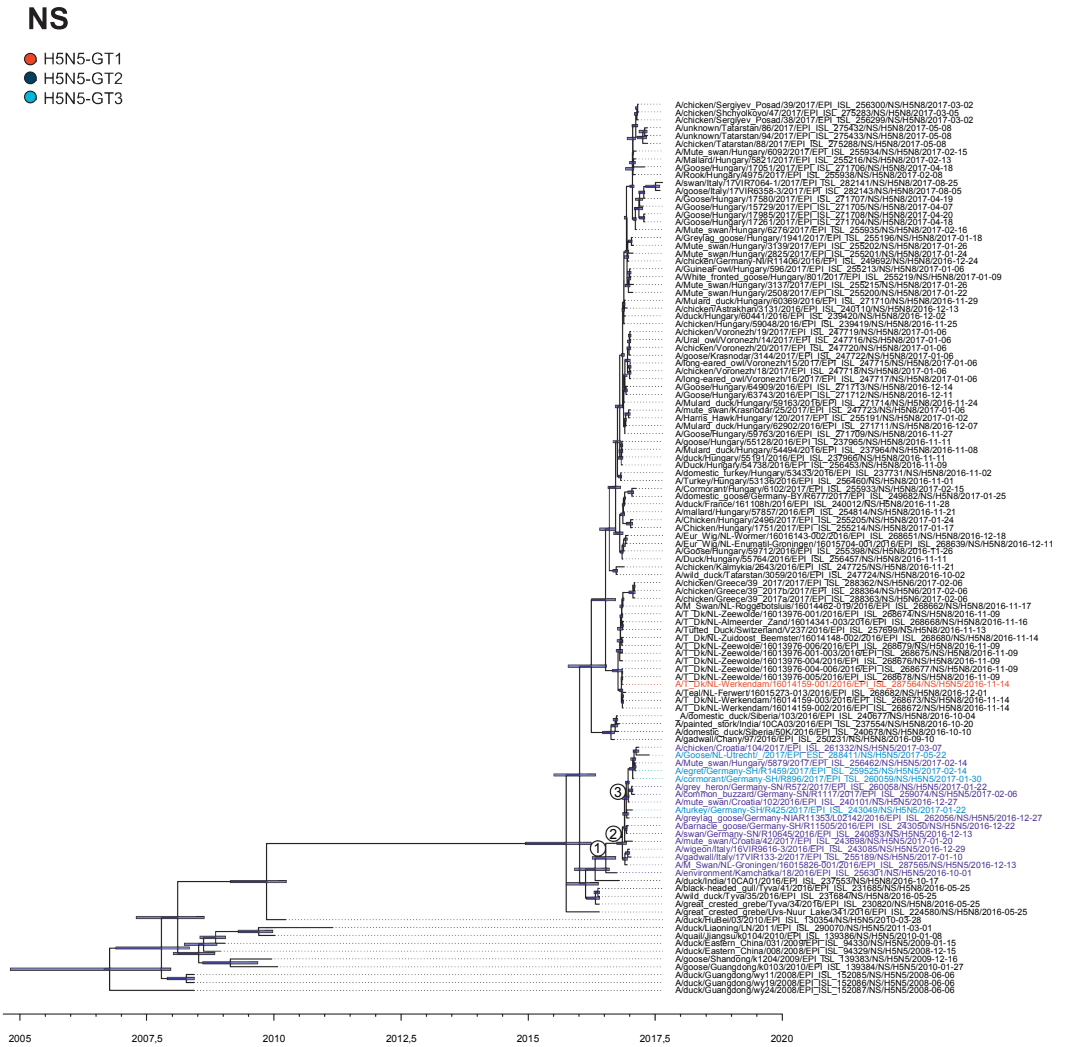
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A/Duck/Hungary/54738/2016/EPI_ISL_236453/NS/H5N8/2016-11-09
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A/Turkey/Hungary/53136/2016/EPI_ISL_236460/NS/H5N8/2016-11-01
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A/Eur_Wig/NL-Wormer/16016143-002/2016/EPI_ISL_268651/NS/H5N8/2016-12-18
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A/Goose/Hungary/5912/2016/EPI_ISL_255398/NS/H5N8/2016-11-26
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A/chicken/Greece/39_2017b/2017/EPI_ISL_288364/NS/H5N8/2017-02-06
A/chicken/Greece/39_2017a/2017/EPI_ISL_288363/NS/H5N8/2017-02-06
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A/T_Dk/NL-Almeerder_Zand/16014341-003/2016/EPI_ISL_268668/NS/H5N8/2016-11-16
A/Tufted_Duck/Switzerland/V237/2016/EPI_ISL_257699/NS/H5N8/2016-11-13
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A/wild_duck/Tyva/35/2016/EPI_ISL_231684/NS/H5N8/2016-05-25
A/great_crested_grebe/Tyva/34/2016/EPI_ISL_230820/NS/H5N8/2016-05-25
A/great_crested_grebe/Uvs-Nuur_Lake/341/2016/EPI_ISL_224580/NS/H5N8/2016-05-25
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A/duck/Liaoning/LN/2011/EPI_ISL_290070/NS/H5N5/2011-03-01
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A/duck/Eastern_China/031/2008/EPI_ISL_94330/NS/H5N5/2009-01-15
A/duck/Eastern_China/008/2008/EPI_ISL_94329/NS/H5N5/2008-12-15
A/goose/Shandong/k1204/2009/EPI_ISL_139383/NS/H5N5/2009-12-16
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CHAPTER 6

Histopathology and tissue distribution of highly pathogenic avian influenza (HPAI) H5N6 virus in chickens and Pekin ducks

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In preparation

Abstract

In 2017-2018, a highly pathogenic avian influenza (HPAI) H5N6 virus caused outbreaks of severe disease and high mortality in commercial chickens and Pekin ducks in the Netherlands. Here, we analysed histopathology and virus distribution in both poultry species after natural infection and experimental intravenous inoculation with HPAI H5N6 virus. In chickens, microscopic lesions and viral antigens were present in multiple organs, whereas in Pekin ducks severe histopathological changes and viral antigens were particularly detected in the lung. Virus replication in the respiratory tract, especially the lung, acute pneumonia and subsequent respiratory failure may have contributed to severe disease and mortality among Pekin ducks.

Keywords: influenza A virus; highly pathogenic avian influenza; H5N6; poultry; chickens; Pekin ducks; histopathology; immunohistochemistry

Introduction, Methods, and Results

During the 2017-2018 winter season, outbreaks of a novel reassortant highly pathogenic avian influenza (HPAI) H5N6 virus belonging to HPAI H5 clade 2.3.4.4 group B occurred in Asia and Europe, infecting various poultry and wild bird species. The virus was first detected in November-December 2017 in Japan, South Korea [1,2] and the Netherlands [3], and subsequently also detected in several other European countries [4]. Previous genetic analysis demonstrated that the virus emerged from the Eurasian HPAI H5N8 clade 2.3.4.4 group B viruses that circulated in 2016-2017, but obtained novel polymerase basic 2 (PB2) and neuraminidase (NA) genes by reassortment with co-circulating low pathogenic avian influenza (LPAI) viruses in wild birds [3,4].

The virus is a descendent of the A/Goose/Guangdong/1/1996 (GsGd) H5N1 lineage, which was first detected in domestic geese in China in 1996 [5]. HPAI H5 clade 2.3.4.4 group B viruses of subtype H5N8 were detected initially in Asia in 2013-2014 [6-8], but re-emerged during spring 2016 [9,10]. In the autumn and winter of 2016–2017, the H5N8 viruses spread to Europe, Africa and the Middle East and caused numerous outbreaks of severe disease and high mortality in poultry and wild birds [11-14]. Although the number of outbreaks was limited compared to H5N8, H5N6 virus infections have also been associated with acute disease and mortality among various wild bird and poultry species. HPAI H5 viruses may also present a considerable threat to human health because of their zoonotic potential [15]. However, the Asian and European H5N6 clade 2.3.4.4 group B viruses are genetically distinct from the H5N6 clade 2.3.4.4 group C and D viruses that were detected in the same period in Asia with occasional transmissions to humans [16,17].

In the Netherlands, several HPAI H5N6 virus infections were identified in wild birds [3,18]. In addition, two hobby holdings, two commercial duck farms and one commercial chicken farm became infected. Severe clinical symptoms and sudden deaths were recorded at the infected poultry farms [3]. Virus pathogenicity was assessed in a previous study using intravenous pathogenicity index (IVPI) tests in chickens and Pekin ducks [18], according to the standard protocol described by the World Organisation for Animal Health (OIE) [19]. This revealed IVPI scores of 2.99 and 3.00 in chickens and Pekin ducks, respectively, confirming high pathogenicity of the HPAI H5N6 virus in both poultry species. However, in the IVPI test mortality is used as a measure of pathogenicity, but information on what determines pathogenicity is limited. Moreover, as intravenous inoculation does not represent the natural route of infection, it is valuable to study the pathogenicity of HPAI H5N6 virus in naturally infected birds to better understand infection-related mortality under field conditions. To increase our understanding of HPAI H5N6 virus infection dynamics and pathogenicity among poultry species, we investigated histopathologic changes and the localization of viral antigen in chickens and Pekin ducks after natural infection and experimental intravenous inoculation.

We performed a semi-quantitative histopathological and limmunohistochemical (IHC) analysis of various tissues collected from chickens (*Gallus gallus domesticus*) and Pekin ducks (*Anas platyrhynchos domesticus*) after natural infection and experimental intravenous inoculation with HPAI H5N6 virus. The naturally infected chickens (CN1-5) were obtained from a parent breeding flock in Oldekerk, the Netherlands, testing positive for HPAI H5N6 virus on 25 February 2018 (A/chicken/Netherlands/18003041-001-005/2018; EPI_ISL_332430) [18]. The

TABLE 1. Histopathological changes in tissues of chickens and Pekin ducks after natural infection and experimental intravenous inoculation with HPAI H5N6 virus.

	Chicken		Pekin duck		
Organ	**Natural**	**Experimental**	**Natural**	**Experimental**	**Morphology of histopathologic changes**
Brain	+	+	-/+	+	Gliosis with glia nodules
Conchae	+++	+/++	++	++	Heterophilic rhinitis with edema, epithelial degeneration and ulceration
Trachea	+++	++	++	nd	Heterophilic tracheitis with epithelial degeneration and necrosis
Lung	+	++	++/+++	+++	Heterophilic and fibrinonecrotic interstitial pneumonia with edema
Air sac	++/+++	+	++	++/+++	Heterophilic airsacculitis with epithelial degeneration and necrosis
Ileum	+	-/+	-/+	+	Lymphoplasmacytic ileitis with depletion and necrosis of lymphocytes in gut-associated lymphoid tissue
Cloaca	++	++	+	-/+	Degeneration and necrosis of lymphocytes
Spleen	+/++	+++	++	+++	Depletion and necrosis of lymphocytes with sinushistiocytosis
Pancreas	++/+++	-	++	+	Lymphohystiocytic pancreatitis with acinar degeneration and necrosis

Haematoxylin and eosin (HE) slides were scored for severity of histopathological changes: none (-), mild (+), moderate (++), severe (+++). The mean histopathological scores are calculated based on the individual scores of five birds in each group, as presented in Table S1. nd, not done.

naturally infected Pekin ducks (DN1-5) were obtained from a duck farm in Biddinghuizen, the Netherlands, with confirmed HPAI H5N6 infection on 7 December 2017 (A/duck/Netherlands/17017237-001-005/2017; EPI_ISL_287907) [3]. The two isolates had the same gene constellation, but differed at several amino acid positions [18]. From both farms, animals with advanced disease, as indicated by moderate to severe clinical signs typically observed during HPAI virus infection [20], were submitted for necropsy. The experimentally infected chickens (CE1-5) and Pekin ducks (DE1-5) were inoculated intravenously with 0.1 ml of 1:10 diluted allantoic fluid containing HPAI H5N6 virus (A/duck/Netherlands/17017237-001-005/2017; EPI_ISL_287907) according to the standard protocol described by the OIE [19], as described in our previous study [18].

All experimentally inoculated birds died within 24 hours and organs (brain, conchae, trachea, lung, air sac, ileum, cloaca, spleen and pancreas) were collected shortly after death. For histopathologic and IHC analysis, tissue samples were fixed in 10% neutral-buffered formalin, embedded in paraffin and sectioned at 4-µm-thickness. Haematoxylin and eosin (HE) slides were scored (0-3) for severity (mild, moderate or severe) of the histopathologic changes. In addition, the morphology of histopathologic changes was described. IHC staining was performed using monoclonal mouse HB65 antibodies to the influenza virus A nucleoprotein, as previously described [21]. Tissues from birds known to be HPAI virus positive and negative, as well as a negative control of the primary antibody staining, were included in each staining procedure. The IHC stains were scored (0-4) based on the number of cells expressing viral antigen in the nucleus (negative, sparse, moderate, abundant or excessive), and cell types expressing viral antigen (epithelial, endothelial or mononuclear cells) were identified. Histopathological analysis was performed in a blinded fashion by a certified veterinary pathologist, and two independent scorings with consensus were performed for IHC analysis.

The overall morphology of histopathological changes in the different organs of the HPAI H5N6 virus infected birds was comparable between poultry species and infection routes, however variations in severity of changes and affected tissues were observed (Table 1 and Supplemental Table S1). In naturally infected chickens, severe histopathological changes were found in the upper respiratory tract (conchae and trachea) and the air sac, characterized by multifocal degeneration and necrosis of epithelial cells with infiltrates of heterophils and fewer macrophages and lymphocytes in underlying connective tissue. The histopathological changes in the conchae were particularly prominent in the nasal mucosal epithelium (Figure 1A). The pancreas showed moderate to severe histopathological changes, with areas of acinar degeneration and necrosis in the peripheral parts of the organ (Figure 1B). Histopathological changes were also observed in most other organs, but were mainly mild to moderate with individual variation between chickens.

In naturally infected Pekin ducks, histopathological changes were most noticeable in the lung. The lung displayed heterophilic and fibrinonecrotic interstitial pneumonia with edema (Figure 1C). The spleen showed depletion of the white pulp characterized by multifocal lymphocytic necrosis (Figure 1D). Interestingly, the lesions that were observed in the lung were more severe compared to those seen in the naturally infected chickens, whereas histopathological changes in the upper respiratory tract, ileum, cloaca and pancreas were milder.

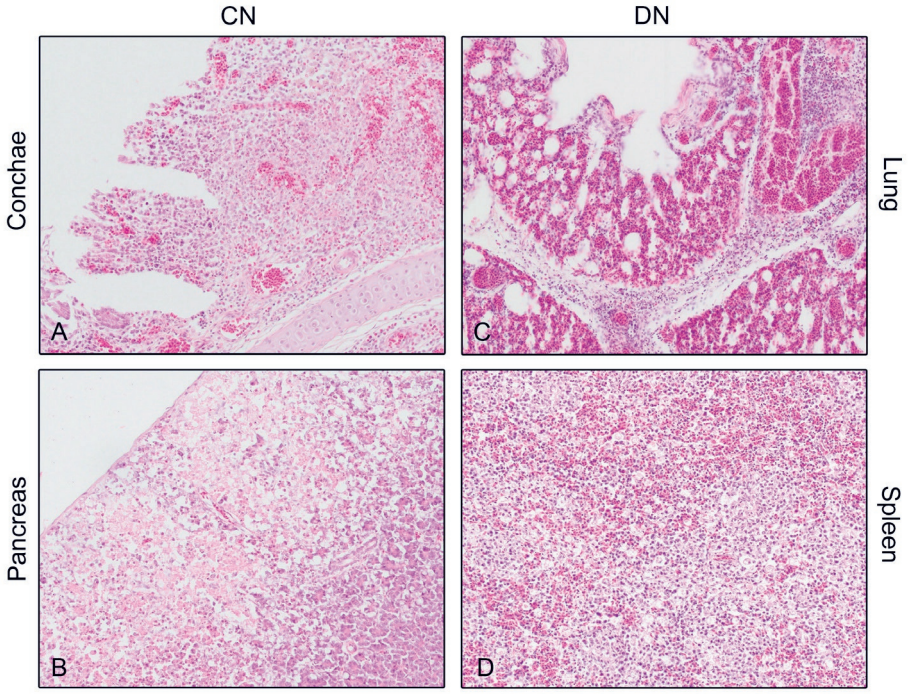

FIGURE 1. HISTOPATHOLOGICAL CHANGES IN TISSUES OF CHICKENS AND PEKIN DUCKS AFTER NATURAL INFECTION AND EXPERIMENTAL INTRAVENOUS INOCULATION WITH HPAI H5N6 VIRUS. Histopathological changes in selected tissues of chickens (CN) and Pekin ducks (DN) naturally infected with highly pathogenic avian influenza (HPAI) H5N6 virus, analysed by haematoxylin and eosin (HE) staining. (A) Conchae of a naturally infected chicken showing multifocal degeneration and necrosis of mucosal epithelium with infiltrates of heterophils, macrophages and lymphocytes. (B) Pancreas of a naturally infected chicken showing multifocal acinar degeneration and necrosis. (C) Lung of a naturally infected duck showing fibrinonecrotic interstitial pneumonia with excessive perivascular infiltration of heterophils, macrophages and lymphocytes. (D) Spleen of a naturally infected duck showing depletion of the white pulp with necrosis of lymphocytes (20x objective).

