Highly Pathogenic Avian Influenza in Wild Birds

Towards evidence-based surveillance

M.J. Poen

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Towards evidence-based surveillance

Maria Johanna Poen

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Highly Pathogenic Avian Influenza In Wild Birds:
Towards evidence-based surveillance

Hoog pathogene vogelgriep in wilde vogels:
Aanzet tot een wetenschappelijk onderbouwd surveillance systeem

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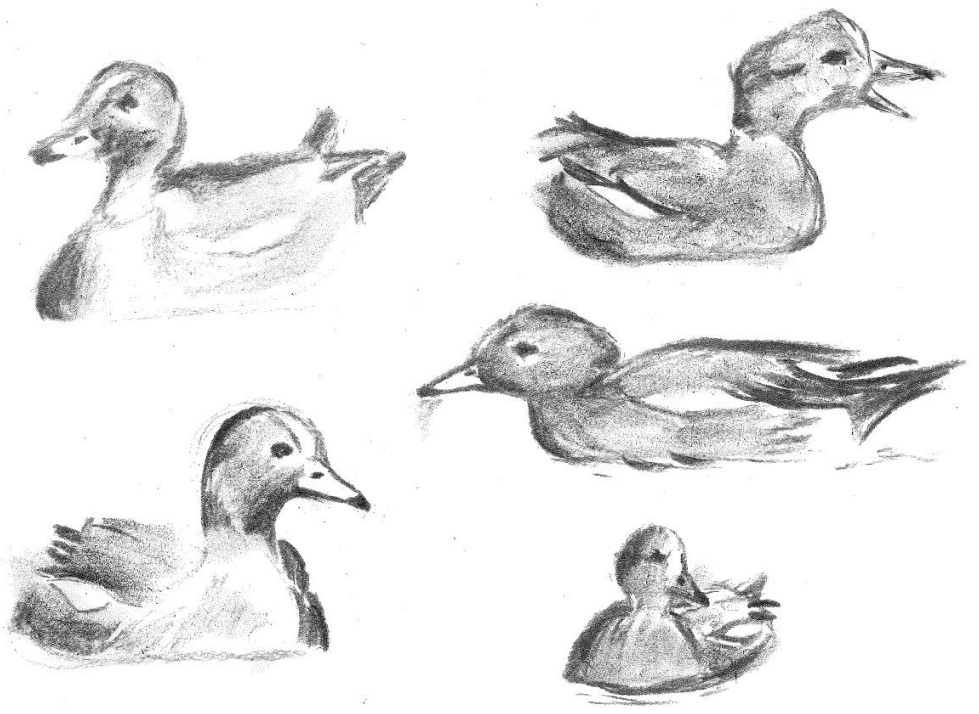

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Preface

No duty is more urgent than that of returning thanks
-James Allen (1864-1912)

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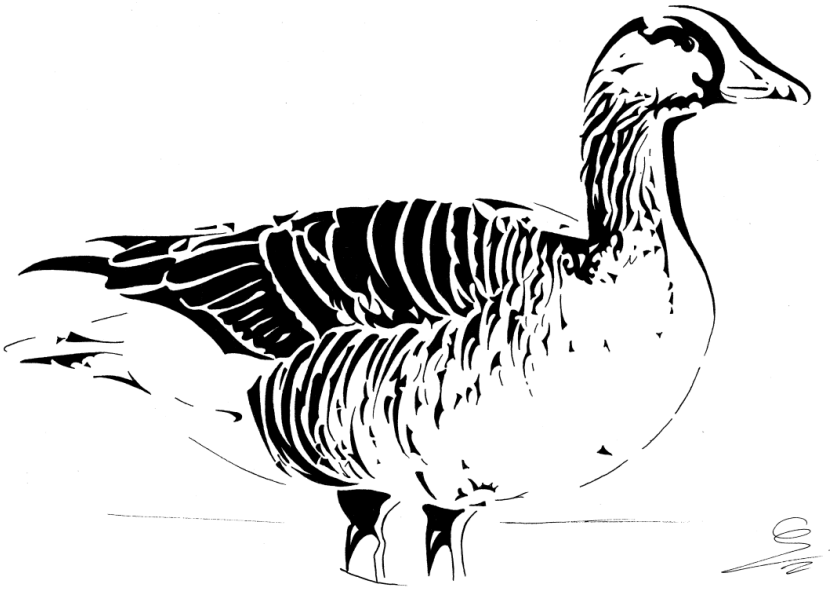

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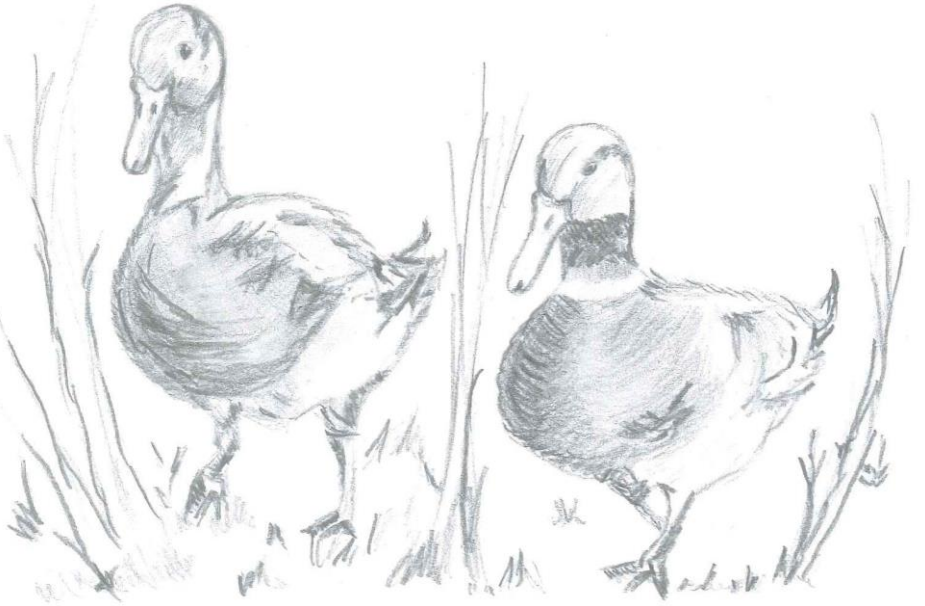
Chapter 1

General introduction



Vic

Chapter 1

Introduction

Avian influenza viruses

Avian influenza (AI) viruses are negative sense single-stranded RNA viruses of the Orthomyxovirus family. The genome comprises eight gene segments [1]. Influenza A viruses are classified based on two surface glycoproteins, the haemagglutinin (HA) and neuraminidase (NA), encoded by the HA gene and the NA gene respectively. The internal gene cassette consists of six gene segments that code for the polymerase complex (polymerase basic 2 [PB2], polymerase basic 1 [PB1] and polymerase acidic [PA]), the nucleoprotein (NP), the matrix proteins (M1 and M2), and the non-structural proteins (NS1 and NS2) [1]. Currently, 18 HA subtypes (H1 – H18) and 11 NA subtypes (N1 – N11) have been recognised [2-7]. The wild bird reservoir, in particular wild waterfowl, harbours most of the combinations of 16 HA (H1 – H16) and 9 NA (N1 – N9) subtypes [6, 8-10]. Viruses of the subtypes H17 and H18 together with N10 and N11 have been detected solely in bats [7]. Influenza viruses evolve rapidly by mutation or reassortment, i.e. the exchange of gene segments.

Highly pathogenic avian influenza viruses

Avian influenza viruses pose a constant threat to both animal and human health and are therefore pathogens of major global concern. Avian influenza viruses exist in two forms, as low pathogenic avian influenza (LPAI) or highly pathogenic avian influenza (HPAI) viruses. Wild birds, mainly wild waterfowl of the orders *Anseriformes* (mainly ducks, geese and swans) and *Charadriiformes* (mainly gulls and shorebirds) [8] are the natural hosts for LPAI viruses, that circulate enzootically in these species with main tropism for the gastro-intestinal tract [11-13] without obvious signs of disease [14]. Occasionally, these LPAI viruses are introduced into poultry, with mild or no signs of disease [15-17]. LPAI viruses of the subtypes H5 and H7 can become highly pathogenic upon introduction in poultry [8, 18], by the insertion of several nucleotides coding for basic amino acids at the cleavage site of the HA protein, resulting in a so-called multi-basic cleavage site. This multi-basic cleavage site enables the viral HA to be cleaved (activated) by ubiquitous furin-like proteases, leading to systemic virus replication [19-22] with severe signs of disease in poultry [23], and to

variable extent in wild birds [24-29]. In contrast, the cleavage of the HA protein of LPAI viruses is dependent on the presence of host proteases such as trypsin-like enzymes, and thus restricted to locations where these proteases are present, i.e. the intestinal tract and respiratory tract. The definition of HPAI versus LPAI was formerly based on the lethality in chickens inoculated experimentally via the intravenous route [30], but currently the molecular criterion of the presence of a multi-basic cleavage site is sufficient. The exact mechanism of this LPAI to HPAI virus transformation is still unrevealed, but there is strong evidence this transformation is related to multiple nucleotide insertions and substitutions or by recombination [31] that is thought to occur in poultry (chickens and turkeys) hosts only [32-34]. Thus, it is generally accepted that HPAI viruses detected in wild birds are spill-over infections from poultry. The economic impact and animal welfare issues that are associated with outbreaks of HPAI in poultry are tremendous, hence detections of viruses of the H5 and H7 subtype in poultry are notifiable [30]. In addition, the introduction of these viruses into the wild bird population can result in a fast and wide geographical spread [35, 36]. Several avian influenza virus subtypes have been associated with human infections. HPAI H5N1 viruses caused 800-900 human infections between 1997 and 2017, part of which resulted in severe disease or death [37, 38], but recently HPAI and LPAI H7N9 and HPAI H5N6 viruses have become the biggest concern for human health, having caused approximately 1,567 [39] and 23 [38] recent human infections respectively, since 2013.

Global emergence of highly pathogenic avian influenza viruses of the H5 subtype

One of the first HPAI H5 virus detections dates back to 1959, when an HPAI H5N1 virus was detected in chickens in Scotland [40]. Until 1996, six additional detections of HPAI H5 virus have been reported from South Africa, Canada, the United States of America, Ireland, the United Kingdom, and Mexico. Most likely, these were all separate transformations from LPAI to HPAI H5 viruses, without subsequent spread [41, 42]. In 1996, an HPAI H5N1 virus called A/Goose/Guangdong/1/1996 (GSGD) was detected in China [43]. In contrast to the earlier single detections of HPAI H5 viruses, descendants of this 1996 virus, referred to as GSGD-lineage viruses, were occasionally detected in Asia between 1996 and 2003 [44]. From 2003, these viruses have circulated enzootically in poultry in several countries in South and Southeast Asia, the Middle East and Africa [45]. Periodically, these HPAI H5 viruses have been introduced into wild birds with subsequent spread to other geographical areas, likely through bird migration [35, 36]. Viruses of the GSGD-lineage have genetically diversified into different genetic "clades" leading also to antigenic differences. In 2008, 10 different main clades (clade 0 to clade 9) were identified [46]. Viruses of most of these clades have circulated only for a limited time frame and with limited geographical spread. However, some, like clade 2, have evolved into several

subclades with subsequent subdivisions, e.g. clade 2.1.1.1. Since 2012, only viruses of clade 1, 2, and 7 have been detected [44].

It is likely that specific aspects of the poultry production sector in Asia have contributed to the conditions where the GSGD-lineage viruses could be maintained and spread in poultry populations. The poultry sector in Asia has greatly expanded in recent decades. Although chickens are a common poultry type, Asia has by far the largest number of domestic ducks in the world. Farms in Asia range in size from small backyard farms to large commercial farms, which combination might facilitate a good environment for HPAI H5 viruses to be maintained [47]. Many poultry farms keep livestock outside, free-ranging in close contact with wild waterfowl and their environment [48]. Furthermore, there is a lively poultry trade in Asia where farmers from diverse regions bring live poultry to local and regional wet markets. This aggregation of live poultry from different geographical locations facilitates virus transmission and dissemination among poultry populations from different locations [49, 50]. GSGD-lineage viruses have been detected in (migratory) wild birds frequently, most likely as a consequence of mingling with free-ranging poultry. The combination of a dense and diverse poultry sector, wet markets with live poultry trade, high contact rates with wild migratory waterfowl, suboptimal veterinary service and poor biosecurity forms an ideal environment for influenza viruses to be maintained, evolve and disperse [51]. HPAI H5N1 viruses of the GSGD-lineage clade 2.2 emerged after 2003, leading to a massive number of outbreaks in Asia in 2003/2004 with subsequent spread to Europe in 2005 [45, 52], infecting poultry and wild bird populations. This virus clade disappeared from Europe in 2009/2010 [45, 53]. From 2008 onwards a new subclade of HPAI H5N1 virus, clade 2.3.2.1c, gained prevalence in China and Southeast Asia [53], subsequently expanding from China to Mongolia, Russia and Eastern Europe [54] in early 2010 [55]. In 2015, clade 2.3.2.1c viruses were again detected in Eastern Europe, China, Russia and Africa [56, 57]. Since 2014, HPAI H5 clade 2.3.4.4 viruses with different NA subtypes have emerged (e.g. H5N8, H5N6, H5N3, H5N2, H5N5 [58-60]), which have been circulating in Southeast Asia alongside HPAI viruses of other clades like 2.3.2 [56] and 2.2. These novel clade 2.3.4.4 viruses caused three waves of intercontinental spread, starting in 2014 and still ongoing.

Diagnostics and virus characterization

Traditionally, influenza virus diagnostics depended on virus isolation, by inoculating clinical material (e.g. oropharyngeal or cloacal swab material) into 11-day-old embryonated chicken eggs (or Madin-Darby Canine Kidney cells) to obtain a virus isolate. The virus isolate's HA and NA subtypes were determined with haemagglutination inhibition (HI) assays and neuraminidase inhibition assays,

respectively. Nowadays, newer, faster and more sensitive viral detection methods based on genetics are commonly used, like Polymerase Chain Reaction (PCR) techniques that are able to specifically detect the presence of the influenza virus genes (e.g. matrix, H5 gene or H7 gene). Subsequently, the genetic code of the full viral genome can be obtained by sequencing methods like Sanger sequencing or next-generation sequencing (NGS) methods. In contrast to Sanger sequencing that generates a consensus (i.e. majority) sequence, NGS methods can obtain sequences of individual genomic segments in a sample, enabling the identification of minority variants [61, 62]. The increasing popularity and decreasing costs of NGS methods have led to the development of many different sequencing platforms (sample preparation and sequencing machines) with different bioinformatics workflows to process the raw sequence data.

Antibodies are markers for immune response to infections, allowing the diagnosis of past infections. In response to an influenza virus infection, the immune system generates responses that help eliminate the virus and provide a certain level of protection for future encounters with similar viruses. Serological assays like HI assays, enzyme-linked immunosorbent assays (ELISA), and microneutralisations (MN) assays are commonly used to detect antibodies in the blood that are formed upon infection with an influenza virus in humans and domestic animals. However, there are currently no validated serological assays for testing wild bird sera, although NP-ELISAs, HI assays and MN assays are most commonly used with proteins micro-arrays gaining popularity. In addition to the ability to distinguish for subtype-specific antibodies, the rapid diversification of HPAI H5 viruses have led to the ability to distinguish between H5 clade and subclade specific antibodies [63]. However, taking into consideration that wild birds' initial antibody responses are weak and may be short-lived, antibodies may be only detectable for a limited timeframe of months [64].

Avian influenza surveillance in wild birds

Since 1997, an increasing number of countries have established avian influenza surveillance programmes in wild birds. Some of these national wild bird surveillance programmes were set up to serve as an early warning system for the presence of HPAI viruses, in order to prevent further spread to poultry. Most of those programmes cover passive surveillance activities, i.e. testing of diseased or dead animals. In some countries, like the United States (Delaware Bay), Canada (Alberta) [8, 65, 66], Germany, Sweden (Ottenby [67]), and the Netherlands [68], additional more continuous active surveillance programmes are implemented in which living and clinically healthy birds are tested for virus and/or antibody presence. In the Netherlands, active surveillance is performed by the Department of Viroscience of

the Erasmus MC in collaboration with ornithologists, testing approximately 10,000-15,000 birds in the Netherlands and 2,000 birds from other countries annually. To date, it is unknown which wild bird species are involved in long-distance dispersal of HPAI viruses, although the involvement of terrestrial birds is less likely [69-71]. The flyways used by migratory birds to migrate between wintering and breeding sites further complicate influenza surveillance studies in wild birds. Although eight major flyways have been described these are only rough abstractions, and are highly variable due to factors like the weather, availability of food or human activities [72-74]. Despite these challenges, active avian influenza surveillance projects in wild birds have proved to be valuable in providing new information with regard to host species, seasonal trends, population dynamics, and virus subtype diversity for both HPAI and LPAI viruses.

Thesis outline

It is important to characterise and understand the emergence and dynamics of avian influenza virus infections in wild birds, that are able to transport these viruses over large distances. In this thesis, we investigated the involvement of wild birds and discussed the virus dynamics in three subsequent incursions of emerging HPAI H5 clade 2.3.4.4 viruses in Europe after 2014 based on virological and serological results (chapter 2.1, 2.2 and 3). In addition, we studied the applicability of NGS for epidemiological studies in outbreak situations by evaluating the repeatability and comparability of NGS results from HPAI H5N8 viruses (chapter 4). The information we gathered by studying these outbreaks have contributed to the knowledge of HPAI circulation in wild birds, and to our vision on an evidence-based optimal combination of national and international surveillance efforts to serve as a system that would better fulfil the purpose of an early warning system for these HPAI viruses entering Europe (chapter 5).

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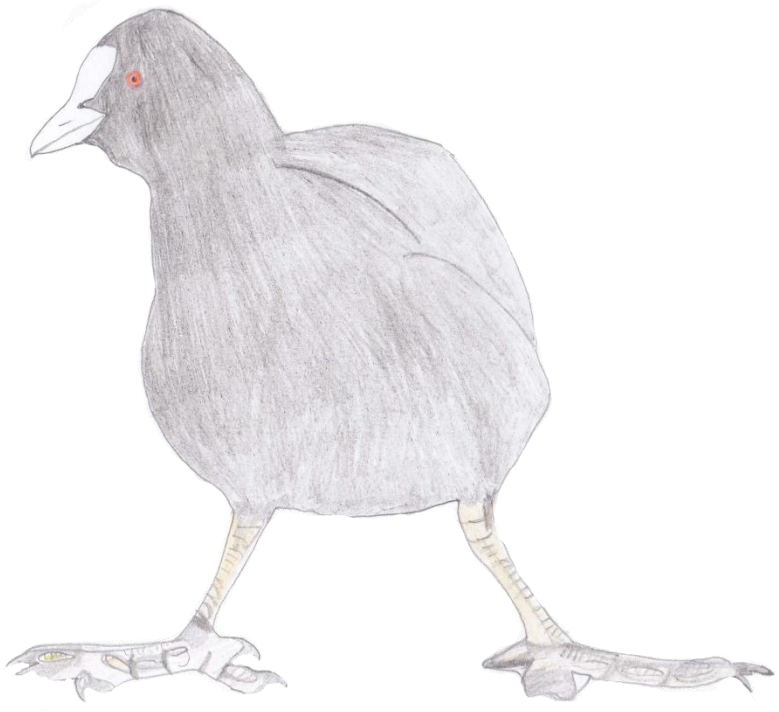

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Chapter 2

Highly pathogenic avian influenza virus A/H5N8 in Europe

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Chapter 2.1

Lack of virological and serological evidence for continued circulation of highly pathogenic avian influenza H5N8 virus in wild birds in the Netherlands, 14 November 2014 to 31 January 2016

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Abstract

In 2014, H5N8 clade 2.3.4.4 highly pathogenic avian influenza (HPAI) viruses of the A/Goose/Guangdong/1/1996 lineage emerged in poultry and wild birds in Asia, Europe and North America. Here,wild birds were extensively investigated in the Netherlands for HPAI H5N8 virus (real-time polymerase chain reaction targeting the matrix and H5 gene) and antibody detection (haemagglutination inhibition and virus neutralisation assays) before, during and after the first virus detection in Europe in late 2014.Between 21 February 2015 and 31 January 2016, 7,337 bird samples were tested for the virus. One HPAI H5N8 virus-infected Eurasian wigeon (Anas penelope) sampledon 25 February 2015 was detected. Serological assays were performed on 1,443 samples, including 149 collected between 2007 and 2013, 945 between 14 November 2014 and 13 May 2015, and 349 between 1 September and 31 December 2015. Antibodies specific for HPAI H5 clade 2.3.4.4 were absent in wild bird sera obtained before 2014 and present in sera collected during and after the HPAI H5N8 emergence in Europe, with antibody incidence declining after the 2014/15 winter. Our results indicate that the HPAI H5N8 virus has not continued to circulate extensively in wild bird populations since the 2014/15 winter and that independent maintenance of the virus in these populations appears unlikely.