In both experimentally infected chickens and Pekin ducks, severe histopathological changes were found in the spleen, with microscopic lesions comparable to those described for the naturally infected ducks. Similar to the naturally infected ducks, the experimentally inoculated ducks showed more severe histopathological changes in the lung compared to the chickens, with more extensive perivascular infiltration of heterophils and more prominent depositions of fibrin and cellular debris in the parabronchi.

IHC analysis demonstrated variation in tissue and cell tropism of HPAI H5N6 virus between poultry species and infection routes (Table 2 and Supplemental Table S2). In naturally infected chickens, influenza antigen was clearly expressed in all tissues examined, except the brain and ileum, in which staining was only sparse or undetectable. The conchae and pancreas showed

TABLE 2. Expression of influenza A antigen in tissues of chickens and Pekin ducks after natural infection and experimental intravenous inoculation with HPAI H5N6 virus.

Species	Infection route	Organ	No. birds expressing viral antigen	Mean score	Cell types expressing antigen
Chicken	Natural	Brain	1/5	+	Mononuclear cells
		Conchae	5/5	+++	Epithelial cells, endothelial cells, mononuclear cells
		Trachea	2/2	+/++	Endothelial cells, mononuclear cells
		Lung	5/5	+/++	Epithelial cells, mononuclear cells, (endothelial cells)
		Air sac	4/4	++/+++	Epithelial cells, mononuclear cells, (endothelial cells)
		Ileum	1/5	-/+	Mononuclear cells
		Cloaca	4/5	++/+++	Epithelial cells, endothelial cells, mononuclear cells
		Spleen	4/5	+/++	Mononuclear cells
		Pancreas	5/5	+++	Epithelial cells
	Experimental	Brain	5/5	+/++	Endothelial cells, mononuclear cells
		Conchae	5/5	++/+++	Endothelial cells, mononuclear cells
		Trachea	5/5	+++	Endothelial cells, mononuclear cells
		Lung	5/5	++++	Epithelial cells, endothelial cells, mononuclear cells
		Air sac	2/2	++/+++	Epithelial cells, endothelial cells, mononuclear cells
		Ileum	5/5	+++	Endothelial cells, mononuclear cells
		Cloaca	5/5	+++	Endothelial cells, mononuclear cells
		Spleen	5/5	++++	Endothelial cells, mononuclear cells
		Pancreas	5/5	++	Epithelial cells, endothelial cells, mononuclear cells

TABLE 2. (continued)

Species	Infection route	Organ	No. birds expressing viral antigen	Mean score	Cell types expressing antigen
Pekin duck	Natural	Brain	3/5	-/+	Endothelial cells, mononuclear cells
		Conchae	4/5	-/+	Epithelial cells, mononuclear cells, (endothelial cells)
		Trachea	3/5	-/+	Epithelial cells, mononuclear cells
		Lung	5/5	++	Epithelial cells, mononuclear cells, (endothelial cells)
		Air sac	5/5	+/++	Epithelial cells
		Ileum	2/5	-/+	Mononuclear cells
		Cloaca	2/4	-/+	Epithelial cells (mononuclear cells)
		Spleen	2/5	+/++	Mononuclear cells
		Pancreas	1/4	-/+	Epithelial cells, mononuclear cells
	Experimental	Brain	1/5	-/+	Mononuclear cells
		Conchae	5/5	+/++	Endothelial cells, mononuclear cells
		Trachea	nd	nd	-
		Lung	5/5	+++	Epithelial cells, mononuclear cells
		Air sac	3/3	++	Epithelial cells, (endothelial cells)
		Ileum	4/5	-/+	Mononuclear cells
		Cloaca	1/2	-/+	Mononuclear cells (epithelial cells)
		Spleen	5/5	+++	Mononuclear cells
		Pancreas	0/5	-	-

the most widespread expression of viral antigen in all naturally infected chickens. In the conchae, viral antigen was frequently found in both mucosal (Figure 2A) and glandular epithelium (Figure 2A inset), but was also present in mononuclear cells and few endothelial cells. In the pancreas, staining was restricted to acinar epithelial cells and locally abundant in the peripheral parts of the organ (Figure 2E). The expression of viral antigen in the lung, air sac, cloaca and spleen was variable between chickens. Influenza antigen was predominantly expressed in epithelial and mononuclear cells in the lung (Figure 2B) and the air sac (Figure 2C), equally expressed between epithelial, endothelial and mononuclear cells in the cloaca, and mainly expressed in mononuclear cells in the spleen (Figure 2D).

In naturally infected Pekin ducks, influenza antigen was predominantly expressed in epithelial and mononuclear cells in the lung (Figure 2G), and in epithelial cells of the air sac (Figure 2H). In the lung, viral antigen expression was similar to naturally infected chickens, as viral antigen was detected in all animals examined and comparable numbers of cells were stained. Similar to the naturally infected chickens, virus was rarely detected in the brain and ileum of naturally infected ducks. Only one naturally infected duck (DN1) showed moderate to abundant staining in the spleen (Figure 2I), whereas no to limited staining was observed in the other ducks. Also, the expression of viral antigen was lower in the conchae (Figure 2F), trachea, cloaca and pancreas (Figure 2J) compared to naturally infected chickens, to low or undetectable levels.

The experimentally inoculated chickens showed expression of viral antigen in all tissues examined. Similar to the naturally infected chickens, viral antigen was frequently detected in mononuclear cells. However, significant tropism for endothelial cells was observed, as viral antigen was detected throughout endothelial cells of small and larger blood vessels in all tissues examined. Excessive viral antigen expression was observed particularly in the lung and spleen, which are highly vascularised tissues. In the lung, viral antigen was concentrated in the air capillaries of the parabronchus, whereas no staining of the squamous epithelium and underlying smooth musculature of the parabronchus was observed (Figure 2L). In the spleen, viral antigen was highly expressed in the blood capillaries of the red pulp (Figure 2N). Viral antigen expression in epithelial cells was largely restricted to the lung, air sac and pancreas (Figures 2L, M, O).

The experimentally inoculated ducks demonstrated predominant influenza antigen expression in epithelial cells in the lung (Figure 2Q) and mononuclear cells in the spleen (Figure 2S). In contrast to chickens, minimal viral antigen expression was observed in endothelial cells of small blood vessels in these organs. Staining was also present in other parts of the respiratory tract: in epithelial cells of the air sac (Figure 2R), and few endothelial and mononuclear cells in the conchae (Figure 2P). Some ducks showed viral antigen in the brain, ileum and cloaca, but the number of cells was much lower compared to the experimentally infected chickens, and predominantly restricted to mononuclear cells. No viral antigen was detected in the pancreas of the experimentally infected ducks (Figure 2T).

Discussion

Together, the histopathological and IHC analysis revealed variations in severity of microscopic lesions and tissue distribution of HPAI H5N6 virus between chickens and Pekin ducks, after both natural infection and experimental intravenous inoculation. The results demonstrated a

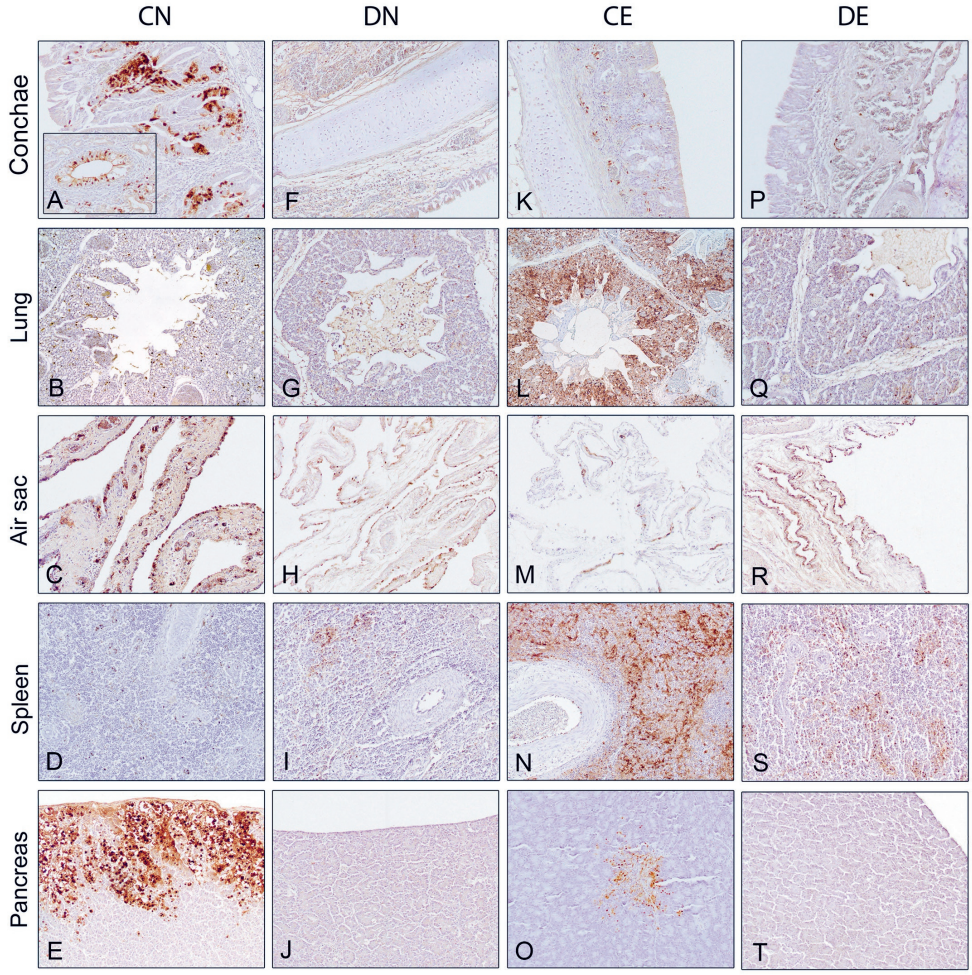

FIGURE 2. EXPRESSION OF INFLUENZA A ANTIGEN IN TISSUES OF CHICKENS AND PEKIN DUCKS AFTER NATURAL INFECTION AND EXPERIMENTAL INTRAVENOUS INOCULATION WITH HPAI H5N6 VIRUS. The distribution of viral antigen expression in the conchae, lung, air sac, spleen and pancreas of naturally infected chickens (CN), naturally infected ducks (DN), experimentally inoculated chickens (CE), and experimentally inoculated ducks (DE). Tissue sections were stained for influenza virus A nucleoprotein by immunohistochemistry (IHC) (20x objective).

more extensive distribution of HPAI H5N6 virus in chickens compared to Pekin ducks. This is in line with previous experimental studies showing attenuated and less invasive infection with HPAI H5 GsGd lineage viruses in ducks compared to chickens [22-24]. The presence of viral antigen was often associated with dispersed necrosis and inflammation in both species, which is consistent with the acute stage of HPAI virus infection [25]. The broad range of infected tissues corresponds with the ability of HPAI viruses to induce a systemic infection. The detection of viral antigen in various cell types further suggests a broad cell tropism.

In naturally infected chickens, viral antigen was expressed predominantly in mononuclear cells in most tissues, but also frequently detected in epithelial cells of the respiratory tract and cloaca, which represent the primary sites of viral entry and shedding under field conditions. Viral antigen expression in the glandular epithelial cells in the conchae of the naturally infected chickens indicates the possibility of virus excretion through mucus. The locally abundant expression of viral antigen in the peripheral parts of the pancreas suggests extension per continuitatem from the air sacs. The detection of viral antigen in endothelial cells, which was particularly abundant in experimentally inoculated chickens, confirms that HPAI viruses can readily infect endothelial cells in a wide range of organs in chickens [23-26]. Infection of endothelial cells can increase the permeability of blood vessels, resulting in virus spread via the bloodstream and severe pathological effects, such as edema, haemorrhage and necrosis, with multiple organs being involved [20]. The severely progressive clinical manifestation in chickens generally causes multiple organ failure and death. The administration of a high viral dose directly into the bloodstream has facilitated the rapid and excessive virus replication in endothelial cells of the experimentally infected chickens.

In contrast to chickens, no significant tropism for endothelial cells was observed in Pekin ducks infected via both routes, supporting the hypothesis that endothelial cells have a limited contribution to the pathogenesis of HPAI viruses in ducks [25]. Like in previous experimental studies using HPAI H5N1 clade 2.1 and H5N8 clade 2.3.4.4 group A viruses [23,24], viral antigen was often present in epithelial cells in the respiratory organs of the HPAI H5N6 virus infected ducks, irrespective of the infection route. Viral antigen was also detected in mononuclear cells in the spleen of most ducks, indicating systemic spread of the virus occurred. Viral antigen expression in other internal organs of ducks, including the pancreas and brain, has been reported after experimental infection with many other HPAI GsGd-lineage viruses of diverse subtypes [24,27], but was not observed for the HPAI H5N6 virus in Pekin ducks in this study. Several other studies also reported no to limited expression of viral antigen in the brain and pancreas of ducks [22,23,28,29], indicating tissue distribution varies between strains.

Although the morphology of histopathological findings were comparable between poultry species and infection routes, variations in severity were observed. Most tissues from naturally infected chickens showed more severe histopathological changes compared to naturally infected ducks, except in the lung, which showed less severe histopathological changes. These findings correlate with the broad expression of viral antigen in naturally infected chickens. In naturally infected ducks, histopathological changes were also present in most organs, despite viral antigen expression was lacking in most organs other than the lung and air sac. The discrepancies between the histopathological and IHC results in these birds, as well as variations between individual ducks, may be attributed to unknown variations in field conditions, age or co-infections with other avian pathogens. After intravenous inoculation, severe histopathological changes were found in the spleen of both species, which correlated with abundant viral antigen expression.

Interestingly, more severe histopathological changes were found in the lung of Pekin ducks compared to chickens, while influenza virus antigen expression was comparable or less abundant. Moreover, fibrinous exudates were abundant in parabronchi of the lung of both naturally and experimentally infected ducks, despite no viral antigen was expressed in endothelial cells. These changes have probably compromised oxygen exchange, which may have led to acute respiratory failure and death. The combined histopathological and IHC results indicate a higher pathogenicity of the virus in lung tissue of Pekin ducks compared to

chickens. Viral replication in the respiratory tract, especially the lung, may have contributed to the pathogenesis of HPAI H5N6 virus in Pekin ducks.

In conclusion, this study demonstrates a more extensive distribution of HPAI H5N6 virus in chickens compared to Pekin ducks. In chickens, microscopic lesions and viral antigens were present in multiple organs, whereas in Pekin ducks severe histopathological changes and viral antigens were particularly detected in the lung. Acute pneumonia and subsequent respiratory failure may have contributed to severe disease and mortality among Pekin ducks. Even though intravenous inoculation is not the natural mode of infection and field conditions were not fully controlled, the study contributes to an increased understanding about factors that may contribute to severe disease and death among poultry species infected with HPAI H5N6 virus. Experimental studies using more natural infection routes are required to further investigate virus pathogenicity among avian species.

ETHICAL STATEMENT

The animal experiment and associated procedures were conducted in accordance with the national regulations on animal experimentation under the approval of the Dutch Central Authority for Scientific Procedures on Animals (CCD) (permit number AVD4010020172824; experiment number 2017.D-0054.001), as part of a previous study [18]. The experiment was performed conform the guidelines from the European Union directive 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes [30]. The experiment was performed in biosafety level 3 facilities at Wageningen Bioveterinary Research (WBVR, Lelystad, the Netherlands).