Introduction

Wild birds are the natural hosts of low pathogenic avian influenza (LPAI) viruses, which generally do not cause clinical signs of disease in these host species [1]. So far, virus subtypes H1 to H16 and N1 to N9 have been detected in wild birds, of which viruses of subtypes H5 and H7 have shown the ability to evolve to highly pathogenic avian influenza (HPAI) viruses in poultry, causing severe disease with high mortality in such animals. These HPAI viruses were historically mainly detected in rapidly contained sporadic outbreaks in poultry, until H5N1 viruses of the A/Goose/Guangdong/1/1996 (GsGd) lineage emerged in Asia in 1997. Subsequently, these viruses have continuously circulated in poultry with frequent detections in wild birds [2] and with significant expansion in global range.

HPAI H5N8 viruses of the GsGd lineage of clade 2.3.4.4 emerged in poultry and wild birds on multiple continents in 2014. The ancestral influenza H5N8 virus to the strains causing outbreaks from 2014 onwards was first detected in China in 2010 in a captive-held mallard *(Anas platyrhynchos)* [3]. In early 2014, HPAI H5N8 GsGd virus of clade 2.3.4.4 occurred for the first time in poultry in South Korea, soon after causing outbreaks also in Japan [4]. From late 2014 onwards, this virus spread to other areas of the world including Europe, North America, Russia and Taiwan [5-8]. The HPAI H5N8 virus detections in Europe were limited to sporadic cases in wild birds and a relatively small number of unrelated outbreaks in poultry. However in North America HPAI H5N8 viruses reassorted with co-circulating LPAI viruses, giving rise to new HPAI H5N1 and H5N2 virus subtypes that caused a large number of outbreaks in poultry with numerous detections in wild birds [9]. Despite mild clinical symptoms caused by infection with HPAI H5N8 viruses of clade 2.3.4.4 in experimentally infected mammals [10-12] and ducks [11], the widespread detection and rapid global spread of HPAI H5 clade 2.3.4.4 viruses pose a potential threat to domestic and wild animals and should be studied further.

The major challenges in understanding the epidemiology of emerging influenza viruses in wild birds are the large numbers of potential host species and the usually short period of viral shedding, combined with the difficulty of catching and sampling representative numbers per species. For instance, mallards that were experimentally infected with HPAI H5N8 virus shed infectious virus in tracheal swabs for only up to 5 days post infection [11]. These impediments result in a low probability of detecting newly emerging avian influenza viruses in wild birds through active virological surveillance and result in a delay of implementation of effective control measures. Nevertheless, to date HPAI H5N8 virus has been detected in 30 wild bird species. In addition to the host species previously described [13,14], HPAI H5N8 viruses have been detected in wild bird species belonging to the orders *Anseriformes* in Asia (*Aythya spp.*) and North America (*Branta spp.*) [6]. In Europe, HPAI H5N8 viruses

have been detected in bird species of the orders *Anseriformes* (Anas spp. and Cygnus spp.) and Charadriiformes (Larus spp.) [5,6,14].

To estimate the likelihood of the involvement of live wild birds in local and long distance movement of HPAI H5 viruses, information on recent exposure of wild bird populations to HPAI H5N8 viruses using serology, in addition to virology, would add substantial power to surveillance programmes. Studies with ferret sera have shown serological tests to have substantial discriminative power between antibodies directed to HPAI H5 viruses of different clades and LPAI H5 viruses using haemagglutination inhibition (HI) assays [12,15]. Although less is known about serology in wild birds, a study on wild birds sampled in Europe and Mongolia showed that antigenic differences between the haemagglutinin (HA) of classical Eurasian LPAI H5 viruses and GsGd lineage HPAI H5 viruses can be used to define bird populations in which HPAI viruses have previously been circulating [16]. With regard to HPAI H5N8 viruses specifically, a 2014 South Korean serology study showed evidence of a rise of H5 virus antibodies occurring in long distance migratory duck species after the onset of the HPAI H5N8 virus emergence in South Korea [4].

In this study, in response to the emergence of HPAI H5N8 virus in Europe, we present data on wild bird surveillance activities in the Netherlands, including results of virological and serological assays.

Methods

Ethical statement

The capture of free-living birds was approved by the Dutch Ministry of Economic Affairs based on the Flora and Fauna Act (permit number FF/75A/2009/067 and FF/75A/2014/054). Handling and sampling of free-living birds was approved by the Animal Experiment Committee of the Erasmus Medical Centre (permit number 122–11–31). Free-living birds were released into the wild after sampling and all efforts were made to minimise animal suffering throughout the studies.

Study population

Immediately after the first detection of HPAI H5N8 virus in poultry in Europe, ongoing influenza surveillance activities in migrating and overwintering wild birds in the Netherlands were intensified (14 November 2014–13 May 2015). Hereafter, this period will be referred to as 'during the outbreak'. Surveillance activities in wild birds in the Netherlands were again intensified from the onset of the arrival of wild migrating birds a year after the initial HPAI H5N8 virus detection in Europe (1 September–31 December 2015). This period will be referred to as 'after the outbreak'.

Sampled populations consisted of resident birds, partial migrants and long distance migrants. During both periods of intensified surveillance, blood samples were obtained in addition to samples for virus detection. A matching historical set of serum samples was compiled based on similarity in species and family, hereafter referred to as 'before the outbreak' (2007–2013).

Sample collection

Wild birds were captured using duck decoys, clap nets, cannon nets, mist nets, leg-nooses, swan hooks, or manually. Birds were sampled routinely for virus detection using cloacal and/or oropharyngeal swabs as described elsewhere [14]. In addition, faecal samples were collected from a limited number of species for virus detection. Blood samples were collected for antibody detection. Blood samples were collected from the brachial or metatarsal vein and centrifuged at 3,000 rpm for 10 min in 0.8 mL gel separation tubes (MiniCollect tubes, Greiner). Serum samples were stored below -20 °C until analysis.

Virus detection, isolation and characterisation

Samples for virus detection were analysed for the presence of HPAI H5(N8) virus using matrix- and H5-specific real-time polymerase chain reaction (RT-PCR) assays followed by H5 and neuraminidase sequencing as previously described [14]. Samples testing positive in matrix specific RT-PCR were inoculated in embryonated chicken eggs as described previously [17].

Antibody detection

Serum samples were first tested for the presence of H5-specific antibodies in an HI assay according to standard procedures [18]. Briefly, serum samples were incubated for 16 hours at 37 °C with Vibrio cholerae filtrate containing receptor-destroying enzyme to remove non-specific inhibitors of haemagglutination activity, followed by incubation for 1 hour at 56 °C. Twofold serial dilutions of serum samples with a start dilution of 1:20 were prepared using phosphate-buffered saline (PBS) in U-bottomed 96 well microtitre plates. Serum dilutions were incubated with four haemagglutinating units (HAU) of Madin–Darby canine kidney (MDCK) (all HPAI H5 clade viruses) or egg (A/Mallard/Netherlands/3/1999) cultured virus for 30 min at 37 °C. A suspension of 1% turkey red blood cells (TRBC) was added to the serum-virus dilutions. After incubation for 1 hour at 4 °C, haemagglutination patterns were read. Negative controls, based on serum incubation without virus, were used to measure non-specific haemagglutination of each serum sample. Sera showing high

background (i.e. high non-specific haemagglutination) were pre-treated with 10% TRBC for 1 hour at 4 °C and retested for the presence of H5-specific antibodies as described above. Serum samples from experimentally inoculated ferrets [12,15], a domestic duck, and a domestic goose were used as positive controls.

All serum samples were initially screened for antibodies specific for classical Eurasian LPAI H5N2 virus A/Mallard/Netherlands/3/1999 and clade 2.3.4.4 HPAI H5N8 virus A/Chicken/Netherlands/EMC-3/2014. Serum samples that tested positive for HPAI H5 clade 2.3.4.4-specific antibodies were further tested against HPAI viruses of the H5 clades 1 (A/Viet Nam/1194/2004), 2.1 (A/Indonesia/5/2005), 2.2 (A/Turkey/Turkey/1/2005), and 2.3 (A/Anhui/1/2005), and retested against the clade 2.3.4.4 virus. Samples showing more than threefold differences in titre or testing negative in the second assay after showing initial titres were tested a third time. The viruses used were recombinant viruses based on an A/PR/8/34 virus backbone, containing the HA and neuraminidase (NA) of the representative H5 strains. The sequences of the HA genes were modified to remove the multi-basic cleavage site to enable this study within biosafety level 2 laboratories. HPAI H5 virus of clade 0 was excluded from the analyses due to high overall reactivity with all avian positive control sera as previously described [16] and thus of limited discriminative value.

A representative selection (based on titre and serum availability) of serum samples that tested positive for HPAI H5 clade 2.3.4.4 antibodies were sent to the Animal and Plant Health Agency (APHA) (Weybridge, UK) for confirmation of HPAI H5 clade 2.3.4.4-specific antibodies using an HI assay. The HI assay procedure used by the APHA differed from the HI assay described above and was carried out in accordance to the World Organisation for Animal Health (OIE) [19]. In short, twofold serial dilutions of serum samples with a start dilution of 1:12 were made using phosphate-buffered saline (PBS) and prepared in V-bottomed microtitre plates. Serum dilutions were incubated with four HAU of egg cultured virus for 30 min at room temperature. A solution of 1% chicken red blood cells (CRBC) was added to the serum–virus dilutions. After incubation for 30 min at room temperature, haemagglutination patterns/streaming of red cells were read. Polyclonal chicken sera raised against the same clade 2.1, 2.2, 2.3, and 2.3.4.4 viruses as mentioned above were used as positive controls, supplemented with LPAI H5N3 virus A/Teal/England/7394–2805/2006 and clade 2.3.4.4 HPAI H5N8 virus A/Duck/England/36254/2014.

All samples that tested positive for HPAI H5 clade 2.3.4.4-specific antibodies in the initial HI assay were tested in a virus neutralisation (VN) assay if sufficient amounts of serum were available. The VN assay was performed as described previously [20], using titrated virus stocks of clade 2.1, 2.3, and 2.3.4.4. Briefly, serum was heat inactivated for 30 min at 56 °C and twofold serial dilutions of the sera starting at a 1:20 dilution were prepared and 100 median tissue culture infectious dose (TCID50) was added. After incubating antigen and serum for 1 hour at 37 °C with 5% CO_2, the

mixtures were transferred to 96 well flat bottom plates containing MDCK cells, which were washed once with infection medium before inoculation. The plates were incubated for 1 hour at 37 °C with 5% CO2, after which the cells were washed once with 100 µL infection medium and the medium was replaced by 200 µL infection medium. Three days later, a haemagglutination assay was performed with the supernatant to determine the antibody titres.

Results

Study population

A total of 11,355 birds were sampled for virus detection during and after the first detection of HPAI H5N8 viruses in poultry and wild birds in Europe. Of those, 5,387 birds were sampled during the outbreak and 5,968 after the outbreak. This report describes the results on 7,337 samples obtained from 21 February 2015 onwards in addition to the previously reported 4,018 samples obtained until 20 February 2015 [14]. Sampled species mainly belonged to the orders Anseriformes, Charadriiformes and Gruiformes (Table 1).

For antibody detection, 1,443 serum samples were analysed. Among these, 945 samples from 25 avian species were obtained during the outbreak, while 349 samples from 15 species originated from after the outbreak. A total of 149 serum samples from 15 species sampled before the HPAI H5N8 virus emergence, obtained between 2007 and 2013, served as controls (Table 2). The majority of these samples were collected from birds wintering in Dutch wetlands.

Table 1. Wild bird species sampled for virus detection during and after the emergence of highly pathogenic avian influenza H5N8 virus in Europe, the Netherlands, 21 February 2015–31 January 2016 (n = 7,337 animals)

Order	Family	Species	During outbreak: 21 Feb 2015–13 May 2015				After outbreak: 14 May 2015–31 Jan 2016			
			Birds sampled (N)	AIV-positive birds (N)	H5-positive birds (N)	Pathotype	Birds sampled (N)	AIV-positive birds (N)	H5-positive birds (N)	Pathotype
Anseriformes	Ducks	Common pochard *(Aythya ferina)*	0	0	0	NA	1	0	0	NA
		Common teal *(Anas crecca)*	8	0	0	NA	221	39	4	LPAI
		Egyptian goose *(Alopochen aegyptiaca)*	58	0	0	NA	136	0	0	NA

Order	Family	Species	During outbreak: 21 Feb 2015–13 May 2015				After outbreak: 14 May 2015–31 Jan 2016			
			Birds sampled (N)	AIV-positive birds (N)	H5-positive birds (N)	Pathotype	Birds sampled (N)	AIV-positive birds (N)	H5-positive birds (N)	Pathotype
		Eurasian wigeon (*Anas penelope*)	175	1	1	HPAI	1,034	101	2	LPAI
		Gadwall (*Anas strepera*)	1	0	0	NA	175	15	0	NA
		Mallard (*Anas platyrhynchos*)	748	50	0	NA	2,464	354	15	LPAI
		Mandarin duck (*Aix galericulata*)	2	0	0	NA	0	0	0	NA
		Northern pintail (*Anas acuta*)	0	0	0	NA	7	3	0	NA
		Northern shoveler (*Anas clypeata*)	0	0	0	NA	17	2	0	NA
		Tufted duck (*Aythya fuligula*)	0	0	0	NA	1	0	0	NA
	Geese	Barnacle goose (*Branta leucopsis*)	96	5	4	LPAI	926	3	0	NA
		Bean goose (*Anser fabalis*)	0	0	0	NA	8	0	0	NA
		Brent goose (*Branta bernicla*)	54	0	0	NA	0	0	0	NA
		Canada goose (*Branta canadensis*)	3	0	0	NA	72	0	0	NA
		Greylag goose (*Anser anser*)	59	0	0	NA	239	0	0	NA
		Pink-footed goose (*Anser brachyrhynchus*)	0	0	0	NA	1	0	0	NA
		Greater white-fronted goose (*Anser albifrons*)	0	0	0	NA	55	0	0	NA
	Swans	Mute swan (*Cygnus olor*)	3	0	0	NA	31	1	0	NA
Charadriiformes	Gulls	Black-headed gull (*Chroicocephalus ridibundus*)	84	0	0	NA	392	53	0	NA
		Caspian gull (*Larus cachinnans*)	4	0	0	NA	4	0	0	NA
		Common gull (*Larus canus*)	1	0	0	NA	18	0	0	NA
		Great black-backed gull (*Larus marinus*)	1	0	0	NA	0	0	0	NA
		Herring gull (*Larus argentatus*)	15	0	0	NA	32	2	0	NA
		Lesser black-backed gull (*Larus fuscus*)	0	0	0	NA	33	2	0	NA
		Mediterranean gull (*Larus melanocephalus*)	1	0	0	NA	3	1	0	NA
		Yellow-legged gull (*Larus michahellis*)	0	0	0	NA	1	0	0	NA
	Lapwings	Northern lapwing (*Vanellus vanellus*)	6	0	0	NA	0	0	0	NA
	Terns	Black tern (*Chlidonias niger*)	0	0	0	NA	0	0	0	NA
		Common tern (*Sterna hirundo*)	0	0	0	NA	0	0	0	NA

Order	Family	Species	During outbreak: 21 Feb 2015–13 May 2015				After outbreak: 14 May 2015–31 Jan 2016			
			Birds sampled (N)	AIV-positive birds (N)	H5-positive birds (N)	Pathotype	Birds sampled (N)	AIV-positive birds (N)	H5-positive birds (N)	Pathotype
Columbiformes	Pigeons	Common wood-pigeon *(Columba palumbus)*	1	0	0	NA	0	0	0	NA
Gruiformes	Coots	Common coot *(Fulica atra)*	46	0	0	NA	92	0	0	NA
	Rails	Little crake *(Porzana parva)*	0	0	0	NA	1	0	0	NA
		Common moorhen *(Gallinula chloropus)*	3	0	0	NA	4	0	0	NA
Total			1,369	56	5	NA	5,968	576	21	NA

AIV: avian influenza virus; HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza; N: number; NA: not applicable. Surveillance activities were intensified from 21 February to 13 May 2015 (n = 1,369) and 1 September to 31 December 2015 (n = 3,736).

Table 2. Wild bird species sampled for H5-specific antibody detection before, during and after the emergence of highly pathogenic avian influenza H5N8 virus in Europe, the Netherlands, 2007–2015 (n = 1,443)

Order	Family	Species	Number of individuals sampled		
			Before outbreak (before 2014)	During outbreak (14 Nov 2014 – 13 May 2015)	After outbreak (1 Sep 2015 – 31 Dec 2015)
Anseriformes	Ducks	Common teal *(Anas crecca)*	0	15	111
		Egyptian goose *(Alopochen aegyptiaca)*	9	62	28
		Eurasian wigeon *(Anas penelope)*	0	78	46
		Gadwall *(Anas strepera)*	1	3	1
		Mallard *(Anas platyrhynchos)*	21	93	18
		Mandarin duck *(Aix galericulata)*	1	2	0
		Northern pintail *(Anas acuta)*	0	0	1
		Northern shoveler *(Anas clypeata)*	0	2	3
		Ruddy shelduck *(Tadorna ferruginea)*	1	0	0
	Geese	Barnacle goose *(Branta leucopsis)*	20	19	0
		Bean goose *(Anser fabalis)*	5	0	0
		Brent goose *(Branta bernicla)*	0	19	0
		Greylag goose *(Anser anser)*	0	2	0
		Lesser white-fronted goose *(Anser erythropus)*	0	3	0
		Pink-footed goose *(Anser brachyrhynchus)*	0	1	0
		Greater white-fronted goose *(Anser albifrons)*	20	77	0

Order	Family	Species	Number of individuals sampled		
			Before outbreak (before 2014)	During outbreak (14 Nov 2014 – 13 May 2015)	After outbreak (1 Sep 2015 – 31 Dec 2015)
	Swans	Bewick's swan *(Cygnus columbianus bewickii)*	0	20	0
		Mute swan *(Cygnus olor)*	10	90	29
		Whooper swan *(Cygnus cygnus)*	0	1	0
Charadriiformes	Gulls	Black-headed gull *(Chroicocephalus ridibundus)*	20	262	31
		Caspian gull *(Larus cachinnans)*	0	6	3
		Common gull *(Larus canus)*	12	34	17
		Great black-backed gull *(Larus marinus)*	0	1	0
		Herring gull *(Larus argentatus)*	7	61	28
		Lesser black-backed gull *(Larus fuscus)*	1	3	8
		Mediterranean gull *(Ichthyaetus melanocephalus)*	2	1	0
		Yellow-legged gull *(Larus michahellis)*	0	0	1
Gruiformes	Rails	Common coot *(Fulica atra)*	19	84	24
		Moorhen *(Gallinula chloropus)*	0	6	0
Total			149	945	349

Virus detection, isolation and characterization

In addition to the two previously reported HPAI H5N8 virus-infected Eurasian wigeons detected in the Netherlands in November 2014 [14], the virus was detected in a third Eurasian wigeon faecal sample obtained on 25 February 2015 (1/1,369 birds sampled in 21 February–13 May 2015), near Ilpendam (52°28′N 4°57′E) (GenBank accession numbers: AKH14448–AKH14459). Since then, no HPAI H5N8 virus has been detected in any of the samples tested (0/5,968 birds sampled in 14 May 2015–31 January 2016) (Table 1).