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AUTHOR CONTRIBUTIONS

Conceptualization: S.B., N.S., N.B.; Methodology: S.B., N.S., N.B.; Validation: S.B., R.Z.; Formal Analysis: S.B., S.V., R.Z.; Investigation: S.B., S.V.; Resources: N.B.; Data Curation: S.B.; Visualization: S.B., S.V.; Supervision: N.B.; Project Administration: N.B.; Funding Acquisition: N.B.; Writing – Original Draft Preparation: S.B.; Writing – Review & Editing: S.V., R.Z., N.S., N.B.

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CONFLICTS OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Supplementary information

SUPPLEMENTARY TABLES

S1 Table. Histopathological scores per bird.
S2 Table. Immunohistochemical scores per bird.

S1 TABLE. HISTOPATHOLOGICAL SCORES PER BIRD.

Histopathological analysis scores of various organs of chickens (n=5) and Pekin ducks (n=5) after natural infection and experimental intravenous inoculation with highly pathogenic avian influenza (HPAI) H5N6 virus. Sections of poor quality were excluded for analysis, indicated as not done (nd).

Organ	CN1	CN2	CN3	CN4	CN5	CE1	CE2	CE3	CE4	CE5	DN1	DN2	DN3	DN4	DN5	DE1	DE2	DE3	DE4	DE5
Brain	1.0	1.5	1.0	1.0	0.0	1.0	1.5	1.0	1.0	1.5	0.0	0.5	0.0	0.0	1.0	1.0	0.0	1.0	1.0	1.5
Conchae	3.0	3.0	3.0	3.0	3.0	1.0	2.5	1.0	1.0	1.5	1.0	2.5	3.0	1.5	1.5	1.0	2.0	2.0	2.0	2.0
Trachea	nd	nd	nd	3.0	3.0	2.0	2.5	1.5	2.5	2.5	2.0	3.0	1.5	2.0	1.0	nd	nd	nd	nd	nd
Lung	1.0	1.0	1.0	1.0	1.0	nd	2.0	2.0	2.0	2.0	2.0	3.0	2.0	3.0	2.0	3.0	3.0	3.0	3.0	3.0
Air sac	nd	1.0	2.5	3.0	3.0	1.5	nd	0.5	nd	nd	2.0	3.0	0.5	2.0	1.5	2.0	2.0	nd	3.0	nd
Ileum	0.0	0.0	2.0	1.0	1.0	nd	0.0	0.0	0.0	1.0	3.0	0.0	0.0	0.0	0.0	1.0	2.0	1.0	1.0	1.0
Cloaca	2.0	2.0	2.0	2.0	2.0	nd	2.0	1.5	3.0	2.0	nd	1.0	2.0	1.0	0.0	nd	nd	0.5	0.0	nd
Spleen	2.0	2.0	1.0	2.0	1.0	nd	3.0	3.0	3.0	3.0	2.0	2.0	2.0	2.0	1.0	3.0	3.0	2.0	3.0	3.0
Pancreas	2.5	2.0	3.0	2.0	2.0	nd	0.0	0.0	0.0	0.0	3.0	nd	2.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0

Scores: none (0), mild (1), moderate (2), severe (3). Abbreviations: CE, experimentally infected chicken; CN, naturally infected chicken; DE, experimentally infected duck; DN, naturally infected duck; nd, not done.

S2 TABLE. IMMUNOHISTOCHEMICAL SCORES PER IRD.

Immunohistochemical analysis scores of various organs of chickens (n=5) and Pekin ducks (n=5) after natural infection and experimental intravenous inoculation with highly pathogenic avian influenza (HPAI) H5N6 virus. Sections of poor quality were excluded for analysis, indicated as not done (nd).

Organ	CN1	CN2	CN3	CN4	CN5	CE1	CE2	CE3	CE4	CE5	DN1	DN2	DN3	DN4	DN5	DE1	DE2	DE3	DE4	DE5
Brain	1.0	0.0	0.0	0.0	0.0	0.5	2.0	1.5	2.0	2.5	0.5	0.5	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.5
Conchae	3.0	3.0	3.5	3.0	3.0	2.0	2.5	2.5	2.5	3.0	0.0	0.5	1.0	0.5	0.5	0.5	2.0	2.0	1.0	2.5
Trachea	nd	nd	nd	2.0	1.0	3.0	3.0	2.5	2.5	3.0	0.5	0.0	0.0	1.0	0.5	nd	nd	nd	nd	nd
Lung	2.5	1.0	0.5	1.0	1.5	4.0	4.0	4.0	4.0	4.0	2.0	2.0	2.0	2.0	1.0	3.0	3.0	3.0	2.5	3.0
Air sac	nd	1.0	1.5	3.0	3.5	2.0	nd	2.5	nd	nd	2.5	2.0	1.5	2.0	0.5	3.0	1.5	nd	1.0	nd
Ileum	0.5	0.0	0.0	0.0	0.0	2.5	3.0	3.0	3.0	3.0	0.5	0.0	0.5	0.0	0.0	0.5	0.0	0.5	0.5	1.0
Cloaca	3.0	3.0	0.0	1.5	1.5	3.5	3.0	3.0	3.0	3.0	nd	0.0	0.5	0.0	0.0	nd	nd	0.5	0.0	nd
Spleen	3.0	0.5	0.0	1.0	2.0	3.5	4.0	4.0	4.0	4.0	2.0	0.5	0.0	0.0	0.0	3.0	2.5	3.0	3.0	3.5
Pancreas	2.5	2.5	3.5	3.0	2.5	1.0	1.5	3.0	2.5	2.5	0.0	nd	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Scores: negative (0), sparse (1), moderate (2), abundant (3), excessive (4). Abbreviations: CE, experimentally infected chicken; CN, naturally infected chicken; DE, experimentally infected duck; DN, naturally infected duck; nd, not done.

CHAPTER 7

Summarizing discussion

Saskia A. Bergervoet

Summarizing discussion

Avian influenza (AI) is an infectious viral disease of birds caused by influenza A viruses that belong to the family of *Orthomyxoviridae*. AI viruses have been isolated from a wide range of bird species, but wild waterfowl belonging to the orders *Anseriformes* (including ducks, geese and swans) and *Charadriiformes* (including gulls, terns and waders) are considered the natural reservoir hosts [1]. The viral genome is composed of eight negative-sense RNA gene segments that encode the surface proteins hemagglutinin (HA) and neuraminidase (NA), eight internal proteins, and several nonessential accessory proteins (**Chapter 1**) [2]. Currently, 16 HA (H1-H16) and 9 NA (N1-N9) subtypes have been identified in many different combinations in birds [3-6]. Frequent mutations and reassortment of the segmented genomes of AI viruses have led to a high genetic diversity and drives the constant emergence of novel virus strains [7-9].

Most AI viruses are low pathogenic avian influenza (LPAI) viruses that circulate in wild birds without signs of disease. Periodically, AI viruses spill over from wild birds to other hosts, including mammals and poultry (chickens, domestic turkeys and domestic ducks). In poultry, some LPAI strains can cause mild clinical signs, such as mild respiratory disease, a reduction in egg production or low mortality [10]. Once introduced into poultry, LPAI viruses of subtypes H5 and H7 can mutate into highly pathogenic avian influenza (HPAI) viruses [11]. HPAI virus infections can cause variable clinical signs in wild birds, and are characterized by severe disease and high mortality in poultry. For this reason, viruses of subtypes H5 and H7 in poultry are considered notifiable by the World Organisation for Animal Health (OIE) [12,13]. Outbreaks of AI viruses can have a major impact on animal health and economic consequences for poultry industries. In addition, some AI viruses can be transmitted to humans, and thus pose a substantial threat to public health. The recurrence of AI virus outbreaks highlights the importance of the early detection, control and prevention of AI introductions into poultry.

This thesis

This thesis aimed to gain more insight in the spread of AI viruses at the wild bird-poultry interface, thereby contributing to the knowledge on how to efficiently monitor and prevent the introduction and spread of AI viruses from wild birds to poultry. Based on routinely collected surveillance data in the Netherlands, we found that LPAI viruses of certain subtypes were frequently detected in poultry, but only sporadically in wild birds (**Chapter 2**), indicating undetected circulation of these subtypes in the wild bird population. In contrast, LPAI virus subtypes that were common in wild birds, were rarely detected in poultry. The observation that chickens are susceptible for these wild bird-biased subtypes indicated that LPAI viruses are likely not transmitted directly from frequently sampled wild bird species to poultry (**Chapter 3**). In addition, we demonstrated that poultry flocks in the Netherlands were not only infected with LPAI viruses from wild birds, but also by transmission between farms (**Chapter 4**). Finally, we investigated the potential contribution of the timing of emergence of novel HPAI reassortant viruses (**Chapter 5**), and variations in HPAI virus pathogenicity between species (**Chapter 6**), in determining the spread of HPAI viruses at the wild bird-poultry interface.

LPAI VIRUSES AT THE WILD BIRD-POULTRY INTERFACE

In the last two decades, the circulation of AI viruses in wild birds and poultry has been studied intensively in many countries worldwide, including the Netherlands. Surveillance programs have been implemented for the early detection of emerging AI viruses, but have also been used to study the spatiotemporal distribution, subtype diversity and evolution of AI viruses. These surveillance programs have provided many insights into the complex epidemiology of AI viruses. Still, wild bird species that act as source of infection for poultry and transmission routes often remain unidentified. Previous studies have shown that wild birds carry a wide variety of AI viruses, but the distribution of subtypes varies between the wild bird species. *Charadriiformes* species, in particular gulls, are predominantly infected with LPAI viruses of subtypes H13 and H16, which are only occasionally found in other birds [4,6]. Among *Anseriformes* species, of which mallards have been most extensively studied, LPAI viruses of subtypes H3, H4 and H6 are most frequently detected, while other subtypes (H8-H12) are rare [14-18]. Analyses of the subtype distribution and the genetic compositions of wild bird and poultry viruses can be used in efforts to identify wild bird species at source of AI virus introductions into poultry. Illuminating sources and transmission routes of AI viruses into poultry is pivotal to the design of effective strategies to prevent introductions and manage further spread.

Undetected circulation of LPAI viruses in the wild bird population

As part of this thesis, we explored the circulation of LPAI viruses in wild birds and poultry in the Netherlands between 2006-2016 using routinely collected surveillance data (**Chapter 2**). We found that wild birds in the Netherlands were frequently infected with LPAI viruses and infection of poultry was not uncommon. Most LPAI viruses in wild birds were isolated from mallards and gulls. LPAI virus infections in poultry were most frequently detected in outdoor chickens, turkeys and domestic ducks. A wide range of LPAI virus subtypes was identified, with differences in subtype incidence between *Charadriiformes* and *Anseriformes* wild bird species corresponding with previous studies [4,6,14-17]. The study also revealed differences in the subtype distribution between wild bird species and poultry. While LPAI viruses of subtypes H8 and H9 were frequently detected in poultry in the Netherlands, these subtypes were rarely detected in wild birds. This is remarkable, given the fact that wild birds are considered the primary source of AI virus infection for poultry. We hypothesized that the transmission of viruses of certain subtypes from wild birds to poultry is selective, and may depend on viral factors that determine host specificity. H8 and H9 viruses may be able to infect poultry more easily and exhibit a higher fitness in poultry. For example, certain H9 strains have been circulating enzootically in poultry in Asia [19,20], indicating infectivity and efficient transmission of the virus in poultry. However, phylogenetic analysis showed that H9 strains isolated from poultry in the Netherlands are not related to the Asian virus lineages, but share clades with viruses isolated from wild birds, particularly from mallards in the Netherlands and Sweden (**Chapter 2**). Experimental infection of chickens with H8 and H9 viruses isolated from wild ducks in the Netherlands resulted in limited and strain-dependent replication (**Chapter 3**). These findings indicate that LPAI viruses may not directly transmitted from mallards and other frequently sampled wild bird species to poultry. This is supported by the fact that no direct genetic links were identified between wild bird and poultry viruses in the Netherlands, indicating prolonged undetected circulation and frequent reassortment of LPAI viruses in wild birds before entry

into poultry (**Chapter 2**). The results suggest that LPAI viruses of subtypes detected in poultry may be more prevalent in avian species or periods of the year for which sampling activities are low.

Mallards are considered important hosts for the maintenance and dispersal of LPAI viruses, as they are the most abundant dabbling duck species, infected with LPAI virus at high prevalence, carry a wide range of LPAI virus subtypes and migrate over large geographical distances. Mallards have therefore been the focus of influenza surveillance studies in many countries [1,5,16-18,21,22], including the Netherlands (**Chapter 2**) [23]. A study in Sweden indicated that mallards have a role in the maintenance of rare subtypes in wild birds, including H8 and H9 [15]. It was shown that the internal genes of LPAI viruses of rare subtypes fall into clades with LPAI viruses of subtypes that are detected at high frequencies in mallards. However, the infrequent detection of certain LPAI virus subtypes suggests that mallards may not be the primary reservoir for all subtypes [18], and may not be the primary reservoir for LPAI viruses that infect poultry. It has been hypothesized that viruses of rare subtypes, including H8 and H9, have a reservoir host other than mallards [15], which have not been sampled frequently during surveillance, e.g. because these birds are harder to catch. *Anseriformes* species that also carry LPAI viruses include other dabbling ducks, such as Eurasian wigeons, common teals and gadwalls [21,24], diving ducks [25,26], and non-duck species, such as geese [23,27] and swans [28-31]. In addition, seabirds that belong to the *Charadriiformes* order, in particular common murres, were relatively frequently infected with the antigenically related influenza A group 1 viruses, which include viruses of subtypes H8 and H9 [32]. Thus, certain wild bird species may be predominant hosts for LPAI virus subtypes and strains that infect poultry, but remain unidentified due to limited sampling. It is also possible that certain unfrequently sampled wild bird species act as intermediate (bridge) hosts between mallards and poultry. If the bridge host is susceptible and able to transmit H8 and H9 viruses, and comes into direct contact or share their habitat with both mallards and poultry [33], then these viruses would be likely detected more frequently in chickens, even if the subtypes are rare in mallards. We observed that geese were frequently infected with LPAI viruses of the same subtype combinations as poultry, e.g. H6N2, H6N8 and H7N7 (**Chapter 2**), indicating a role for geese in the transmission of LPAI viruses to poultry. However, no viruses of subtypes H8 and H9 have been isolated from this species, and genetic evidence for direct transmission of LPAI viruses from geese to poultry is lacking. Increased sampling of non-traditional species is needed to identify potential wild bird species of importance for virus transmission to poultry.

The infrequent detection of H8 and H9 viruses in wild birds may also be affected by the time at which sampling activities were performed. In most surveillance studies, sampling of wild birds is done during autumn migration, when the LPAI virus prevalence in wild birds is high and sample collection is easy. LPAI viruses of common subtypes may be detected more frequently due to a higher fitness in mallards, while subtypes with a lower fitness are less frequently detected due to competition [15], producing a biased picture of which subtypes circulate in wild birds year-round. After the prevalence peak, re-infections with the same or related HA subtypes are uncommon due to host population immunity [34,35], enabling infection of more distantly related viruses. Indeed, viruses of subtype H8 were detected most frequently outside the prevalence peak during a year-round surveillance study in mallards in Sweden [15]. A bias in the timing of sampling may also be a reason why LPAI viruses of subtypes H8 and H9 were only sporadically detected in wild birds in the Netherlands (**Chapter 2**). Increased sampling outside the prevalence peak may allow the detection of rare subtypes.

Common LPAI virus subtypes in wild birds are rarely detected in poultry

In contrast to the H8 and H9 subtypes, viruses of subtypes H3 and H4 were frequently detected in wild birds in the Netherlands and other European countries [14-16], but rarely in poultry (**Chapter 2**). It has been suggested that subtypes that are frequently detected in mallards may have increased host specificity and therefore may be less able to infect other hosts [15], such as chickens. However, an infection experiment demonstrated that chickens are susceptible to infection with wild duck-origin LPAI viruses of subtypes H3 and H4, despite their infrequent detection in poultry in surveillance studies (**Chapter 3**). The results suggest that the infrequent detection of H3 and H4 viruses in poultry is not caused by host range restrictions of the virus or a lower susceptibility of chickens for these subtypes. If virus transmission from mallards to chickens occurs via another avian (bridge) host, this species may act as a barrier for transmission of H3 and H4 viruses. However, the fact that mallards have been identified as frequent visitors of free-range areas of Dutch chicken farms [23,36] indicates that transmission between mallards and chickens could take place without the intervention of a bridge host. Although no direct contact between mallards and poultry was observed, transmission from mallards to poultry may occur via indirect contact, e.g. via faeces-contaminated water or soil [36]. In this situation, the environment provides a bridge for LPAI virus transmission from wild birds to poultry. It is possible that the persistence of LPAI viruses in the environment varies between subtypes and strains [37] and might be reduced for the H3 and H4 viruses, resulting in a low number of introductions of these subtypes into poultry flocks.

Another explanation for the rare detection of subtypes H3 and H4 in poultry could be that virus introduction and circulation of these subtypes in poultry remained undetected during routine screening due to subtype-dependent variations in antibody responses. Poultry farms are tested at least once a year for the presence of antibodies against AI viruses. These antibodies are generally detectable for several weeks or months [38]. However, the antibody response of chickens against viruses of subtypes H3 and H4 may be lower or waning earlier than for viruses of subtypes H8 and H9. If so, viruses of subtypes H3 and H4 may not be detected in poultry due to insufficient sampling. This would also mean that the number of LPAI virus introductions into poultry is higher than expected based on currently available surveillance data. Long-term experimental studies that investigate antibody responses against different subtypes and strains in poultry are needed to determine potential variations between LPAI viruses.

The role of between-farm transmission in LPAI virus spread

Although wild birds are considered the primary source of LPAI virus infections in poultry, flocks may also become infected by transmission between farms. Continuous monitoring and control of LPAI viruses in poultry is particularly important to prevent the large-scale spread of LPAI viruses of subtypes H5 and H7, which can eventually mutate into HPAI viruses in poultry. Previous HPAI outbreaks have demonstrated the devastating consequences of what happens without controlling LPAI H5 and H7 viruses in poultry, of which the HPAI H7N1 outbreak in Italy in 2000-2001 is a prime example [39]. The HPAI H7N7 virus that caused a large outbreak in the Netherlands in 2003 likely also emerged after the introduction of a LPAI virus in poultry [40]. The spread of LPAI H5 and H7 viruses in poultry increases the likelihood of LPAI-to-HPAI transition events and the occurrence of other virulence mutations. Sustained between-farm transmission of LPAI viruses has most frequently been reported for viruses of subtypes H5 and H7 [39,41-46]. However, recurrent outbreaks of H9N2 virus infections in Asia and the Middle East

since 1990 [19,20] and a recent outbreak of H3N1 virus in Belgium in 2019 [47] demonstrated that LPAI viruses of other (non-notifiable) subtypes can also spread rapidly in poultry with major socio-economic impact on the poultry industries. Therefore, it is important not only to prevent LPAI virus introductions from the wild bird reservoir, but also to prevent virus transmission between poultry farms.

Genetic analysis of poultry viruses in the Netherlands demonstrated that between-farm transmission likely contributed considerably to the incidence of LPAI virus infections in poultry in the Netherlands between 2006-2016 (**Chapter 4**). Contrary to previous LPAI outbreaks with sustained transmission between farms, the between-farm transmission events in the Netherlands have not led to massive spread of LPAI viruses. The spread of LPAI viruses may be affected by many factors that determine transmission between poultry flocks, such as biosecurity measures, poultry density, poultry production types, between-farm contacts and trading movements, and virus characteristics. Decreased biosecurity in poultry dense areas was probably the most important factor in the spread of LPAI H7N1 virus in Italy [48]. Strict biosecurity is considered the most effective measure to prevent outbreaks and subsequent spread in poultry. However, knowledge on transmission routes is fundamental to design effective preventive measures. Various routes of between-farm transmission have been suggested, including direct contact between poultry, indirect contact via the movement of persons, contaminated materials, or non-avian vectors between farms [49-54], and transmission of virus via water, air or dust [55-58]. The risk of virus transmission likely depends on the distance between farms [59,60] and is thought to occur more frequently between farms of the same poultry type due to a higher probability of contacts [51]. Indeed, we identified clusters of farms infected with the same virus, but also showed that between-farm transmission was not restricted to holdings of the same poultry production type (**Chapter 4**). Unfortunately, the routes of transmission of LPAI viruses between the infected farms in the Netherlands could not be resolved, which is partly due to the limited number of virus isolations from poultry and the lack of contact tracing.

Between-farm transmission may also depend on the characteristics of the virus. Large-scale spread of viruses is influenced by the ability of the virus to replicate and be shed for a sufficient period, which allows transmission between hosts. For example, the rapid spread of HPAI H5N2 virus in turkeys in the USA in 2014-2015 was likely associated with an unusually long pre-clinical period, high levels of virus shedding, and increased adaptation of the virus to turkeys [61]. For some of the LPAI viruses that were transmitted between poultry holdings in the Netherlands, we identified a deletion in the stalk region of the NA protein (**Chapter 4**), which is an important adaption marker in *Galliformes* hosts (chickens and turkeys) [62,63] and may influence virus replication, excretion and pathogenicity [64-66]. An experimental study on the transmission dynamics of LPAI H5N7 and H7N7 viruses in chickens indicated that transmission characteristics can vary considerably between strains [67]. Experiments comparing transmission efficiencies of different LPAI virus strains in various poultry types are limited, in particular for non-notifiable subtypes.

Optimisation of LPAI virus surveillance efforts

A reduction in time and resources for AI virus surveillance in live wild birds has been observed in recent years. In addition, surveillance in poultry will likely not be expanded, since the newly adopted regulation on transmissible animal diseases ('Animal Health Law') no longer obliges European Union (EU) member states to take immediate eradication measures, while control

measures and surveillance remain required for LPAI H5 and H7 viruses from April 2021 onwards [68]. However, continued or even expanded monitoring with more targeted surveillance approaches is needed in order to better understand how LPAI viruses are spread at the wild bird-poultry interface. The undetected circulation of LPAI viruses in the wild bird population is likely due to biases for target species, locations and times in the surveillance studies, hampering the identification of wild bird risk species for LPAI virus introduction into poultry. Surveillance may be improved by determining which wild bird species are common near farms, allowing targeted sampling of species within specific periods of the year. Video-camera monitoring has recently been used to quantify wild fauna visits in free-range facilities of an outdoor chicken farm in the Netherlands, providing round-the-clock monitoring data [36]. Although this study is limited to one farm, it confirms previous observations that mallards are common near farms [23], and also identifies a wide range of other species that may be involved in the transmission of viruses to poultry. There have also been research efforts to develop a method to investigate (indirect) contacts between wild birds and poultry based on faecal microbiome compositions [69]. These studies contribute to the identification of potential wild bird risk species for infection of poultry, and a better understanding of the role of wild birds in the transmission of LPAI viruses to poultry. It remains unclear why certain subtypes that are common in mallards are not transmitted to poultry, or remain undetected, while indirect contact between the two bird groups has been observed. Experimental studies on the persistence of LPAI viruses in the environment, the within-flock transmissibility of LPAI viruses, and the induction of adaptive immune responses upon LPAI virus infections in poultry are limited, but would provide more information on subtype-dependent differences in LPAI virus dynamics.

Surveillance for LPAI virus infections in poultry in the Netherlands is more stringent than required by the EU programme [70,71]. For example, outdoor chickens and turkeys are tested more frequently than other poultry types because of the assumed higher risk for LPAI virus infections [72-74]. In addition, multiplex serological methods have been used for rapid diagnostic screening for all subtypes [75]. In contrast, in most EU member states, serological monitoring in poultry is limited to LPAI viruses of subtypes H5 and H7. Heightened surveillance of high-risk poultry species and the implementation of multiplex serological methods in other European countries would provide more insight into the occurrence of non-notifiable LPAI virus subtypes in poultry outside the Netherlands, allowing larger-scale comparison between subtype distributions in wild birds and poultry. Poultry surveillance in the Netherlands could be improved by increasing routine sampling of domestic ducks, which also have a higher risk for LPAI virus introduction [74,76,77], and were relatively frequently infected with LPAI viruses in the Netherlands despite their relatively small population size (**Chapter 2**). In addition, LPAI virus infections often remain unnoticed until routine serological screening due to a lack of clinical signs, while virus isolation and subsequent genetic characterisation can be used to trace viruses beyond antigenic subtype and allows comparative analysis between wild bird and poultry viruses. Therefore, increased virological screening for non-notifiable LPAI viruses in poultry would be valuable to increase our understanding of the transmission of LPAI viruses from wild birds to poultry and between farms. Also, contact tracing and tracking of secondary infections is generally not performed during LPAI virus outbreaks of non-notifiable subtypes, but would be valuable to further gain clarity on virus transmission routes between farms.

HPAI VIRUSES AT THE WILD-BIRD POULTRY INTERFACE

HPAI outbreaks do not only occur after LPAI viruses of subtype H5 or H7 mutate into HPAI variants in poultry with subsequent transmission between farms, but also when HPAI viruses of poultry spill over into wild birds, circulate in wild birds, and eventually enter poultry farms elsewhere. In recent years, many countries worldwide have encountered outbreaks of HPAI viruses in poultry that were introduced by wild birds. These outbreaks were caused by HPAI viruses belonging to the H5 A/Goose/Guangdong/1/96 (GsGd) lineage, which emerged in China in 1996 [78], and have caused numerous outbreaks in poultry in Asia since 1997. The HPAI H5 GsGd lineage viruses continued to circulate in poultry and evolved into multiple genetic clades and subtype combinations (i.e. H5N2, H5N3, H5N5, H5N6 and H5N8) [79]. A role for wild birds in the spread of these viruses was already suggested during outbreaks of HPAI H5N1 clade 2.2 virus in poultry in Asia during 2001-2004 [80], but became more evident during a large-scale HPAI H5N1 outbreak in migratory birds in Qinghai Lake, China, in spring 2005 [81,82], and the subsequent global expansion of HPAI H5N1 viruses during the 2005-2006 epizootic [5]. These HPAI H5N1 viruses were highly pathogenic for some wild bird species [81,82], while prior to the detection of HPAI H5 GsGd lineage viruses, HPAI viruses were rarely detected in wild birds, and outbreaks of severe disease and high mortality were confined to poultry [83]. The spread of HPAI viruses in wild birds can be influenced by many factors, such as wild bird species infected, pre-existing immunity due to previous infections, the timing of reassortment events, or alterations in virus characteristics. With introductions and subsequent circulating of HPAI viruses in the wild bird population, it has become increasingly important to prevent re-introductions into poultry. A better understanding of HPAI virus circulation in wild birds will help to predict virus spread in wild birds and the risk of exposure of these viruses to poultry.

The timing of reassortment influences HPAI virus spread in wild migratory birds

In late 2014, outbreaks of HPAI H5N8 clade 2.3.4.4 group A viruses in Europe [84,85] and North America [61] occurred simultaneously, preceding a detection of the virus in South Korea in early 2014 [86]. In a comprehensive global analysis based on genetic, epidemiological and ornithological data, long-distance migratory birds were identified as the most likely ancestral hosts for both outbreaks [87], confirming previous outbreak studies [88-90]. The affected poultry farms were often located in wild bird areas, and indirect contact with materials contaminated with wild bird faeces was considered the most likely route of introduction into poultry farms [87]. Other routes, such as virus introduction via trade of infected poultry or poultry products, were considered less likely. The analysis also identified main routes of large-scale geographical spread of the H5N8 virus by migratory birds, from Southeast Asia to northern breeding grounds during spring, and then in autumn from these breeding grounds to wintering sites in North America and Europe [87].

Two years later, in 2016-2017, the most extensive HPAI outbreak occurred in Europe [91]. This outbreak was characterized by the detection of many different reassortment viruses. In total, five different HPAI H5N8 viruses of clade 2.3.4.4 group B reassortant viruses were detected in Europe [92-95], containing variable genome segment constellations by reassortment with co-circulating LPAI viruses. In addition, three HPAI H5N5 reassortant viruses and one HPAI H5N6 virus were identified during this epizootic [92-99] (**Chapter 5**), which were detected at lower frequencies compared to H5N8. Like the H5N1 clade 2.2 viruses and H5 clade 2.3.4.4 group A viruses, the H5 clade 2.3.4.4 group B viruses were detected at northern breeding grounds in

spring [100,101], prior to their introduction into Europe during autumn. Presumably, the viruses were introduced into Europe by migratory wild birds that travel over long distances from northern breeding grounds to wintering sites in Europe, similar to the HPAI H5N8 clade 2.3.4.4 group A virus in 2014 [87]. The detection of a HPAI H5N5 virus in the Russian Far East also indicated that the viruses were disseminated by migratory birds via a separate flyway towards the Beringia region [96] (**Chapter 5**). However, in contrast to the HPAI H5N8 clade 2.3.4.4 group A virus [88], H5N5 viruses were not detected in North America. Phylogenetic analysis also indicated similarities in the spread of HPAI viruses within Europe, with multiple independent introductions of H5N5 viruses into Europe followed by local spread (**Chapter 5**), similar to H5N8 clade 2.3.4.4 group B viruses [94,102].

Information on patterns of global HPAI virus spread, combined with estimates of the timing of reassortment events, can be used to estimate when (i.e. before, during or after the migration of birds from their breeding grounds) and where (i.e. at breeding grounds or along migration routes to Europe) reassortment events occurred, providing more insight into the evolutionary history of the viruses. The emergence of novel strains just before or during the breeding season may enable virus spread to many birds at breeding sites, and subsequent widespread dispersal to other (wintering-) areas, while strains that emerge at the end of the breeding season, after the start of the autumn migration, may be transmitted only to a relatively low number of wild birds. Indeed, the most frequently detected and geographically widespread HPAI H5N8 reassortant probably emerged in spring, while the less frequently detected H5N8 reassortant likely emerged in summer [92]. Likewise, the most frequently isolated HPAI H5N5 reassortant probably emerged in summer, while the H5N5 reassortant that was detected at low frequencies likely emerged in autumn [94] (**Chapter 5**). Although alterations in the virus genome may also influence HPAI virus spread, e.g. by affecting virus infectivity, transmissibility and pathogenicity, infection of primary chicken and duck cells revealed only minor differences in cytopathogenicity and replication kinetics between H5N5 variants and H5N8 viruses (**Chapter 5**). Thus, the timing of emergence may be an important driver of variations in the number of detections of different virus subtypes and reassortant variants, and may also explain the limited distribution of H5N5 viruses compared to H5N8 viruses. Comprehensive analysis of the viral genome increases our understanding of the evolution and spread of HPAI viruses in the wild bird population.

Differences in HPAI virus pathogenicity among wild bird and poultry species

The number of recorded HPAI virus infections varied between the outbreaks of H5N8 clade 2.3.4.4 group A viruses 2014-2015 [84], H5N8 clade 2.3.4.4 group B viruses in 2016-2017 [92], and H5N6 clade 2.3.4.4 group B viruses in 2017-2018 in Europe [103,104]. In addition, apparent differences in mortality were observed in wild birds [91]. The H5N8 clade 2.3.4.4 group A virus was detected in only a limited number sick or dead wild birds, and in faecal samples of Eurasian wigeons in the Netherlands [105]. Also, HPAI virus-biased antibodies were detected in wild birds in the Netherlands, mainly Eurasian wigeons and swans [106]. In contrast, during the H5N8 clade 2.3.4.4 group B outbreak, mass deaths of wild birds were reported, of which most involved tufted ducks and Eurasian wigeons [107]. During this outbreak, virus was also detected in apparently healthy birds, mainly mallards [102], indicating that these species may be more resistant and therefore can act as a reservoir. HPAI virus-biased antibodies were also detected in several wild bird species, including mallards, gulls, Eurasian wigeons, and swans [102]. The

H5N6 clade 2.3.4.4 group B virus also caused lethal infections in wild birds of various species, but the number of detections was low compared to the H5N8 2.3.4.4 group B outbreak [104].

Previous experimental studies in mallards and Pekin ducks have demonstrated that HPAI GsGd viruses of diverse subtypes have reduced virulence compared to the parental H5N1 virus [108-111], showing that virus pathogenicity varies between subtypes and strains. Also, the susceptibility to disease and the ability to efficiently shed virus differs between species [111]. Corresponding with the field observations, H5N8 clade 2.3.4.4 group A virus infection was subclinical in common waterfowl species [110]. Eurasian wigeons showed the highest excretion of virus [110], which is in line with the detection of virus in faeces and antibodies in this species during the outbreak. In contrast, an experimental study with H5N8 clade 2.3.4.4 virus group B virus showed high mortality among mallards [112], which corresponds with the high number of deaths among wild birds during the outbreak. Pre-existing immunity may have existed in the mallard population, explaining the detection of this virus in apparently healthy mallards during the outbreak.