Influenza A H5 virus clade-specific antibody detection

As shown previously, ferret antisera raised against prototype strains representing LPAI and HPAI H5 viruses of various clades showed almost exclusive reactivity with homologous viruses in HI assays [12] (Table 3). Importantly, a ferret antiserum raised against the clade 2.3.4.4 virus did not react with other H5 viruses, and antisera raised against other prototype H5 strains did not react with the clade 2.3.4.4 virus A/Chicken/Netherlands/EMC-3/2014. Sera obtained upon inoculation of a domestic duck and a domestic goose with the clade 2.3.4.4 virus A/Turkey/Germany/AR2487/2014 reacted similar to the ferret clade 2.3.4.4 antiserum; no cross-reactivity was seen with other prototype H5 strains (Table 3). These data

indicate that the antigenic differences between clade 2.3.4.4 HA and HA of LPAI and HPAI viruses belonging to other clades were sufficiently large to allow serological discrimination by HI assay.

Influenza A virus H5-specific antibody detection in wild birds

Haemagglutination inhibition assays

Of the serum samples initially tested in the HI assay with LPAI H5N2 (A/Mallard/Netherlands/3/1999) and HPAI H5 clade 2.3.4.4 H5N8 (A/Chicken/Netherlands/EMC-3/2014) virus, LPAI H5-specific antibodies were detected in 31 of 1,443 serum samples and HPAI H5 clade 2.3.4.4-specific antibodies in 53 of 1,443 serum samples (Table 4). Among these, seven samples tested positive for both LPAI H5- and HPAI H5 clade 2.3.4.4-specific antibodies. The incidence of LPAI H5-specific antibodies was similar before, during and after the HPAI H5N8 virus emergence in Europe (Fisher exact test, $p = 0.76$ before vs during the outbreak; $p = 0.39$ during vs after the outbreak), while HPAI H5 clade 2.3.4.4-specific antibodies were detected exclusively in sera from five bird species, obtained during and after the HPAI H5N8 virus emergence in Europe (Table 4, Table 5). The incidence of HPAI H5 clade 2.3.4.4-specific antibodies a year after the outbreak (10/329 (20 samples with high background excluded), 3.0%) was lower than during the outbreak (43/940 (5 samples with high background excluded), 4.6%) (Fisher exact test, $p = 0.27$).

Serum samples obtained during (43/940 (5 samples with high background excluded), 4.6%) and after (10/329 (20 samples with high background excluded), 3.0%) the outbreak that tested positive for HPAI H5 clade 2.3.4.4-specific antibodies were subsequently tested in an HI assay against prototype viruses of clades 1, 2.1, 2.2, 2.3, and 2.3.4.4. Of the sera collected during the outbreak, 29/90 mute swans (*Cygnus olor*), 12/78 Eurasian wigeons, 1/3 lesser white-fronted geese (*Anser erythropus*) and 1/84 common coots (*Fulica atra*) tested positive for HPAI H5 clade 2.3.4.4-specific antibodies (Table 5). In these HPAI H5 clade 2.3.4.4-specific antibody positive sera, no cross-reactivity was observed in sera of Eurasian wigeons (12/12) and the lesser white-fronted goose (1/1). In contrast, the common coot (1/1) serum showed an additional titre to the clade 2.3 virus and sera of mute swans showed cross-reactivity to clade 2.3 (27/29), 2.1 (23/29), 1 (9/29) and 2.2 (4/29) viruses. In the majority of samples (22/29), titres to clade 2.1 and 2.3 exceeded those detected to clade 2.3.4.4 (Table 6).

Table 3. Details of positive control sera titres from experimentally infected ferrets, a domestic duck, and a domestic goose with one low pathogenic (LPAI) H5 and different highly pathogenic avian influenza (HPAI) H5 clades (n = 8 antisera)

Antiserum raised against	Characteristics	Species	Haemagglutination inhibition assay						Virus neutralisation assay		
			Viruses						Viruses		
				HPAI clade					HPAI clade		
			LPAI	1[a]	2.1[b]	2.2[c]	2.3[d]	2.3.4.4[e]	2.1[b]	2.3[d]	2.3.4.4[e]
A/Mallard/Netherlands/3/1999	LPAI H5N2	Ferret	**160**	< 10	< 10	< 10	< 10	< 10	ND	ND	ND
A/Viet Nam/1194/2004	HPAI H5N1 clade 1	Ferret	< 10	**80**	< 10	< 10	< 10	< 10	ND	ND	ND
A/Indonesia/5/2005	HPAI H5N1 clade 2.1	Ferret	< 10	< 10	**120**	< 10	60	< 10	**80**	< 10	< 10
A/Turkey/Turkey/1/2005	HPAI H5N1 clade 2.2	Ferret	< 10	< 10	< 10	**1,280**	60	< 10	ND	ND	ND
A/Anhui/1/2005	HPAI H5N1 clade 2.3	Ferret	< 10	< 10	< 10	20	**320**	< 10	< 10	**160**	< 10
A/Chicken/Netherlands/EMC-3/2014	HPAI H5N8 clade 2.3.4.4	Ferret	< 10	< 10	< 10	< 10	< 10	**160**	< 10	< 10	**40**
Turkey/Germany/AR2487/2014	HPAI H5N8 clade 2.3.4.4	Domestic duck	< 10	< 10	< 10	< 10	< 10	160	ND	ND	ND
Turkey/Germany/AR2487/2014	HPAI H5N8 clade 2.3.4.4	Domestic goose	< 10	< 10	< 10	< 10	< 10	80	ND	ND	ND

HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza; ND: not determined. Lowest serum dilution tested was 10. Titres indicating the reactivity of sera to viruses homologous to the viruses, which the sera were raised against are in bold. [a] A/Viet Nam/1194/2004, [b] A/Indonesia/5/2005, [c] A/Turkey/Turkey/1/2005, [d] A/Anhui/1/2005, [e] A/Chicken/Netherlands/EMC-3/2014.

Table 4. Detected haemagglutination inhibition antibody titres to low pathogenic avian influenza H5 virus[a] and to highly pathogenic avian influenza H5 clade 2.3.4.4 H5N8 virus[b] in birds, before, during, and after detection of the highly pathogenic avian influenza H5N8 virus in Europe, the Netherlands, 2007–2015 (n = 1,443 birds)

Strain	Period relative to the outbreak[c]	Haemagglutination inhibition titre							High background	Total tested	Total positives
		BLD	10 – 40	40 – 80	80 – 160	160 – 320	320 – 640	≥ 640			
LPAI H5N2[a]	Before	121	1	0	1	0	0	0	26	149	2
	During	903	16	5	2	1	0	0	18	945	24
	After	324	2	1	0	2	0	0	20	349	5
HPAI H5N8[b]	Before	123	0	0	0	0	0	0	26	149	0
	During	897	7	20	6	4	5	1	5	945	43
	After	319	4	3	2	1	0	0	20	349	10

BLD: below limit of detection; LPAI: low pathogenic avian influenza; HPAI: highly pathogenic avian influenza. Lowest serum dilution tested was 10. [a] A/Mallard/Netherlands/3/1999, [b] A/Chicken/Netherlands/EMC-3/2014, [c] The 'outbreak' refers to the six months following the detection of the highly pathogenic avian influenza H5N8 virus in Europe and this extends from 14

November 2014 to 13 May 2015. The period before the 'outbreak' is from 2007 to 2013, while the period after the 'outbreak' is from 1 September to 31 December 2015.

Table 5. Birds species with antibodies to highly pathogenic avian influenza H5 clade 2.3.4.4 H5N8 virus[a], and number of respective animals, according to their haemagglutination inhibition antibody titres to the virus, during and after detection of highly pathogenic avian influenza H5N8 virus in Europe, the Netherlands, 14 November 2014–31 December 2015 (n = 382 birds)

Species	Period relative to the outbreak[b]	HI titre to HPAI H5 clade 2.3.4.4 (H5N8) virus							High back ground	Total tested
		BLD	10 – 40	40 – 80	80 – 160	160– 320	320 – 640	≥ 640		
Eurasian wigeon (*Anas penelope*)	During	66	6	4	2	0	0	0	0	78
Lesser white-fronted goose (*Anser erythropus*)	During	2	0	1	0	0	0	0	0	3
Mute swan (*Cygnus olor*)	During	59	1	14	4	4	5	1	2	90
Common coot (*Fulica atra*)	During	83	0	1	0	0	0	0	0	84
Eurasian wigeon (*Anas penelope*)	After	42	2	1	0	0	0	0	1	46
Egyptian goose (*Alopochen aegyptiaca*)	After	27	1	0	0	0	0	0	0	28
Mute swan (*Cygnus olor*)	After	19	1	2	2	0	0	0	5	29
Common coot (*Fulica atra*)	After	21	0	0	0	1	0	0	2	24

BLD: below limit of detection; HI: haemagglutination inhibition; HPAI: highly pathogenic avian influenza. Lowest serum dilution tested was 10. a A/Chicken/Netherlands/EMC-3/2014. b The 'outbreak' refers to the six months following the detection of the highly pathogenic avian influenza H5N8 virus in Europe and this extends from 14 November 2014 to 13 May 2015. The period after the 'outbreak' is from 1 September to 31 December 2015.