Virus pathogenicity of HPAI H5 GsGd lineage viruses also differed between poultry species, with attenuated and less invasive infection in ducks as compared to chickens [113,114]. Intravenous pathogenicity index (IVPI) tests confirmed high pathogenicity for all three outbreak strains in chickens. However, interestingly, IVPI tests in Pekin ducks, the most common domestic duck species, revealed a lower pathogenicity score for the H5N8 2.3.4.4 group A virus compared to the 2.3.4.4 group B viruses of subtypes H5N8 and H5N6 [104]. Although the H5N6 virus showed similar high pathogenicity in both chickens and Pekin ducks, analysis of the virus distribution and associated histopathology between various organs still revealed less extensive virus distribution in ducks as compared to chickens (**Chapter 6**). Also, histopathological changes and viral antigen was detected in most organs of chickens, whereas in ducks the presence of microscopic lesions and the expression of antigen were more prominent in respiratory organs, especially the lung (**Chapter 6**), indicating that the presentation of the disease varies between the two poultry species. Moreover, previous experimental studies with HPAI H5 GsGd lineage viruses have demonstrated viral antigen expression in the pancreas and brain [108,109,115-117], but this was not observed for the HPAI H5N6 virus in Pekin ducks (**Chapter 6**), indicating that the tissue distribution can also vary between strains. Changes in virus pathogenicity may influence HPAI virus infection dynamics in wild birds and poultry. If the virus does not cause serious disease, infected wild birds will likely continue to migrate, allowing virus dispersal over large geographical distances. In addition, the viruses may remain undetected due to a lack of overt clinical signs, but pose a high risk for susceptible bird populations, such as poultry. Experimental studies determining virus infectivity, transmissibility and pathogenicity of novel HPAI reassortant viruses in different bird species are of great value to better understand the role of viral characteristic changes in HPAI virus spread.

Optimisation of HPAI virus surveillance efforts

Based on the global patterns of HPAI virus spread that have been identified during previous outbreaks, recommendations can be made with regard to wild bird surveillance sampling location and timing. Increased sampling activities in Southeast Asia before the breeding season in early spring, in northern breeding areas in the breeding season in spring and early summer, and/or sampling at (better accessible) stopover sites along major migration routes in late summer and during autumn, would help to predict the spread of HPAI viruses to Europe.

Sampling at sites where large amount of wild birds from different migration routes come in contact may be most efficient. In addition, local sampling of wild birds in Europe during the arrival of migratory birds in autumn and early winter would be advisable for the early detection of HPAI viruses entering Europe. Increased sampling along major wild bird flyways will increase available data to investigate viral dispersal in migratory birds, allowing a more detailed analysis of virus evolution in time.

Mallards have been the focus of many surveillance programs, but monitoring should also be targeted towards other key species in the dispersal of HPAI viruses that migrate over large distances, are less susceptible for disease, and therefore may be responsible for the global spread of HPAI viruses. Furthermore, variations in pathogenicity between virus strains among avian species must be taken into account during the design of surveillance strategies for HPAI viruses. Passive monitoring has been put in place by EU member states for the detection of HPAI viruses in sick or dead wild birds [70,71], but active monitoring is required for the detection of HPAI viruses in wild bird species that survive HPAI virus infection without presenting overt signs of disease. The latter approach would be valuable for the timely detection of HPAI viruses when no wild bird mortality is observed, and to identify wild bird species that are responsible for long-distance spread. Active surveillance by serological monitoring can also provide information on previous HPAI virus infections and the immune status of wild birds. Passive surveillance will remain useful for the detection of HPAI viruses in species that become sick or die upon infection, thereby acting as an early warning system for local virus circulation.

Although the current passive surveillance program for AI viruses in poultry is sufficient for the early detection of HPAI virus introductions into poultry, the threat of HPAI viruses circulating in wild birds highlights the importance of early control measures to prevent HPAI transmission to poultry. Control measures include keeping poultry indoors to prevent contact with wild birds when HPAI virus detections are reported in the Netherlands or other European countries, and enhancing farmer awareness and implementation of biosecurity measures during high-risk periods for HPAI virus introductions.

CONCLUDING REMARKS

Concluding, the studies described in this thesis provide novel insights into the spread of AI viruses at the wild bird-poultry interface. We demonstrated that detailed genetic analysis of AI viruses, including viruses of non-notifiable subtypes, is a valuable tool to investigate the origin, evolution and transmission patterns of viruses, thereby improving our understanding of AI virus circulation at the wild bird-poultry interface. The studies also emphasized the value of combining genetic analysis with information on the spatiotemporal distribution or phenotypic traits, such as the capacity of viruses to replicate or cause disease in specific hosts. The studies have led to recommendations regarding current national and international surveillance programs, including more targeted monitoring based on sampling location, timing and species. More targeted monitoring is pivotal to identify sources of virus infections in poultry and to elucidate potential routes of virus transmission into poultry and between farms. The implementation of more efficient monitoring and effective prevention of introduction and spread of AI virus in poultry is important to reduce the threat to both animal and human health.

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CHAPTER 8

Samenvattende discussie

Saskia A. Bergervoet

8

Samenvattende discussie

Aviaire influenza (AI), ook wel vogelgriep genoemd, is een besmettelijke vogelziekte die wordt veroorzaakt door het influenza A virus, dat behoort tot de familie *Orthomyxoviridae*. AI virussen komen van nature voor bij wilde vogels. De belangrijkste gastheren behoren tot de orders *Anseriformes* (waaronder eenden, ganzen en zwanen) en *Charadriiformes* (waaronder meeuwen, sternen en steltlopers). Het AI virus bevat een negatief enkelstrengs RNA genoom dat bestaat uit acht gensegmenten (**Hoofdstuk 1**). Twee gensegmenten coderen voor de hemagglutinine (HA) en neuraminidase (NA) eiwitten, die aan het oppervlakte van het virus tot expressie komen. Deze HA en NA eiwitten zijn respectievelijk betrokken bij het vasthechten aan en het verlaten van de gastheercel, en worden gebruikt om AI virussen onder te verdelen in subtypen. In vogels komen 16 HA (H1-H16) subtypen en 9 NA (N1-N9) subtypen voor, in variërende combinaties. De overige segmenten van het virale genoom bestaan uit de zogenoemde interne genen, die coderen voor eiwitten die onder andere verantwoordelijk zijn voor de replicatie van het virale genoom, eiwitexpressie en modulatie van het afweermechanisme van de gastheer. AI virussen kunnen in een hoog tempo veranderen. Tijdens de replicatie van het virus treden kleine veranderingen (mutaties) op in het virale genoom, wat resulteert in genetische variatie in de viruspopulatie. Daarnaast kunnen gensegmenten worden uitgewisseld tussen twee of meerdere AI virussen, waardoor grote genetische veranderingen optreden binnen een zeer korte tijdsperiode. Dit verschijnsel wordt ook wel reassortment genoemd. Door de snelle evolutie ontstaan continu nieuwe virusvarianten van nieuwe HA en NA subtype combinaties, waardoor het virus zich eenvoudig kan aanpassen aan zijn omgeving en de afweermechanismen van de gastheer omzeilt.

Naast de HA en NA subtypen worden AI virussen ook onderverdeeld op basis van het ziekmakend vermogen van het virus, ofwel de pathogeniteit. Er wordt onderscheid gemaakt tussen laagpathogene aviaire influenza (LPAI) virussen en hoogpathogene aviaire influenza (HPAI) virussen. De meeste AI virussen zijn LPAI virussen die in wilde vogels circuleren zonder ziekte te veroorzaken. Wilde vogels kunnen het AI virus overdragen naar andere dieren, waaronder zoogdieren en pluimvee (kippen, kalkoenen en eenden). In pluimvee kunnen sommige LPAI virussen milde ziekteverschijnselen veroorzaken, bestaande uit verminderde voeropname, eilegdaling of lage mortaliteit. LPAI virussen van subtypen H5 en H7 kunnen in pluimvee muteren naar de zeer besmettelijke HPAI variant. HPAI virussen veroorzaken wisselende ziekteverschijnselen in wilde vogels, en ernstige klinische verschijnselen en massale sterfte onder pluimvee. Om die reden is, ter voorkoming van grootschalige vogelgriepuitbraken, het melden en bestrijden van AI virussen van subtypen H5 en H7 in pluimvee verplicht gesteld door de Wereldorganisatie voor Diergezondheid (OIE), ook als het een LPAI variant betreft. Uitbraken van vogelgriep leveren niet alleen een direct gevaar op voor de diergezondheid, maar kunnen ook grote sociale en economische gevolgen hebben voor de pluimveehouderij. Bovendien kunnen sommige varianten mensen infecteren, en vormen daarmee een bedreiging voor de volksgezondheid. Het vroegtijdig herkennen, controleren en voorkomen van uitbraken is daarom van groot belang.

Dit proefschrift

Het in dit proefschrift beschreven onderzoek beoogt meer inzicht te verkrijgen in de verspreiding van AI virussen in wilde vogels en pluimvee. Deze kennis is belangrijk voor de ontwikkeling van efficiënte monitoringsstrategieën en effectieve maatregelen, ter voorkoming van AI virusintroductie en -verspreiding in pluimvee. Uit een langdurige monitoringsstudie naar AI virussen in wilde vogels en commercieel gehouden pluimvee in Nederland is gebleken dat LPAI virussen van bepaalde subtypen regelmatig in pluimvee worden gedetecteerd, maar slechts sporadisch in wilde vogels (**Hoofdstuk 2**). Dit suggereert dat LPAI virussen die pluimvee infecteren in wilde vogels circuleren, maar niet worden gedetecteerd. LPAI virussen die veel in wilde vogels voorkomen werden daarentegen zelden in pluimvee gevonden. De bevinding dat kippen experimenteel vatbaar zijn voor LPAI virussubtypen die een sterkte associatie laten zien met wilde vogels (**Hoofdstuk 3**) suggereert dat LPAI virussen waarschijnlijk niet rechtstreeks van de vaak bemonsterde wilde vogelsoorten op pluimvee worden overgedragen. Daarnaast laat de studie zien dat pluimveekoppels in Nederland niet alleen besmet zijn geraakt met LPAI virussen uit wilde vogels, maar ook door overdracht van virussen tussen pluimveebedrijven (**Hoofdstuk 4**). Tenslotte onderzochten we de bijdrage van het moment van ontstaan van nieuwe HPAI virusvarianten (**Hoofdstuk 5**), en variaties in de pathogeniteit van HPAI virussen tussen vogelsoorten (**Hoofdstuk 6**), in de verspreiding van HPAI virussen in wilde vogels en pluimvee.

DE VERSPREIDING VAN LPAI VIRUSSEN IN WILDE VOGELS EN PLUIMVEE

Wereldwijd wordt al ruim twee decennia intensief gemonitord op de aanwezigheid van AI virussen in wilde vogels en pluimvee. Ook Nederland kent intensieve monitoringprogramma's, die niet alleen gebruikt worden voor de vroege detectie van nieuw opkomende AI virussen in wilde vogels en pluimvee, maar ook om de verspreiding, diversiteit en evolutie van AI virussen te bestuderen. De monitoring voor AI virussen in wilde vogels bestaat uit actieve monitoring in levende vogels en passieve monitoring in ernstig zieke of dode vogels. In beide gevallen vindt het aantonen van infectie meestal plaats op basis van virologische methoden, waarbij bepaald wordt of viraal genetisch materiaal aanwezig is in monsters uit de keel en cloaca van vogels. De monitoring voor AI virussen in pluimvee bestaat ook uit actieve als passieve monitoring. Tijdens de actieve monitoring worden alle pluimveebedrijven in Nederland tenminste één keer per jaar serologisch getest op de aanwezigheid influenza virus-specifieke antistoffen in het bloed. De passieve monitoring op basis van virologische methoden wordt uitgevoerd ter bevestiging van positieve serologie of wanneer er sprake is van een verdenking op basis van klinische verschijnselen. De monitoringstudies hebben reeds belangrijke inzichten geleverd in de complexe epidemiologie van AI virussen. Echter, vaak wordt de bron van AI virusinfecties in pluimvee en verspreidingsroutes niet geïdentificeerd. Eerdere studies hebben laten zien dat wilde vogels veel verschillende AI virussen bij zich dragen, en dat de detectie van verschillende subtypen varieert tussen vogelsoorten. *Charadriiformes* soorten, waaronder meeuwen, zijn voornamelijk geïnfecteerd met LPAI virussen van subtypen H13 en H16, die zelden in andere vogelsoorten worden gevonden. In *Anseriformes* soorten, waarvan de wilde eend het meest intensief is bemonsterd, worden LPAI virussen van subtypen H3, H4 en H6 vaak gedetecteerd, terwijl andere subtypen (H8-H12) zelden worden gevonden. Analyses van de subtypeverdeling en genoomsamenstelling van virussen uit wilde vogels en pluimvee

kunnen worden gebruikt om wilde vogelsoorten te identificeren die de bron zijn van virusinfecties in pluimvee. Het bepalen van de oorsprong en verspreidingspatronen van AI virussen naar pluimvee is cruciaal voor het ontwerpen van effectieve strategieën om introducties en verspreiding te voorkomen.

Onopgemerkte circulatie van LPAI virussen in de wilde vogelpopulatie

Als onderdeel van dit proefschrift is de genetische diversiteit en verspreiding van LPAI virussen in wilde vogels en commercieel pluimvee in Nederland tussen 2006 en 2016 in kaart gebracht (**Hoofdstuk 2**). Hiervoor zijn de gegevens uit monitoringsstudies gebruikt. Uit deze studie blijkt dat in Nederland een grote diversiteit aan LPAI virussen in wilde vogels circuleert. Bovendien vinden er regelmatig introducties in pluimvee plaats. De meeste LPAI virussen uit wilde vogels zijn geïsoleerd uit monsters van wilde eenden en meeuwen. LPAI virusinfecties in pluimvee werden het vaakst aangetoond in legkippen met vrije uitloop, kalkoenen en eenden. Er is een breed scala aan LPAI virussubtypen geïdentificeerd, met verschillen in subtypeverdeling tussen *Charadriiformes* en *Anseriformes* vogelsoorten die overeenkomen met eerdere studies. De studie onthulde ook verschillen in de subtypeverdeling tussen wilde vogels en pluimvee. Zo werden virussen van subtypen H8 en H9 regelmatig in pluimvee gedetecteerd, maar slechts sporadisch in wilde vogels. Dit is opvallend aangezien wilde vogels als de belangrijkste bron voor AI virussen worden beschouwd. De resultaten doen vermoeden dat de verspreiding van LPAI virussen van wilde vogels naar pluimvee selectief is, en mogelijk afhankelijk is van virale factoren die de gastheerspecificiteit van het virus bepalen. H8 en H9 subtypen zijn mogelijk beter in staat om pluimvee te infecteren dan subtypen die minder vaak in pluimvee worden gevonden. Bepaalde H9 virusstammen circuleren endemisch in pluimvee in Azië, wat duidt op een efficiënte verspreiding van het virus in pluimvee. Fylogenetische analyses toonden echter aan dat H9 virusstammen geïsoleerd uit pluimvee in Nederland niet verwant zijn aan de Aziatische virusstammen, maar voorouders delen met virussen geïsoleerd uit wilde vogels, met name wilde eenden in Nederland en Zweden (**Hoofdstuk 2**). Experimentele infectie van kippen met H8 en H9 virussen geïsoleerd uit wilde eenden in Nederland liet zien dat de vatbaarheid van kippen sterk varieerde tussen de verschillende H8 en H9 virusstammen (**Hoofdstuk 3**). Deze bevindingen suggereren dat LPAI virussen niet rechtstreeks worden overgedragen van wilde eenden en andere vaak bemonsterde wilde vogelsoorten naar pluimvee. Dit wordt ondersteund door het feit dat er geen directe genetische verwantschappen zijn aangetroffen tussen virussen uit wilde vogels en pluimvee (**Hoofdstuk 2**), wat aangeeft dat LPAI virussen die pluimvee infecteren in wilde vogels circuleren zonder te worden gedetecteerd.