Table 6. Titres of confirmatory haemagglutination inhibition and virus neutralisation assays for sera positive for highly pathogenic avian influenza H5 clade 2.3.4.4-specific antibodies in the initial screening, the Netherlands, 14 November 2014–31 December 2015 (n = 53 serum samples)

Period	Species[a]	Haemagglutination inhibition assay											Virus neutralisation assay		
		Initial						Confirmatory							
		LPAI H5	HPAI clade					LPAI H5	HPAI clade				HPAI clade		
			1	2.1	2.2	2.3	2.3.4.4[b]		2.1	2.2	2.3	2.3.4.4	2.1	2.3	2.3.4.4
During the outbreak: 2014/15	Eurasian wigeon	< 10	< 10	< 10	< 10	< 10	50	ND	ND	ND	ND	ND	ND	ND	ND
	Eurasian wigeon	< 10	< 10	< 10	< 10	< 10	100	ND	ND	ND	ND	ND	ND	ND	ND
	Eurasian wigeon	20	< 10	< 10	< 10	< 10	15	ND	ND	ND	ND	ND	ND	ND	ND
	Eurasian wigeon	< 10	< 10	< 10	< 10	< 10	60	ND	ND	ND	ND	ND	ND	ND	80
	Eurasian wigeon	< 10	< 10	< 10	< 10	< 10	20	< 6	< 6	< 6	< 6	< 6	ND	ND	20
	Eurasian wigeon	< 10	< 10	< 10	< 10	< 10	40	< 6	< 6	< 6	< 6	< 6	ND	ND	20
	Eurasian wigeon	< 10	< 10	< 10	< 10	< 10	25	ND	ND	ND	ND	ND	ND	ND	40

Period	Species[a]	Haemagglutination inhibition assay											Virus neutralisation assay		
		Initial						Confirmatory							
		LPAI H5	HPAI clade					LPAI H5	HPAI clade				HPAI clade		
			1	2.1	2.2	2.3	2.3.4.4[b]		2.1	2.2	2.3	2.3.4.4	2.1	2.3	2.3.4.4
	Eurasian wigeon	< 10	< 10	< 10	< 10	< 10	15	ND	ND	ND	ND	ND	ND	ND	20
	Eurasian wigeon	< 10	< 10	< 10	< 10	< 10	15	ND	ND	ND	ND	ND	ND	ND	10
	Eurasian wigeon	< 10	< 10	< 10	< 10	< 10	20	ND	ND	ND	ND	ND	ND	ND	20
	Eurasian wigeon	< 10	< 10	< 10	< 10	< 10	40	< 6	< 6	< 6	< 6	< 6	ND	ND	40
	Eurasian wigeon	< 10	< 10	< 10	< 10	< 10	120	ND	ND	ND	ND	ND	ND	ND	160
	Common coot	< 10	40	< 10	< 10	30	40	ND	ND	ND	ND	ND	ND	ND	< 10
	Lesser white-fronted goose	20	< 10	< 10	< 10	< 10	70	ND	ND	ND	ND	ND	< 10	< 10	< 10
	Mute swan	< 10	120	320	< 30	640	40	ND	ND	ND	ND	ND	< 10	< 10	< 10
	Mute swan	< 10	160	160	< 30	640	200	ND	ND	ND	ND	ND	< 10	< 10	< 10
	Mute swan	< 30	< 180	320	< 180	960	60	ND	ND	ND	ND	ND	ND	ND	ND
	Mute swan	< 120	< 120	120	< 120	320	240	ND	ND	ND	ND	ND	< 10	< 10	80
	Mute swan	< 30	30	160	40	640	480	ND	ND	ND	ND	ND	< 10	< 10	60
	Mute swan	< 60	< 60	< 40	< 40	60	480	ND	ND	ND	ND	ND	< 10	< 10	240
	Mute swan	< 60	< 40	240	< 30	640	70	ND	ND	ND	ND	ND	< 10	< 10	< 10
	Mute swan	< 120	< 60	160	< 60	640	960	12	< 6	< 6	< 6	192	< 10	10	240
	Mute swan	< 10	< 40	320	< 40	1,280	70	ND	ND	ND	ND	ND	< 10	< 10	< 10
	Mute swan	< 10	60	480	30	2,560	60	ND	ND	ND	ND	ND	< 10	< 10	< 10
	Mute swan	< 30	< 120	240	< 120	480	70	ND	ND	ND	ND	ND	ND	ND	< 10
	Mute swan	< 60	< 120	320	< 120	640	50	ND	ND	ND	ND	ND	< 10	< 10	< 10
	Mute swan	< 60	< 120	320	< 120	640	80	ND	ND	ND	ND	ND	< 10	< 10	20
	Mute swan	< 10	< 60	320	< 60	960	70	ND	ND	ND	ND	ND	< 10	< 10	< 10
	Mute swan	< 120	160	640	30	2,560	240	< 6	< 6	< 6	< 6	< 6	< 10	< 10	< 10
	Mute swan	< 60	40	320	30	1,280	120	ND	ND	ND	ND	ND	< 10	< 10	< 10
	Mute swan	< 30	30	160	< 30	640	50	ND	ND	ND	ND	ND	< 10	< 10	< 10
	Mute swan	< 10	ND	ND	ND	ND	50	ND	ND	ND	ND	ND	ND	ND	20
	Mute swan	< 120	< 120	160	< 120	640	70	ND	ND	ND	ND	ND	20	< 10	< 10
	Mute swan	< 10	160	320	< 120	1,280	70	ND	ND	ND	ND	ND	ND	ND	ND
	Mute swan	< 60	< 120	160	< 120	640	50	ND	ND	ND	ND	ND	< 10	< 10	< 10
	Mute swan	< 30	40	160	< 60	640	50	ND	ND	ND	ND	ND	< 10	< 10	< 10
	Mute swan	< 30	< 30	160	< 30	320	35	ND	ND	ND	ND	ND	< 10	< 10	< 10
	Mute swan	< 10	< 180	320	< 180	640	100	ND	ND	ND	ND	ND	< 10	< 10	< 10
	Mute swan	40	< 60	160	< 60	640	80	ND	ND	ND	ND	ND	ND	ND	ND
	Mute swan	< 60	< 60	< 60	< 60	160	240	ND	ND	ND	ND	ND	< 10	< 10	10
	Mute swan	< 60	< 240	< 240	< 240	< 240	480	12	< 6	< 6	< 6	96	< 10	< 10	60
	Mute swan	< 60	< 30	< 30	< 30	60	480	< 6	< 6	< 6	< 6	96	< 10	< 10	240
	Mute swan	< 120	< 120	< 120	< 120	320	480	ND	ND	ND	ND	ND	< 10	< 10	60
After the outbreak:	Eurasian wigeon	< 10	< 10	< 10	< 10	< 10	20	ND	ND	ND	ND	ND	ND	ND	160

Period	Species[a]	Haemagglutination inhibition assay														Virus neutralisation assay
		Initial						Confirmatory								
		LPAI H5	HPAI clade					LPAI H5	HPAI clade				HPAI clade			
			1	2.1	2.2	2.3	2.3.4.4[b]		2.1	2.2	2.3	2.3.4.4	2.1	2.3	2.3.4.4	
2015	Eurasian wigeon	< 10	< 10	< 10	< 10	< 10	10	ND	ND	ND	ND	ND	ND	ND	20	
	Eurasian wigeon	< 10	< 10	< 10	< 10	< 10	40	ND	ND	ND	ND	ND	ND	ND	80	
	Common coot	< 10	80	60	60	320	160	ND	ND	ND	ND	ND	ND	ND	20	
	Egyptian goose	< 10	< 10	< 10	< 10	80	25	ND	ND	ND	ND	ND	ND	ND	< 10	
	Mute swan	160	80	60	< 30	160	120	ND	ND	ND	ND	ND	< 10	< 10	40	
	Mute swan	40	80	80	80	320	45	ND	ND	ND	ND	ND	< 10	< 10	< 10	
	Mute swan	< 10	< 10	< 10	< 10	30	15	ND	ND	ND	ND	ND	< 10	< 10	< 10	
	Mute swan	< 10	80	80	80	320	60	ND	ND	ND	ND	ND	< 10	< 10	< 10	
	Mute swan	< 10	160	160	240	320	80	ND	ND	ND	ND	ND	80	20	< 10	

HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza; ND: not determined. Lowest serum dilution tested was 10 for the initial haemagglutination inhibition (HI) and virus neutralisation assay and 6 for the confirmatory HI assay. [a] Species included common coot (*Fulica atra*), Egyptian goose (*Alopochen aegyptiaca*), Eurasian wigeon (*Anas penelope*), lesser white-fronted goose (*Anser erythropus*), mute swan (*Cygnus olor*). [b] Mean titre of in duplo tested samples.

Of the sera collected after the outbreak, 5/29 mute swans, 3/46 Eurasian wigeons, 1/28 Egyptian geese (Alopochen aegyptiaca) and 1/24 common coots tested positive for HPAI H5 clade 2.3.4.4-specific antibodies (Table 5). The sera of the Eurasian wigeons reacted with HPAI H5N8 virus exclusively. However, the common coot as well as 1/5 mute swans showed HI titres to all five H5 clades. The other 3/5 mute swans showed HI titres to multiple but not all H5 clades, while 1/5 mute swans and 1/1 Egyptian goose only showed an additional titre to clade 2.3 (Table 6). Seven of the HPAI H5 clade 2.3.4.4-seropositive bird sera obtained during the outbreak, from four mute swans and three Eurasian wigeons, were retested in an HI assay at the APHA. Here, 3/4 mute swan samples with high initial HI antibody titres against HPAI H5 clade 2.3.4.4 (H5N8) virus were confirmed. However, 1/4 mute swan sera could not be confirmed, and HPAI H5 clade 2.3.4.4-specific antibodies were also not detected in 3/3 sera of the Eurasian wigeons that had low antibody titres in the initial tests (Table 6).

Virus neutralisation assays

For 37/43 HPAI H5 clade 2.3.4.4-positive sera collected during and 10/10 sera collected after the outbreak, sufficient serum volumes were available for retesting in a VN

assay. In this assay, HPAI H5 clade 2.3.4.4-specific antibodies were detected in sera of 9/9 Eurasian wigeons and of 10/26 mute swans obtained during the outbreak. Sera of the mute swans did not react with viruses of other H5 clades. HPAI H5 clade 2.3.4.4-specific antibodies were not detected in the sera of the common coot and the lesser white-fronted goose by VN assay. HPAI H5 clade 2.3.4.4-specific antibodies were confirmed by VN assay in sera from 3/3 Eurasian wigeons, 1/5 mute swans, 1/1 common coot and 0/1 Egyptian goose collected after the outbreak (Table 6).