Wilde eenden worden gezien als belangrijke gastheren voor het behoud en de verspreiding van LPAI virussen, omdat zij de meest voorkomende eendensoort zijn, vaak geïnfecteerd zijn met LPAI virussen, een grote diversiteit aan LPAI virussen bij zich dragen en over grote geografische afstanden kunnen migreren. Veel monitoringsprogramma's richten zich daarom op wilde eenden, zo ook in Nederland (**Hoofdstuk 2**). Een studie in Zweden liet zien dat wilde eenden waarschijnlijk een rol hebben in het behoud van zeldzame LPAI virussubtypen, waaronder H8 en H9. Zij toonden aan dat de interne genen van zeldzame subtypen genetisch verwant zijn aan LPAI virussen van subtypen die vaak worden gedetecteerd in wilde eenden. Echter, het feit dat bepaalde LPAI virussubtypen zelden in wilde eenden worden gedetecteerd suggereert dat wilde eenden niet de belangrijkste gastheer zijn

voor alle subtypen, en mogelijk ook niet de gastheer zijn voor LPAI virussen die pluimvee infecteren. Het is mogelijk dat virussen van zeldzame LPAI virussubtypen, waaronder H8 en H9, in wilde vogelsoorten circuleren die minder vaak worden bemonsterd tijdens monitoringsprogramma's, bijvoorbeeld omdat deze vogels moeilijker te vangen zijn. *Anseriformes* vogelsoorten die ook LPAI virussen bij zich dragen zijn bijvoorbeeld andere zwemeenden (waaronder smienten, wintertalingen en krakeenden), duikeenden, ganzen en zwanen. Bovendien zijn zeevogels die tot de *Charadriiformes* orde behoren, in het bijzonder de zeekoet, relatief vaak geïnfecteerd met influenza A groep 1 virussen, waar subtypen H8 en H9 onder vallen. Bepaalde wilde vogelsoorten zijn dus mogelijk belangrijke gastheren voor LPAI virussen die pluimvee infecteren, maar worden niet geïdentificeerd door beperkte bemonstering. Het is ook mogelijk dat deze vogelsoorten fungeren als tussengastheren voor de overdracht van virussen van wilde eenden naar pluimvee. Als de tussengastheer vatbaar is voor H8 en H9 virussen, in staat is om deze virussen over te dragen en hun leefomgeving deelt met zowel wilde eenden als pluimvee, dan zouden de virussen waarschijnlijk vaker worden gedetecteerd in pluimvee, zelfs als de subtypen zeldzaam zijn in wilde eenden. De observatie dat ganzen relatief vaak besmet zijn met LPAI virussen van hetzelfde subtype als pluimvee, bijvoorbeeld H6N2, H6N8 en H7N7 (**Hoofdstuk 2**), kan wijzen op een rol voor ganzen in de overdracht van LPAI virussen naar pluimvee. Er zijn echter geen virussen van subtypen H8 en H9 uit deze vogelsoort geïsoleerd en geen genetisch aanwijzingen voor directe overdracht van LPAI virussen van ganzen naar pluimvee gevonden. Bemonstering van niet-traditionele vogelsoorten is nodig om mogelijke wilde vogelsoorten te identificeren die betrokken zijn bij de overdracht van virus naar pluimvee.

De geringe detectie van H8 en H9 virussen in wilde vogels wordt mogelijk ook veroorzaakt door de periode in het jaar waarin de bemonsteringsactiviteiten worden uitgevoerd. In veel monitoringsstudies vindt bemonstering van wilde vogels plaats tijdens de migratieperiode in de herfst, wanneer een hoge prevalentie van virus in wilde vogels wordt waargenomen en het verzamelen van materiaal relatief eenvoudig is. Virussen van veelvoorkomende subtypen worden tijdens deze periode mogelijk vaker gedetecteerd door een hogere fitness in wilde eenden, terwijl subtypen met een lagere fitness door concurrentie minder vaak worden gevonden. Na de prevalentiepiek komen herinfecties met dezelfde of gerelateerde subtypen minder vaak voor vanwege immuniteit in de gastheerpopulatie, wat infectie met minder verwante virussen en subtypen mogelijk maakt. Tijdens een monitoringsonderzoek in wilde eenden in Zweden werden LPAI virussen van subtype H8 inderdaad vaker buiten de prevalentiepiek gedetecteerd. De tijd van bemonstering kan ook een reden zijn waarom subtypen H8 en H9 slechts sporadisch in wilde vogels in Nederland zijn gedetecteerd (**Hoofdstuk 2**). Verhoogde bemonstering van wilde vogels buiten de prevalentiepiek kan de detectie van zeldzame subtypen mogelijk maken.

Veelvoorkomende subtypen in wilde vogels zelden gedetecteerd in pluimvee

In tegenstelling tot subtypen H8 en H9 werden subtypen H3 en H4 regelmatig in wilde vogels in Nederland en andere Europese landen gedetecteerd, maar zelden in pluimvee (**Hoofdstuk 2**). Het is mogelijk dat subtypen die vaker in wilde eenden worden gedetecteerd een verhoogde gastheerspecificiteit hebben en daardoor minder goed in staat zijn om andere gastheren te infecteren, zoals kippen. Een experimentele studie toonde echter aan dat kippen vatbaar zijn voor infectie met LPAI virussen van subtypen H3 en H4 virussen afkomstig uit

wilde eenden, ondanks hun geringe detectie in pluimvee (**Hoofdstuk 3**). Deze resultaten suggereren dat de zeldzame detectie van subtypen H3 en H4 in pluimvee niet wordt veroorzaakt door gastheerrestricties van het virus of een lagere vatbaarheid van kippen voor deze subtypen. Als virusverspreiding tussen wilde eenden en pluimvee via een andere vogel verloopt, kan het zijn dat deze tussengastheer een barrière voor de overdracht van H3 en H4 virussen vormt. Het feit dat wilde eenden in andere studies zijn geïdentificeerd als frequente bezoekers van vrije uitloopgebieden van Nederlandse kippenbedrijven, impliceert echter dat de overdracht tussen wilde eenden en kippen kan plaatsvinden zonder de tussenkomst van een andere vogel. Hoewel geen direct contact tussen wilde eenden en pluimvee is waargenomen, kan overdracht van wilde eenden op pluimvee plaatsvinden via indirect contact, bijvoorbeeld via gecontamineerd water. In deze situatie fungeert de omgeving als brug voor de overdracht van LPAI virussen van wilde vogels naar pluimvee. Het is mogelijk dat de persistentie van LPAI virussen in de omgeving varieert tussen virussubtypen en -stammen. Voor subtypen H3 en H4 is dit mogelijk verminderd, wat resulteert in een lager aantal introducties van deze subtypen in pluimvee.

Een andere verklaring voor de geringe detectie van subtypen H3 en H4 in pluimvee zou kunnen zijn dat virusintroductie en -circulatie van deze subtypen in pluimvee tijdens routinematige screening onopgemerkt is gebleven vanwege subtype-afhankelijke variaties in de antilichaamrespons. Pluimveebedrijven worden ten minste één keer per jaar getest op de aanwezigheid van antilichamen tegen AI virussen. Deze antilichamen zijn over het algemeen enkele weken of maanden detecteerbaar. De antilichaamrespons van kippen tegen virussen van subtypen H3 en H4 kan echter lager zijn of eerder afnemen dan voor virussen van subtypen H8 en H9. In dat geval worden subtypen H3 en H4 niet in pluimvee gedetecteerd vanwege de beperkte bemonstering. Dit zou ook betekenen dat het aantal LPAI virusintroducties in pluimvee hoger ligt dan verondersteld op basis van de huidige monitoringsgegevens. Langlopende experimentele studies naar antilichaamresponsen tegen verschillende virussubtypen- en stammen in pluimvee zijn nodig om mogelijke variaties tussen LPAI virussen te achterhalen.

De rol van de verspreiding van LPAI virussen tussen bedrijven

Hoewel wilde vogels worden beschouwd als de belangrijkste bron van AI virussen, kan pluimvee ook besmet raken door overdracht tussen bedrijven. Monitoring is belangrijk om zicht te houden op LPAI virussen die in Nederland circuleren. Het is belangrijk om de verspreiding van LPAI virussen van subtypen H5 en H7 te controleren om mutaties naar HPAI virussen te voorkomen. Eerdere grootschalige vogelgriepuitbraken in pluimvee, zoals de uitbraak van het HPAI H7N1 virus in Italië in 2000-2001, tonen de verwoestende gevolgen van wat er gebeurt als LPAI virussen van subtypen H5 en H7 niet op tijd worden bestreden. Het HPAI H7N7 virus dat in 2003 een grote uitbraak in Nederland veroorzaakte is vermoedelijk ook ontstaan na de introductie van een LPAI virus in pluimvee. De verspreiding van LPAI H5 en H7 virussen in pluimvee verhoogt het risico op mutatie van LPAI naar HPAI virus en het ontstaan van andere virulentie mutaties. Verspreiding van LPAI virussen tussen pluimveebedrijven wordt meestal gerapporteerd voor virussen van subtypen H5 en H7, die meldings- en bestrijdingsplichtig zijn. Echter, terugkerende uitbraken van H9N2 virussen in Azië en het Midden-Oosten sinds 1990, en een recente uitbraak van het H3N1 virus in België in 2019, tonen aan dat LPAI virussen van andere subtypen ook snel kunnen verspreiden in

pluimvee met grote sociaal-economische gevolgen voor de pluimveehouderij. Het is daarom niet alleen belangrijk om maatregelen te nemen ter voorkoming van nieuwe introducties uit wilde vogels, maar ook om de verspreiding van vogelgriepvirussen tussen pluimveebedrijven tegen te gaan.

Een genetische analyse toonde aan dat virusverspreiding tussen pluimveebedrijven waarschijnlijk aanzienlijk heeft bijgedragen aan het totale aantal LPAI virusintroducties in pluimvee in Nederland tussen 2006-2016 (**Hoofdstuk 4**). In tegenstelling tot eerdere grootschalige LPAI virusuitbraken heeft tussen-bedrijf transmissie in Nederland echter niet geleid tot massale verspreiding van LPAI virussen. De verspreiding van LPAI virussen in pluimvee kan worden beïnvloed door vele factoren, zoals maatregelen om verspreiding van infectieziekten te voorkomen (bioveiligheid), pluimveedichtheid, soorten pluimvee, contacten en transport tussen bedrijven, en eigenschappen van het virus. Verminderde bioveiligheid in gebieden met een hoge pluimveedichtheid was waarschijnlijk een belangrijke factor bij de verspreiding van het LPAI H7N1virus in Italië. Een strenge bioveiligheid wordt beschouwd als de meest effectieve maatregel om uitbraken en de daaropvolgende verspreiding van AI virussen in pluimvee te voorkomen. Kennis van verspreidingsroutes is van fundamenteel belang om effectieve preventieve maatregelen te kunnen nemen. Transmissie tussen bedrijven kan via verschillende routes verlopen, waaronder direct contact tussen pluimvee, indirect contact via personen, besmet materiaal of vectoren, en overdracht van virussen via water, lucht of stofdeeltjes. Het risico van virusoverdracht lijkt afhankelijk te zijn van de afstand tussen bedrijven en vaker voor te komen tussen bedrijven van hetzelfde pluimveesoort vanwege een grotere kans op contacten. In Nederland zijn inderdaad clusters van bedrijven geïdentificeerd die besmet zijn met hetzelfde virus, maar werd ook aangetoond dat de verspreiding tussen bedrijven niet beperkt was tot hetzelfde soort pluimvee (**Hoofdstuk 4**). Helaas konden de verspreidingsroutes van LPAI virussen tussen de besmette bedrijven in Nederland niet worden achterhaald, wat deels te wijten is aan het beperkte aantal virusisolaties en de afwezigheid van contacttracering.

Virusverspreiding tussen bedrijven kan ook afhangen van eigenschappen van het virus. Grootschalige verspreiding van virussen wordt beïnvloed door het vermogen van het virus om zich te repliceren en gedurende een voldoende lange periode uit te scheiden, waardoor overdracht tussen gastheren mogelijk is. De snelle verspreiding van HPAI H5N2 virussen in kalkoenen in de Verenigde Staten in 2014-2015 werd bijvoorbeeld geassocieerd met een ongewoon lange asymptomatische periode, hoge niveaus van virusuitscheiding en verhoogde aanpassing van het virus aan kalkoenen. Voor sommige van de LPAI virussen die zijn gevonden op besmette pluimveebedrijven in Nederland werd een deletie in het NA eiwit aangetoond (**Hoofdstuk 4**), wat een belangrijke aanwijzing is voor aanpassing van het virus aan *Galliformes* soorten (kippen en kalkoenen). Deze mutatie kan de replicatie, excretie en pathogeniteit van virussen beïnvloeden. Een eerdere experimentele studie naar LPAI H5N7 en H7N7 virussen in kippen liet zien dat virale eigenschappen die belangrijk zijn voor efficiënte verspreiding aanzienlijk kunnen verschillen tussen virusstammen. Experimenten die de transmissie-efficiëntie vergelijken van verschillende LPAI virusstammen in verschillende pluimveesoorten zijn beperkt, met name voor virussubtypen die niet meldings- en bestrijdingsplichtig zijn.

Aanbevelingen voor het verbeteren van LPAI virus monitoring

De afgelopen jaren is een verminderde inzet van tijd en middelen voor AI virus monitoring in levende wilde vogels waargenomen. Bovendien zal monitoring in pluimvee naar verwachting niet toenemen, aangezien door een nieuwe verordening in de diergezondheidswet van de Europese Unie (EU) LPAI virussen van subtypen H5 and H7 vanaf april 2021 niet langer meldings- en bestrijdingsplichtig zijn. Voortdurende of zelfs uitgebreidere monitoring met meer gerichte benaderingen is echter nodig om beter te begrijpen hoe vogelgriepvirussen zich verspreiden in wilde vogels en pluimvee. De onopgemerkte circulatie van LPAI virussen in wilde vogels wordt waarschijnlijk veroorzaakt door een bias in de monitoring met betrekking tot de soort, locatie en tijd van bemonsteren, die de identificatie van vogelsoorten als bron voor virusinfectie in pluimvee en het ophelderen van verspreidingsroutes bemoeilijkt. De monitoringsprogramma's kunnen worden verbeterd door te bepalen welke wilde vogelsoorten veel voorkomen in de omgeving van pluimveebedrijven, wat gerichte bemonstering van vogelsoorten binnen specifieke perioden van het jaar mogelijk maakt. In een recente studie zijn videocamera's gebruikt om bezoeken van wilde vogels aan de uitloopruimte van een kippenbedrijf in Nederland te kwantificeren. Hoewel dit onderzoek beperkt is tot één pluimveebedrijf, bevestigt het eerdere waarnemingen dat wilde eenden vaak in de buurt van pluimveebedrijven worden gezien. Tevens is een breed scala aan andere wilde vogelsoorten geidentificeerd die mogelijk een rol hebben in de overdracht van AI virussen naar pluimvee. Er zijn ook onderzoeksinspanningen gedaan om een methode te ontwikkelen voor het onderzoeken van (indirecte) contacten tussen wilde vogels en pluimvee op basis van de samenstelling van het microbioom in de mest van kippen. Deze studies dragen bij aan de identificatie van potentiële wilde vogelsoorten die de bron zijn van virusinfecties in pluimvee en de rol van wilde vogels in de overdracht van LPAI virussen naar pluimvee inzichtelijk maken. Het blijft onduidelijk waarom bepaalde veelvoorkomende virussubtypen uit wilde eenden niet worden overgedragen naar pluimvee of niet in pluimvee worden gedetecteerd, terwijl wel indirect contact tussen de twee vogelgroepen is waargenomen. Experimentele studies naar de persistentie van LPAI virussen in de omgeving, de overdraagbaarheid van LPAI virussen in pluimveekoppels en de inductie van adaptieve immuunresponsen na LPAI virusinfecties bij pluimvee zijn beperkt. Deze experimentele studies kunnen meer informatie geven over subtype-afhankelijke verschillen in transmissiedynamiek in pluimvee.

Het huidige AI virus monitoringsprogramma in pluimvee in Nederland is uitgebreider dan door het EU programma is vereist. Vrije uitloopkippen en kalkoenen worden bijvoorbeeld vaker getest dan andere pluimveesoorten vanwege een hoger risico op LPAI virusinfecties. Bovendien worden in Nederland multiplex-serologische methoden gebruikt voor een snelle diagnostische screening voor alle virussubtypen. In veel EU lidstaten is serologische monitoring bij pluimvee daarentegen beperkt tot LPAI virussen van subtypen H5 en H7. Verhoogde monitoring in risicovolle pluimveesoorten en de implementatie van multiplex-serologische methoden in andere Europese landen zou meer inzicht geven in de verspreiding van niet-meldings- en bestrijdingsplichtige LPAI virussubtypen in pluimvee buiten Nederland, wat vergelijking van virussubtypen- en stammen tussen wilde vogels en pluimvee op grotere schaal mogelijk zou maken. De AI virusmonitoring in pluimvee in Nederland kan worden verbeterd door de bemonstering van gedomesticeerde eenden te verhogen, die ook een hoger risico hebben op introductie van LPAI virussen en relatief vaak zijn besmet met LPAI

virussen, ondanks hun relatief kleine populatie (**Hoofdstuk 2**). Bovendien blijven LPAI virusinfecties vaak onopgemerkt tot serologische screening wordt uitgevoerd, terwijl virusisolatie en daaropvolgende genetische karakterisering kan worden gebruikt om virussen uit wilde vogels en pluimvee te traceren en met elkaar te vergelijken. Om inzicht in de overdracht van LPAI virussen van wilde vogels naar pluimvee en tussen bedrijven te vergroten zou het daarom waardevol zijn om vaker virologisch te testen op LPAI virussen die niet meldings- en bestrijdingsplichtig zijn. Tenslotte wordt het traceren van contacten tussen bedrijven en het opsporen van secondaire virusinfecties niet uitgevoerd voor uitbraken met LPAI virussubtypen anders dan H5 en H7, maar zou dat waardevol zijn om meer duidelijkheid te verkrijgen over mogelijke verspreidingsroutes.

DE VERSPREIDING VAN HPAI VIRUSSEN IN WILDE VOGELS EN PLUIMVEE

Uitbraken van HPAI virussen vinden niet alleen plaats na introductie van LPAI H5 of H7 virussen in pluimvee en de daaropvolgende verspreiding tussen bedrijven, maar ook wanneer HPAI virussen uit wilde vogels naar pluimvee worden overgedragen. In de afgelopen jaren hebben wereldwijd meerdere vogelgriepuitbraken plaatsgevonden na de introductie van HPAI virussen uit wilde vogels. De HA genen van deze HPAI virussen behoren tot de H5N1 A/Goose/Guangdong/1/96 (GsGd) stam, die in 1996 voor het eerst werd geïsoleerd uit een gans in China en sinds 1997 uitbraken heeft veroorzaakt in pluimvee in Azië. Het virus heeft zich vervolgens verder verspreid en ontwikkeld tot diverse fylogenetische subgroepen (clades). Door reassortment zijn er ook verschillende subtypen ontstaan (H5N2, H5N3, H5N5, H5N6 en H5N8). Een rol voor wilde vogels in de verspreiding van deze virussen werd eerder al gesuggereerd tijdens uitbraken van het HPAI H5N1 clade 2.2 virus in pluimvee in Azië in 2001-2004. Deze rol werd nog duidelijker tijdens een grootschalige HPAI H5N1 uitbraak onder trekvogels aan het Qinghaimeer in China in het voorjaar van 2005, en de daaropvolgende wereldwijde verspreiding van HPAI H5N1 virussen in 2005-2006. Deze HPAI H5N1 virussen bleken hoog pathogeen voor sommige wilde vogelsoorten, terwijl voorafgaand aan de detectie van HPAI H5 GsGd virussen HPAI virussen zelden werden gedetecteerd in wilde vogels, en uitbraken van ernstige ziekte en hoge sterfte beperkt waren tot pluimvee. De verspreiding van HPAI virussen in wilde vogels kan worden beïnvloed door uiteenlopende factoren, zoals de vogelsoorten die besmet raken, bestaande immuniteit na eerdere infecties, het moment van het ontstaan van het virus, of veranderingen in eigenschappen van het virus. Met introducties en de daaropvolgende circulatie van HPAI virussen in wilde vogels is het met toenemende mate belangrijk om herintroducties in pluimvee te voorkomen. Een beter begrip van HPAI viruscirculatie in wilde vogels zal bijdragen aan het voorspellen van virusverspreiding en de potentiele blootstelling van pluimvee aan het virus.

Het moment van reassortment beïnvloedt de HPAI virus verspreiding in wilde vogels
Eind 2014 vonden gelijktijdig uitbraken plaats van HPAI H5N8 clade 2.3.4.4 groep A virussen in Europa en Noord-Amerika, na detectie van het virus in Zuid-Korea in het begin van 2014. In een uitgebreide studie op basis van genetische, epidemiologische en ornithologische gegevens werden trekvogels die over lange afstanden migreren geïdentificeerd als de meest waarschijnlijke verspreiders van het virus, hetgeen eerdere uitbraakstudies bevestigde. De getroffen pluimveebedrijven bevonden zich vaak in gebieden met wilde vogels. Indirect

contact met materialen die besmet waren met uitwerpselen van wilde vogels werd beschouwd als de meest waarschijnlijke route van virusverspreiding naar pluimvee. Andere routes, zoals verspreiding via de handel in besmet pluimvee of besmette pluimveeproducten, werden minder aannemelijk geacht. De analyse identificeerde ook belangrijke routes in de verspreiding van het H5N8 virus in trekvogels: in het voorjaar van Zuidoost-Azië naar noordelijke broedplaatsen, en in de herfst van deze broedplaatsen naar overwinteringslocaties in Noord-Amerika en Europa.

Twee jaar later, in 2016-2017, vond de meest omvangrijke HPAI virusuitbraak plaats in Europa. Deze uitbraak werd gekenmerkt door de detectie van veel verschillende HPAI virusvarianten, die variabele samenstellingen van gensegmenten bevatten door reassortment met co-circulerende LPAI virussen. In totaal werden vijf verschillende HPAI H5N8 clade 2.3.4.4 groep B virusvarianten gedetecteerd in Europa. Bovendien werden drie HPAI H5N5 virusvarianten en één HPAI H5N6 virus gevonden (**Hoofdstuk 5**), al was de verspreiding van deze virussen beperkt vergeleken met H5N8. Net als de H5N1 clade 2.2 virussen en H5 clade 2.3.4.4 groep A virussen, werden de H5 clade 2.3.4.4 groep B virussen gedetecteerd op noordelijke broedplaatsen in het voorjaar, voorafgaand aan hun introductie in Europa in de herfstperiode. Vermoedelijk zijn de virussen door trekvogels van noordelijke broedplaatsen naar overwinteringslocaties in Europa gebracht, vergelijkbaar met het HPAI H5N8 clade 2.3.4.4 groep A virus. De detectie van een HPAI H5N5 virus in het Russische Verre Oosten laat zien dat de virussen ook door trekvogels via een andere trekroute naar de Beringstraat regio zijn verspreid (**Hoofdstuk 5**). In tegenstelling tot het HPAI H5N8 clade 2.3.4.4 groep A virus zijn de H5N5 virussen echter niet gedetecteerd in Noord-Amerika. Fylogenetische analyse wees ook op overeenkomsten in de verspreiding van HPAI virussen binnen Europa, met meerdere onafhankelijke introducties van H5N5 virussen in Europa gevolgd door lokale verspreiding (**Hoofdstuk 5**), vergelijkbaar met H5N8 clade 2.3.4.4 groep B virussen.

Informatie over patronen van HPAI virusverspreiding in trekvogels, gecombineerd met schattingen van het moment van ontstaan van virussen, kan worden gebruikt om in te schatten wanneer (vóór, tijdens of na de migratie van wilde vogels vanuit hun broedplaatsen) en waar (op broedplaatsen of langs trekroutes naar Europa) reassortment plaats heeft gevonden, wat meer inzicht geeft in de evolutionaire geschiedenis van de virussen. Het ontstaan van nieuwe virusvarianten vlak voor of tijdens het broedseizoen draagt mogelijk bij aan een efficiënte virusverspreiding tussen wilde vogels op de broedplaatsen, en de daaropvolgende verspreiding naar andere (overwinterings-)gebieden. Virusvarianten die aan het eind van het broedseizoen zijn ontstaan, na de start van de migratie van wilde vogels, infecteren daarentegen waarschijnlijk maar een relatief klein aantal wilde vogels. De meest voorkomende en geografisch wijdverspreide HPAI H5N8 clade 2.3.4.4. groep B virusvariant is inderdaad naar alle waarschijnlijkheid in de lente ontstaan, terwijl de minder frequent gedetecteerde H5N8 virusvariant waarschijnlijk in de zomer is ontstaan. Op eenzelfde manier is de meest voorkomende HPAI H5N5 virusvariant waarschijnlijk in de zomer ontstaan, terwijl de H5N5 virusvariant die beperkt werd gevonden waarschijnlijk pas in het najaar is ontstaan (**Hoofdstuk 5**). De beperkte verspreiding van de H5N5 virussen in vergelijking tot de H5N8 virussen zou ook kunnen worden verklaard doordat er veranderingen in de virale eigenschappen zijn opgetreden, zoals veranderingen in de pathogeniteit of het replicatievermogen. Experimenten met primaire kippen- en eendencellen toonden echter slechts kleine veranderingen aan in het replicatievermogen en het veroorzaken van celdood

tussen de verschillende H5N5 virusvarianten en het H5N8 virus (**Hoofdstuk 5**). Het moment van reassortment in trekvogels heeft mogelijk invloed op de mate van verspreiding van verschillende HPAI virusvarianten in wilde vogels, en kan ook de beperkte verspreiding van H5N5 virussen in vergelijking met H5N8 virussen verklaren. Uitgebreide analyse van het virale genoom vergroot ons begrip van de evolutie en verspreiding van HPAI virussen in wilde vogels.

Verschillen in HPAI virus pathogeniteit tussen wilde vogel- en pluimveesoorten

Het aantal gerapporteerde HPAI virusinfecties varieerde sterk tussen de uitbraken van H5N8 clade 2.3.4.4 groep A virussen in 2014, H5N8 en H5N5 clade 2.3.4.4 groep B virussen in 2016-2017 en H5N6 clade 2.3.4.4 groep B virussen in 2017-2018 in Europa. Ook werden er grote verschillen in sterfte onder wilde vogels waargenomen. Tijdens de H5N8 2.3.4.4 groep A uitbraak in 2014 werd virus aangetroffen in een beperkt aantal zieke en dode wilde vogels en in mestmonsters van smienten in Nederland. Ook werden er HPAI-specifieke antilichamen gedetecteerd bij wilde vogels in Nederland, voornamelijk smienten en zwanen. In tegenstelling tot het H5N8 2.3.4.4 groep A virus veroorzaakte het H5N8 2.3.4.4 groep B virus massale sterfte onder wilde vogels, voornamelijk kuifeenden en smienten. Tijdens deze uitbraak werd ook virus gedetecteerd in ogenschijnlijk gezonde wilde vogels, voornamelijk wilde eenden. Dit geeft aan dat deze vogelsoort mogelijk resistenter is, en als reservoir voor het virus kan fungeren. Er werden ook HPAI-specifieke antilichamen gedetecteerd in verschillende wilde vogelsoorten, waaronder wilde eenden, meeuwen, smienten en zwanen. Het H5N6 2.3.4.4 groep B virus werd ook aangetoond in verschillende dode wilde vogels, maar in veel mindere mate dan het H5N8 clade 2.3.4.4 groep B virus.

Experimentele studies met wilde en gedomesticeerde eenden toonden aan dat HPAI H5 GsGd virussen van verschillende subtypen –en stammen een verminderde virulentie hebben in vergelijking met het H5N1 voorouder virus, waaruit blijkt dat de pathogeniteit varieert tussen de virusvarianten. Ook werden verschillen in de vatbaarheid voor ziekte en permissiviteit tussen wilde vogelsoorten waargenomen. In overeenstemming met waarnemingen uit het veld was infectie met het H5N8 clade 2.3.4.4 groep A virus subklinisch in veelvoorkomende wilde vogelsoorten. Smienten vertoonden de hoogste virusuitscheiding, hetgeen in overeenstemming is met de detectie van virus in mestmonsters en antilichamen bij deze vogelsoort tijdens de uitbraak. Een experimenteel onderzoek met H5N8 clade 2.3.4.4 groep B virus toonde daarentegen een hoge mortaliteit aan onder wilde eenden, wat overeenkomt met de massale sterfte onder wilde vogels tijdens de uitbraak. Bestaande immuniteit door eerdere infecties kan de detectie van virus in ogenschijnlijk gezonde wilde eenden tijdens de uitbraak verklaren.

De pathogeniteit van HPAI H5 GsGd virussen varieerde ook tussen wilde vogels en pluimvee, waarbij eenden een verzwakte en minder invasieve virusinfectie lieten zien vergeleken met kippen. Intraveneuze pathogeniteitindex (IVPI) testen bevestigden een hoge pathogeniteit van de drie uitbraakstammen in kippen. In Pekingeenden werd daarentegen een lagere pathogeniteit gemeten voor het H5N8 clade 2.3.4.4 groep A virus, terwijl een gelijke hoge pathogeniteit werd vastgesteld voor de clade 2.3.4.4 groep B virussen van subtypen H5N8 en H5N6. Hoewel het H5N6 virus een vergelijkbare hoge pathogeniteit bij zowel kippen als Pekingeenden vertoonde, toonde een analyse van de virusverspreiding en histopathologische veranderingen in verschillende organen een minder wijdverspreide

virusinfectie aan bij Pekingeenden vergeleken met kippen (**Hoofdstuk 6**). Ook werden histopathologische veranderingen en viraal antigeen gedetecteerd in de meeste organen van kippen, terwijl in Pekingeenden de aanwezigheid van microscopische laesies en de expressie van antigeen voornamelijk zichtbaar was in de ademhalingsorganen, met name de longen (**Hoofdstuk 6**). Deze bevinding geeft aan dat de virusinfectiedynamiek varieert tussen de twee pluimveesoorten. Eerdere experimentele onderzoeken met HPAI H5 GsGd virussen toonden expressie van viraal antigeen aan in de pancreas en hersenen van eenden, maar dit werd niet waargenomen voor het HPAI H5N6 virus in Pekingeenden (**Hoofdstuk 6**). Dit suggereert dat virusverspreiding naar verschillende organen ook kan variëren tussen virussubtypen en -stammen. Veranderingen in de virale eigenschappen kunnen verschillen in pathogeniteit en virusinfectiedynamiek veroorzaken, en daarmee de verspreiding van HPAI virussen in wilde vogels beïnvloeden. Als het virus geen ernstige ziekte veroorzaakt, zullen besmette wilde vogels waarschijnlijk blijven migreren, wat virusverspreiding over grote geografische afstanden mogelijk maakt. Bovendien kunnen besmette vogels onopgemerkt blijven vanwege de afwezigheid van zichtbare klinische verschijnselen, maar vormen ze wel een hoog risico voor gevoelige vogelpopulaties, zoals pluimvee. Experimentele studies die de pathogeniteit en overdraagbaarheid van nieuwe HPAI virusvarianten bij verschillende vogelsoorten bepalen, zijn van grote waarde om de rol van veranderingen in virale eigenschappen in de verspreiding van HPAI virussen beter te begrijpen.

Aanbevelingen voor het verbeteren van HPAI virus monitoring

Op basis van de wereldwijde patronen van HPAI virusverspreiding in trekvogels, die zijn vastgesteld tijdens eerdere uitbraken, kunnen aanbevelingen worden gedaan met betrekking tot de locatie en tijd van bemonstering van wilde vogels. Verhoogde bemonsteringsactiviteiten in Zuidoost-Azië vóór het broedseizoen in het vroege voorjaar, in noordelijke broedgebieden tijdens broedseizoen in het voorjaar en de vroege zomer, en/of bemonstering op (beter toegankelijke) stopplaatsen langs belangrijke trekroutes in de late zomer en tijdens de herfst, kunnen helpen de verspreiding van HPAI virussen naar Europa te voorspellen. Bemonstering is mogelijk het meest effectief op locaties waar grote hoeveelheden wilde vogels van verschillende trekroutes met elkaar in contact komen. Bovendien zou lokale bemonstering van wilde vogels in Europa tijdens de aankomst van trekvogels in de herfst en vroege winter raadzaam zijn voor de vroege detectie van HPAI virussen die Europa binnenkomen. Verhoogde bemonstering langs de belangrijkste trekroutes van wilde vogels zal de beschikbare gegevens vergroten om de verspreiding van HPAI virussen in trekvogels te onderzoeken, wat een meer gedetailleerde analyse van de virusevolutie in de tijd mogelijk maakt.