Discussion

In this report surveillance data for HPAI H5N8 in birds in the Netherlands are presented. In addition to bird samples previously investigated for the virus from 14 November 2014 to 20 February 2015, a new set of 7,337 samples obtained between 21 February 2015 and 31 January 2016 is analysed. One faecal sample obtained from a Eurasian wigeon (Anas penelope) on 25 February 2015 tested positive for the HPAI H5N8 virus, adding to the previous finding of the virus in two Eurasian wigeons in the country in late 2014 [14]. Virological surveillance moreover suggests that only very limited numbers of wild bird species were identified as potential hosts in Europe. Importantly, to the best of our knowledge, there are no reports of additional findings of HPAI H5N8 viruses in wild birds and poultry in Europe, since the last detection of the virus in February 2015 in the Netherlands.

Given the difficulty of detecting newly emerging HPAI virus strains in wild birds however, the application of a more sensitive and cost-effective method to detect potential host species is warranted. For this purpose, we performed serological assays specifically aimed to detect antibodies specific to HPAI H5 clade 2.3.4.4 viruses in a substantial number of sera obtained before, during, and after HPAI H5N8 emergence in the Netherlands. Three potential HPAI H5N8 host species were identified by HI assays and confirmed by VN assays; Eurasian wigeons, mute swans and common coots. Considering the results of virological studies performed worldwide since the onset of the HPAI H5N8 virus emergence in early 2014, the detection of HPAI H5 clade 2.3.4.4-specific antibodies in these species is not surprising. HPAI H5N8 virus was isolated from Eurasian wigeons in Russia [8] and the Netherlands [14], from mute swans in Sweden [6], and from a common coot in South Korea [21].

The serological results reported here were not entirely consistent between HI and VN assays and between HI assays performed in two different laboratories. Although low HI titres (e.g. in Eurasian wigeons) were reproducible within a laboratory with the same HI assay and a VN assay, they were not detected by HI assay in a second laboratory, potentially due to differences in the methods used and hence differences in sensitivity and specificity. High antibody titres in mute swan sera were reproduced

by HI assay in a second laboratory and by VN assay, but low antibody titres in mute swans were not always reproduced. While it is thus clear that individual HI titres in avian sera obtained from a single test cannot be used reliably for diagnosis, use of serum panels from cohorts of birds, use of multiple tests to cross-validate results, a panel of relevant viruses and use of collections of control antisera may still enable the use of serological tests in support of HPAI H5 surveillance studies.

Previously, HI assays were shown to be discriminative enough to detect antibodies in serum samples collected from free-living wild birds in Europe and Asia to be directed to either HPAI or LPAI H5 viruses. However, widely varying results were obtained as far as HPAI H5 clade-specific antibodies were concerned [16]. In this study, most birds that tested positive for HPAI H5 clade 2.3.4.4-specific serum antibodies showed relative low HI titres. This is in accordance with findings based on experimental HPAI H5N8 virus infections of ferrets [10-12], possibly indicating low immunogenicity upon infection. In addition, there is limited knowledge about the longevity of avian antibodies after naturally occurring infection with avian influenza viruses. Antibodies specific to LPAI viruses were detected up to several months after experimental or natural infection [22-24], whereas little is known about the duration of detection of antibodies specific to HPAI viruses with a reported maximum of detection of 28 days after experimental infection in domestic ducks [25]. To date, there is no knowledge on the effect of a prior exposure to an unrelated subtype or on the phenomena of antigenic sin in avian species. Hypothetically, low immunogenicity in combination with decreasing titres in time could be an explanation for the low incidence and relative low titres of antibodies detected in wild bird sera in this study.

In conclusion, our results provide evidence that clinically unaffected long distance migratory and local wild birds sampled in the Netherlands during the H5N8 outbreak late 2014 and early 2015, and again late 2015, have been exposed to HPAI H5N8 or closely related HPAI H5 clade 2.3.4.4 viruses and seroconverted upon exposure. Since HPAI H5N8 virus has not been detected in Europe since early 2015 and because HPAI H5 clade 2.3.4.4-specific antibody incidence decreased in time, we conclude that the virus has not circulated extensively at the breeding grounds in summer and upon the return of the birds to their wintering areas in the 2015/16 winter. As a consequence, the newly emerging HPAI H5N8 clade 2.3.4.4 virus subtype appears to have already disappeared from European wild birds indicating that sustained transmission and independent maintenance may be less likely. This is an important consideration in the ongoing evolution and ecology of these viruses in wild birds and the potential risks they pose for introduction to poultry and the pathways through which they might spread. Finally we recommend that serological tools be further optimised, harmonised, and validated for avian influenza surveillance studies in wild birds.

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Chapter 2.2

Local amplification of highly pathogenic avian influenza H5N8 viruses in wild birds in the Netherlands, 2016 to 2017

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Abstract

Highly pathogenic avian influenza (HPAI) viruses of subtype H5N8 were re-introduced into the Netherlands by late 2016, after detections in southeast Asia and Russia. This second H5N8 wave resulted in a large number of outbreaks in poultry farms and the deaths of large numbers of wild birds in multiple European countries. **Methods:** Here we report on the detection of HPAI H5N8 virus in 57 wild birds of 12 species sampled during active (32/5,167) and passive (25/36) surveillance activities, i.e. in healthy and dead animals respectively, in the Netherlands between 8 November 2016 and 31 March 2017. Moreover, we further investigate the experimental approach of wild bird serology as a contributing tool in HPAI outbreak investigations. **Results:** In contrast to the first H5N8 wave, local virus amplification with associated wild bird mortality has occurred in the Netherlands in 2016/17, with evidence for occasional gene exchange with low pathogenic avian influenza (LPAI) viruses. **Discussion:** These apparent differences between outbreaks and the continuing detections of HPAI viruses in Europe are a cause of concern. With the current circulation of zoonotic HPAI and LPAI virus strains in Asia, increased understanding of the drivers responsible for the global spread of Asian poultry viruses via wild birds is needed.

Introduction

Highly pathogenic avian influenza (HPAI) viruses of the H5 subtype, originating from the A/Goose/Guangdong/1/1996 (GsGd) lineage, have been circulating continuously in poultry in south-east Asia since 1997 and have also been detected frequently in wild birds [1]. In 2014, a new HPAI H5N8 virus of this GsGd lineage of clade 2.3.4.4 emerged globally. This first intercontinental wave of HPAI H5N8 started with virus detections in south-east Asia from early 2014 onwards in both poultry and wild birds [2-4]. By the end of 2014, this HPAI H5N8 virus simultaneously spread to Europe and North America through long distance migratory birds [5]. In North America, the virus reassorted with local low pathogenic avian influenza (LPAI) viruses causing a massive number of outbreaks and associated economical loss [6]. In Europe, this first wave caused a relative limited number of outbreaks in poultry holdings, and was detected in some wild birds between November 2014 and February 2015 [7,8]. During the spring and summer of 2015, occasional detections of HPAI H5N8 were reported in south-east Asia [9]. To assess the risk of virus re-introduction by wintering birds arriving in Europe by the autumn of 2015, intensified active surveillance (i.e. surveillance in living birds) was performed in the Netherlands from September to December 2015. This surveillance provided virological and serological evidence that the HPAI H5N8 virus had disappeared from the European (wintering) wild bird population with no virus detections in any of the tested birds and a decreased seroprevalence of HPAI H5 clade 2.3.4.4-specific antibodies, suggesting no massive viral replication in the 2015 breeding season [10].

However, in June 2016, the detection of HPAI H5N8 in wild birds of multiple species on their breeding grounds was reported around Uvs-Nuur Lake in Russia [11]. In contrast to the 2014 emerging strains, which belong to group A (A/broiler duck/Korea/Buan2/2014-like), this virus belonged to group B (A/breeder duck/Korea/Gochang1/2014-like) viruses [4,11]. These group B viruses had been detected previously in China and South Korea in 2014, but had not been reported since [3,12]. From mid-October 2016 onwards, group B lineage HPAI H5N8 viruses were detected in both India [13] and in European countries. Unlike the 2014/15 group A viruses, group B viruses caused local die-offs of wild birds in many countries, often resulting in wild bird deaths preceding those in poultry [14-16]. The introduction of these group B HPAI H5N8 viruses in the Netherlands was marked by a die-off of tufted ducks (Aythya fuligula) and great crested grebes (Podiceps cristatus) in the Gouwzee (52°27'09"N, 5°04'07"E), a large fresh water lake, on 8 November 2016 [14,17]. Most of the internal genes of this 'second wave' HPAI H5N8 virus were derived from Eurasian LPAI viruses via reassortment, after their original detection in China and South Korea in 2014 and Russia in May 2016 [11,15]. Occasional reassortment of the neuraminidase (NA) gene also led to a few detections of clade 2.3.4.4 HPAI H5N5 and H5N6 viruses [18].

Wild migratory birds were shown to be the most probable vectors for the first global spreadof HPAI H5N8 in 2014 that coincided with the timing and flyways of the autumn migration, based on a recent worldwide phylogenetic study of HPAI H5N8 viruses [5,19]. These avian influenza viruses constitute a constant animal and human health threat, where the risk in part is determined by the (evolving) genomic constitution of the circulating viruses. It is therefore of crucial importance to actively monitor influenza viruses and their evolution in wild bird populations, to monitor trends and diversity of circulating viruses, and to assess the risk of spread for strains that are unusual in their genetic make-up and/or spread for animal and human health. In this study we have performed intense active surveillance in wild birds in the Netherlands in response to the HPAI H5N8 introduction in Europe in late 2016. We performed both virological and serological studies to attempt to identify wild bird species that might contribute to the spread and maintenance of this virus.

Methods

Ethical statement

The capture of free-living birds was approved by the Dutch Ministry of Economic Affairs based on the Flora and Fauna Act (permit number FF/75A/2009/067, FF/75A/2014/054 and licence number 951 to Vogeltrekstation NIOO-KNAW). Handling and sampling of free-living birds was approved by the Animal Experiment Committee of the Erasmus Medical Center (permit number 122–11–31). Free-living birds were released into the wild after sampling and all efforts were made to minimise animal suffering throughout the procedures.