Veel monitoringsprogramma's zijn gefocust op wilde eenden, terwijl bemonstering ook zou moeten plaatsvinden bij andere vogelsoorten met een potentiële rol in de verspreiding van HPAI virussen, bijvoorbeeld vogelsoorten die over grote afstanden migreren, minder vatbaar zijn voor ziekte en daarom HPAI virussen mogelijk wereldwijd verspreiden. Bovendien is het tijdens het opzetten van monitoringsprogramma's voor HPAI virussen belangrijk om rekening te houden met variaties in de pathogeniteit tussen virusstammen en -subtypen in verschillende vogelsoorten. EU lidstaten hebben passieve monitoring ingevoerd voor de vroege detectie van HPAI virussen, dat met name geschikt voor het detecteren van HPAI virussen in vogelsoorten die ziek worden of sterven door infectie. Actieve monitoring is

nodig voor het detecteren van HPAI virussen in vogelsoorten die de infectie overleven zonder duidelijke tekenen van ziekte. De laatste methode zou waardevol zijn voor de vroege detectie van HPAI virussen wanneer geen sterfte in wilde vogels wordt waargenomen, en om vogelsoorten te identificeren die verantwoordelijk zijn voor verspreiding van virus over lange afstanden. Actieve monitoring door serologische monitoring kan informatie verschaffen over eerdere HPAI virusinfecties en de immuunstatus van wilde vogels. Passieve monitoring zal nuttig blijven voor de detectie van HPAI virussen bij soorten die ziek worden of sterven na infectie, en fungeert daarmee als een vroeg waarschuwingssysteem voor lokale viruscirculatie.

Hoewel het huidige passieve monitoringsprogramma in pluimvee toereikend is voor de vroege detectie van HPAI virusintroducties in pluimvee, onderstreept de dreiging van HPAI virussen uit wilde vogels het belang van vroege controlemaatregelen om overdracht van HPAI virussen van wilde vogels naar pluimvee te voorkomen. Deze controlemaatregelen omvatten onder meer het ophokken van pluimvee om contact met wilde vogels te voorkomen, bijvoorbeeld wanneer HPAI virussen in Nederland of andere Europese landen zijn gedetecteerd, en het vergroten van het bewustzijn van boeren en de implementatie van maatregelen voor een verhoogde bioveiligheid tijdens risicovolle perioden voor HPAI virusintroducties.

CONCLUSIE

Concluderend, het in dit proefschrift beschreven onderzoek levert nieuwe inzichten in de verspreiding van AI virussen in wilde vogels en pluimvee. De studies tonen aan dat het analyseren van het genetische materiaal van AI virussen, inclusief virussen van niet-meldingsplichtige subtypen, zeer waardevol is om onze kennis over de circulatie van AI virussen in wilde vogels en pluimvee te vergroten. Het onderzoek benadrukt bovendien de waarde van het combineren van genetisch onderzoek met informatie over de verspreiding van het virus in plaats en tijd, en fenotypische eigenschappen van het virus, zoals het replicatievermogen en de pathogeniteit. Het onderzoek heeft geleid tot aanbevelingen met betrekking tot huidige nationale en internationale monitoringsprogramma's, waaronder het gerichter bemonsteren van wilde vogels op basis van soort, locatie en tijd. Gerichtere monitoring is van cruciaal belang om gastheersoorten voor infecties in pluimvee te identificeren en mogelijke verspreidingsroutes van wilde vogels naar pluimvee en tussen bedrijven vast te stellen. De implementatie van efficiëntere monitoring en effectievere preventie van de introductie en verspreiding van AI virussen bij pluimvee is belangrijk om de bedreiging voor de gezondheid van zowel dieren als mensen te verminderen.

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About the author

Curriculum Vitae

Saskia A. Bergervoet was born on 11 January 1990 in Wisch, the Netherlands. In 2009, she moved to Utrecht and started a Bachelor's program in Biology with a minor in Biomedical Sciences at Utrecht University. In 2012, she finished her Bachelor's thesis on the role of neutrophils during viral respiratory infections in humans, and decided to continue her education in the area of immunology and infectious diseases. After earning her Bachelor's degree, she started the Master's program Infection and Immunity (I&I) at Utrecht University. As a Master's student, she completed her first research internship at the Laboratory for Translational Immunology in the Wilhelmina Children's Hospital (WKZ) of UMC Utrecht. During this internship, she worked on the identification of cytokines that modulate the expression of the cystic fibrosis transmembrane conductance regulator in human intestinal epithelial organoid cultures. In 2013-2014, she completed two additional research internships at the Dutch National Institute for Public Health and the Environment (RIVM) in Bilthoven. During these internships, she worked at the National Coordination Centre for Outbreak Management, where she was introduced to infectious disease prevention and response programs, and at the Virology department Emerging Infectious Diseases on the development and validation of a serological assay for the differential diagnosis of flavivirus infections in horses. Her Master's thesis was a literature review on the emergence of Middle East respiratory syndrome coronavirus, conducted at AMC Amsterdam. Saskia received her Master's degree by the end of 2014. In 2015, she started her PhD studies at Erasmus MC in Rotterdam. During her PhD studies, she performed her research at Wageningen Bioveterinary Research in Lelystad, supervised by her co-promotors Dr. Nancy Beerens and Dr. Alex Bossers and promotor Prof.dr. Ron A.M. Fouchier. The research was focused on avian influenza viruses at the wild bird-poultry interface, and has resulted in this thesis.

EDUCATION

2012-2014 Master Biomedical Sciences (MSc) Infection and Immunity
Utrecht University, Utrecht, The Netherlands

2009-2012 Bachelor Biology (BSc) with a minor Biomedical Sciences
Utrecht University, Utrecht, The Netherlands

2002-2008 VWO - Nature & Health and Nature & Technology
Almende College Isala Silvolde, The Netherlands

RESEARCH INTERNSHIPS

2014 6-month internship
Development and validation of a protein microarray based on nonstructural protein 1 for the differential diagnosis of serologically cross-reactive flavivirus infections in horses for public and animal health surveillance.
RIVM Dutch National Institute for Public Health and the Environment
Virology department Emerging Infectious Diseases
Bilthoven, The Netherlands

2013 2-month internship
Support of Public Health Departments during preparedness exercises
RIVM Dutch National Institute for Public Health and the Environment
National Coordination Centre for Outbreak Management
Bilthoven, The Netherlands

2012-2013 9-month internship
Identifying cytokines that modulate the expression of the cystic fibrosis transmembrane conductance regulator in intestinal epithelial organoids
WKZ Wilhelmina Children's Hospital - UMC Utrecht
Laboratory for Translational Immunology
Utrecht, The Netherlands

BACHELOR AND MASTER THESIS

2014 Master thesis
A review on Middle East respiratory syndrome (MERS) coronavirus
AMC Amsterdam, The Netherlands

2012 Bachelor thesis
Het nut en het kwaad van neutrofielen tijdens een virale respiratoire infectie
UMC Utrecht, The Netherlands

PhD portfolio

Thesis title	Avian influenza at the wild bird-poultry interface
Research department	Department of Virology, Wageningen Bioveterinary Research, Lelystad, The Netherlands & Department of Viroscience, Erasmus MC, Rotterdam, The Netherlands
Research school	Erasmus Postgraduate School Molecular Medicine (MolMed)
PhD period	2015 - 2019
Promotor	Prof.dr. R.A.M. Fouchier
Copromotors	Dr. N. Beerens Dr. A. Bossers

COURSES AND WORKSHOPS

2018 Course Study Design and Analysis of Animal Experiments
Wageningen Bioveterinary Research, Lelystad, The Netherlands

2017 Course Biomedical Scientific English Writing
MolMed, Rotterdam, The Netherlands

2016 VEME International Bioinformatics Workshop on Virus Evolution and Molecular Epidemiology: Evolutionary Hypothesis Module
Korea University College of Medicine, Seoul, Republic of Korea

EPIZONE Workshop on NGS applications and Bioinformatics
Veterinary and Agrochemical Research Center, Brussels, Belgium

Basic Course on 'R'
MolMed, Rotterdam, The Netherlands

Course Virology
MolMed, Rotterdam, The Netherlands

Annual Course on Molecular Medicine
MolMed, Rotterdam, The Netherlands

Course Integrity in Science
Erasmus MC, Rotterdam, The Netherlands

SNP Course XIII: SNPs and Human Diseases
MolMed, Rotterdam, The Netherlands

Workshop on Microsoft Excel 2010: Basic and advanced
MolMed, Rotterdam, The Netherlands

2015 Course Biomedical Research Techniques
MolMed, Rotterdam, The Netherlands

Basic Introduction Course on SPSS
MolMed, Rotterdam, The Netherlands

INTERNATIONAL CONFERENCES AND SYMPOSIA

2019 ECV European Congress of Virology 2019
Rotterdam, the Netherlands

2018 ISAI 10th International Symposium on Avian Influenza 2018
Brighton, United Kingdom

2017 11th Annual Meeting of EPIZONE
Paris, France

2016 National Reference Lab Meeting for Avian Influenza and New Castle Disease
Copenhagen, Denmark

10th Annual Meeting of EPIZONE
Madrid, Spain

2015 ESVV International Congress of Veterinary Virology and
9th Annual Meeting of EPIZONE
Montpellier, France

NATIONAL CONFERENCES AND SYMPOSIA

2019 NCOH Netherlands Centre for One Health Meeting 2019
Nijmegen, The Netherlands

DAVS Dutch Annual Virology Symposium 2019
Amsterdam, The Netherlands

2018 DAVS Dutch Annual Virology Symposium 2018
Amsterdam, The Netherlands

2016 20th Molecular Medicine Day
Rotterdam, The Netherlands

ORAL PRESENTATIONS

2019 *Young NCOH Pitch*
NCOH Netherlands Centre for One Health Meeting

Nijmegen, The Netherlands

2018 *Genetic analysis of potential between-farm transmitted LPAI viruses*
ISAI 10th International Symposium on Avian Influenza 2018
Brighton, United Kingdom

2017 *Genetic analysis of potential between-farm transmitted LPAI viruses*
11th Annual Meeting of EPIZONE
Paris, France

Diversity of AI virus subtypes in poultry and wild birds in the Netherlands
Wageningen Bioveterinary Research Cees Wensing lecture
Lelystad, The Netherlands

Towards a Global One Health research pitch
Wageningen University and Research Dies Natalis Symposium 2017
Wageningen, The Netherlands

2016 *Circulation of LPAI viruses in wild birds and poultry in the Netherlands, 2006-2016*
National Reference Lab Meeting for Avian Influenza and New Castle Disease
Copenhagen, Denmark

Circulation of LPAI viruses in wild birds and poultry in the Netherlands, 2006-2016
Wageningen Bioveterinary Research Virology meeting
Lelystad, The Netherlands

Er als de kippen bij zijn
Diagnostiek en Onderzoek Aviaire Influenza Blok 3: 'eigen bedrijf'. De organisatie van dierziektebestrijding: Aviaire Influenza.
Faculteit Diergeneeskunde, Utrecht, The Netherlands

POSTER PRESENTATIONS

2019 G*enetic analysis identifies potential transmission of low pathogenic avian influenza viruses between poultry farms*
ECV European Congress of Virology 2019
Rotterdam, The Netherlands

2016 *Diversity of AI virus subtypes in poultry and wild birds in the Netherlands*
10th Annual Meeting of EPIZONE
Madrid, Spain

Diversity of AI virus subtypes in poultry and wild birds in the Netherlands
VEME International Bioinformatics Workshop on Virus Evolution and Molecular Epidemiology
Seoul, Republic of Korea

TEACHING AND SUPERVISION

2018-2019	Supervision 4th year student Biologie en Medisch Laboratoriumonderzoek Avans Hogeschool, Breda, The Netherlands
2018	Supervision 3rd year student Biologie en Medisch Laboratoriumonderzoek Hogeschool Saxion, Deventer, The Netherlands

REGULAR INSTITUTIONAL MEETINGS

2019	PhD meetings Wageningen Bioveterinary Research, Lelystad, The Netherlands
2015-2019	Lab meetings Wageningen Bioveterinary Research, Lelystad, The Netherlands
	Virology meetings Wageningen Bioveterinary Research, Lelystad, The Netherlands
	Cees Wensing lectures Wageningen Bioveterinary Research, Lelystad, The Netherlands

OTHER ACTIVITIES

2015-2019	Young EPIZONE board member

Overview of publications

S.A. Bergervoet, S. Vreman, R. Zwart, N. Stockhofe-Zurwieden, N. Beerens. Histopathology and tissue distribution of highly pathogenic avian influenza (HPAI) H5N6 virus in chickens and Pekin ducks. *In preparation*.

E.A. Germeraad, A.R.W. Elbers, N.D. de Bruijn, R. Heutink, W. van Voorst, R. Hakze van der Honing, **S.A. Bergervoet**, M.Y. Engelsma, W.H.M. van der Poel, N. Beerens. Detection of low pathogenic avian influenza virus subtype H10N7 in poultry and environmental water samples during a clinical outbreak in commercial free-range layers, Netherlands, 2017. 2020 May. *Front Vet Sci.* DOI: 10.3389/fvets.2020.00237

S.A. Bergervoet, E.A. Germeraad, M. Alders, M.M. Roose, M.Y. Engelsma, R. Heutink, R. Bouwstra, R.A.M. Fouchier, N. Beerens. Susceptibility of chickens to low pathogenic avian influenza (LPAI) viruses of wild bird- and poultry-associated subtypes. 2019 Oct. *Viruses*. DOI: 10.3390/v11111010.

S.A. Bergervoet, S. Pritz-Verschuren, J.L. Gonzales, A. Bossers, M.J. Poen, J. Dutta, Z. Khan, D. Kriti, H. van Bakel, R. Bouwstra, R.A.M. Fouchier, N. Beerens. Circulation of low pathogenic avian influenza (LPAI) viruses in wild birds and poultry in the Netherlands, 2006-2016. 2019 Sep. *Scientific Reports*. DOI: 10.1038/s41598-019-50170-8.

S.A. Bergervoet, C.K.Y. Ho, R. Heutink, A. Bossers, N. Beerens. Spread of highly pathogenic avian influenza (HPAI) H5N5 viruses in Europe in 2016-2017 appears related to the timing of reassortment events. 2019 May. *Viruses.* DOI: 10.3390/v11060501.

S.A. Bergervoet, R. Heutink, R. Bouwstra, R.A.M. Fouchier, N. Beerens. Genetic analysis identifies potential transmission of low pathogenic avian influenza viruses between poultry farms. 2019 Apr. *Transbound Emerg Dis*. DOI: 10.1111/tbed.13199.

N. Beerens, R. Heutink, S. Pritz-Verschuren, E.A. Germeraad, **S.A. Bergervoet**, F. Harders, A. Bossers, G. Koch. Genetic relationship between poultry and wild bird viruses during the highly pathogenic avian influenza H5N6 epidemic in the Netherlands, 2017-2018. 2019 Mar. *Transbound Emerg Dis*. DOI: 10.1111/tbed.13169.

N. Beerens, R. Heutink, **S.A. Bergervoet**, F. Harders, A. Bossers, G. Koch. Multiple reassorted viruses as cause of highly pathogenic avian influenza A(H5N8) virus epidemic, the Netherlands, 2016. 2017 Dec. *Emerg Infect Dis*. DOI: 10.3201/eid2312.171062.

N.B. Cleton, K. van Maanen, **S.A. Bergervoet**, N. Bon, C. Beck, G.J. Godeke, S. Lecollinet, R. Bowen, D. Lelli, N. Nowotny, M.P.G. Koopmans, C.B.E.M. Reusken. A serological protein microarray for detection of multiple cross-reactive flavivirus infections in horses for veterinary and public health surveillance. 2017 Dec. *Transbound Emerg Dis.* DOI: 10.1111/tbed.12569.

S.A. Bergervoet, R. Heutink, S. Pritz-Verschuren, M.J. Poen, R. Bouwstra, R.A.M. Fouchier, N. Beerens. Diversity and evolution of avian influenza (AI) viruses in poultry and wild birds. 2017 Mar. *Virus Evol*. DOI: 10.1093/ve/vew036.040.

S.A. Bergervoet, A.J. Jacobi. Opleiden, trainen en oefenen als voorbereiding op infectieziekte-uitbraken. 2014 May. *Infectieziekten Bulletin*.