Study population

A continuous active surveillance programme of resident and migrating wild birds for avian influenza viruses is in place in the Netherlands. The ongoing surveillance efforts were intensified in response to the first detection of HPAI H5N8 virus in the Netherlands in 2016 between 13 November and 31 December 2016, the period during which mortality among wild birds and outbreaks in poultry holdings were occurring in the country, and from 8 February until 19 February 2017, when die-offs of wild birds and outbreaks in poultry had ceased. On 1 March and 23 October 2016, as well as approximately during the first period of intensified surveillance (13 November to 21 December 2016), and on 8 February 2017, blood samples were obtained in addition to samples for virus detection.

Sample collection

Live wild birds were captured using duck decoys, cannon nets, leg nooses, swan hooks, or manually. Birds were sampled routinely for virus detection using oropharyngeal and cloacal swabs as described elsewhere [10]. In addition, fresh faecal samples were collected from Eurasian wigeons (*Anas penelope*) for virus detection. Fresh faecal samples were collected by trained ornithologists able to distinguish species-specific droppings from locations where large homogeneous groups of Eurasian wigeons were foraging in the field. Blood samples were collected for serum antibody detection as described previously [10]. In addition to active surveillance, oropharyngeal and/or cloacal swabs of a limited number of freshly dead wild birds were opportunistically collected for virus detection (i.e. passive surveillance).

Virus detection, isolation and characterisation

Samples for virus detection were analysed for the presence of HPAI H5(N8) virus using matrix- and H5-specific real-time reverse-transcription PCR (RRT-PCR) assays, followed by haemagglutinin (HA) and NA gene sequencing as previously described [7]. Samples testing positive in matrix and H5 specific RRT-PCR were inoculated in Madin–Darby canine kidney (MDCK) cells. Samples were characterised as HPAI H5 virus by detection of a multi-basic cleavage site upon Sanger sequencing of the HA gene.

Virus sequencing and phylogeny

Full length HA and NA sequences of all virus isolates and full genome sequences for a subset of these were obtained by Sanger sequencing. All sequences were deposited in a public database (http://www.gisaid.com). Primer sequences are available upon request. For HA and NA phylogeny, sequences obtained in this study were supplemented with publicly available sequences of HPAI H5 viruses of clade 2.3.4.4 detected globally between 2014 and 2017. These additional sequences were obtained from the Global Initiative on Sharing Avian Influenza Data database (http://www.gisaid.com) on 20 May 2017 (Table 1). Maximum likelihood (ML) phylogenetic trees were constructed based on the HA (1,637 nt: position 49–1,685) and NA (1,227 nt: position 64–1,291) genes. ML trees were generated using PhyML version 3.1 using the general time-reversible (GTR) model, performing subtree pruning and regrafting (SPR) searches [20]. The reliability of the phylogenetic grouping was assessed with 250 bootstrap replicates. Trees were visualised using Figtree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree). ML trees of HA and NA were used in Dendroscope version 3.5.9 (http://dendroscope.org/) [21] to display

a tanglegram between the HA and NA midpoint rooted phylogenies. The twines were colourcoded according to wave and location.

Table 1. Acknowledgements of authors, and originating and submitting laboratories providing the sequences used for phylogenetic analysis

Isolate ID	Isolates name	Collection date	Originating laboratory	Submitting Laboratory	Authors
EPI_ISL_257700	A/Mute_Swan/Czech_Republic/1813–17/2017	5 Feb 2017	Not listed	State Veterinary Institute Prague	Nagy, A.
EPI_ISL_250885	A/Mute_Swan/Czech_Republic/54–17_2/2017	2 Jan 2017	Not listed	State Veterinary Institute Prague	Nagy, A.
EPI_ISL_248663	A/Mallard/Czech_Republic/722–17_2/2017	15 Jan 2017	Not listed	State Veterinary Institute Prague	Nagy, A.
EPI_ISL_247713	A/Tufted_Duck/Denmark/17740–1/2016	8 Nov 2016	Technical University of Denmark	Animal and Plant Health Agency (APHA)	Hjulsager CK, Krog JS, Larsen LE, Kvisgaard LK, Essen S
EPI_ISL_238197	A/Mute_Swan/Croatia/78/2016	12 Nov 2016	Not listed	Croatian Veretinary Institute	Not listed
EPI_ISL_238196	A/Mute_Swan/Croatia/70/2016	30 Oct 2016	Not listed	Croatian Veretinary Institute	Not listed
EPI_ISL_237945	A/Tufted_Duck/Germany/AR8459-L01988/2016	8 Nov 2016	Not listed	Friedrich-Loeffler-Institut	Not listed
EPI_ISL_237944	A/Tufted_Duck/Germany/AR8444-L01987/2016	7 Nov 2016	Not listed	Friedrich-Loeffler-Institut	Not listed
EPI_ISL_237732	A/Tufted_Duck/Germany-SH/R8446/2016	7 Nov 2016	Not listed	Friedrich-Loeffler-Institut	Not listed
EPI_ISL_238039	A/Chicken/Germany-SH/R8758/2016	11 Nov 2016	Not listed	Friedrich-Loeffler-Institut	Not listed
EPI_ISL_237921	A/Wild_Duck/Poland/82A/2016	2 Nov 2016	Not listed	National Veterinary Research Institut Poland	Świętoń E, Śmietanka K
EPI_ISL_175535	A/MuteSwan/Sweden/SVA150313KU0141/SZ543/2015	5 Mar 2015	National Veterinary Institute, Sweden	National Veterinary Institute, Sweden	Zohari S, Ullman K, Olofsson A
EPI_ISL_238896	A/Chicken/Sweden/SVA161122KU0453/SZ0209321/2016	21 Nov 2016	National Veterinary Institute, Sweden	National Veterinary Institute, Sweden	Not listed
EPI_ISL_231685	A/Black-headed_Gull/Tyva/41/2016	25 May 2016	State Research Center of Virology and Biotechnology (VECTOR)	WHO National Influenza Centre Russian Federation	Fadeev A, Komissarov A, Egorova A, Sintsova K, Musaeva T, Susloparov I, Marchenko V, Ryzhikov A
EPI_ISL_231684	A/Wild_Duck/Tyva/35/2016	25 May 2016	State Research Center of Virology and Biotechnology (VECTOR)	WHO National Influenza Centre Russian Federation	Fadeev A, Komissarov A, Egorova A, Sintsova K, Musaeva T, Susloparov I, Marchenko V, Ryzhikov A
EPI_ISL_224580	A/Great_Crested_Grebe/Tyva/341/2016	25 May 2016	Research Institute of Experimental and Clinical Medicine	Research Institute of Experimental and Clinical Medicine	Sharshov K, Kurskaya O, Sobolev I, Alekseev A, Alikina T, Kabilov M, Shestopalov A
EPI_ISL_234057	A/grey_heron/Uvs-Nuur_Lake/20/2016	25 May 2016	Research Institute of Experimental and Clinical Medicine	Research Institute of Experimental and Clinical Medicine	Sharshov K, Kurskaya O, Sobolev I, Alekseev A, Shestopalov A
EPI_ISL_234058	A/common_tern/Uvs-Nuur_Lake/26/2016	25 May 2016	Research Institute of Experimental and Clinical Medicine	Research Institute of Experimental and Clinical Medicine	Sharshov K, Kurskaya O, Sobolev I, Alekseev A, Alikina T, Kabilov M, Shestopalov A
EPI_ISL_26	A/Eurasian_Herring_Gull/Netherlands/2	20 Dec	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD,

Isolate ID	Isolates name	Collection date	Originating laboratory	Submitting Laboratory	Authors
9601	/2016	2016			Fouchier,RAM et al.
EPI_ISL_268916	A/Caspian_Gull/Netherlands/1/2016	20 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_255910	A/Mew Gull/Netherlands/1/2016	23 Nov 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269602	A/Lesser_Black-backed_Gull/Netherlands/1/2016	20 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269697	A/Great_Black-backed_Gull/Netherlands/2/2016	23 Nov 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269599	A/Great_Black-backed_Gull/Netherlands/4/2016	14 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269598	A/Great_Black-backed_Gull/Netherlands/3/2016	23 Nov 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269597	A/Great_Black-backed_Gull/Netherlands/1/2016	23 Nov 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_255892	A/Great Black-backed Gull/Netherlands/2/2016	23 Nov 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269600	A/Great_Crested_Grebe/Netherlands/2/2016	21 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269696	A/Eurasian_Wigeon/Netherlands/23/2016	05 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269694	A/Eurasian_Wigeon/Netherlands/1/2016	04 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ,Müskens,G.J.D.M, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269596	A/Eurasian_Wigeon/Netherlands/13/2016	14 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269595	A/Eurasian_Wigeon/Netherlands/12/2016	14 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269594	A/Eurasian_Wigeon/Netherlands/22/2016	14 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269593	A/Eurasian_Wigeon/Netherlands/11/2016	13 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269592	A/Eurasian_Wigeon/Netherlands/8/2016	09 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269591	A/Eurasian_Wigeon/Netherlands/6/2016	09 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_268937	A/Eurasian_Wigeon/Netherlands/10/2016	08 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_255914	A/Eurasian Wigeon/Netherlands/9/2016	04 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_255912	A/Eurasian Wigeon/Netherlands/4/2016	09 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269703	A/Eurasian_Wigeon/Netherlands/25/2016	05 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269695	A/Eurasian_Wigeon/Netherlands/21/2016	05 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.

Isolate ID	Isolates name	Collection date	Originating laboratory	Submitting Laboratory	Authors
EPI_ISL_269692	A/Mallard/Netherlands/3/2017	11 Jan 2017	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269604	A/Mallard/Netherlands/1/2017	07 Jan 2017	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269603	A/Mallard/Netherlands/51/2016	20 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_255913	A/Mallard/Netherlands/2/2017	07 Jan 2017	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_268927	A/Common_Buzzard/Netherlands/1/2016	07 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_255891	A/Tufted Duck/Netherlands/1/2016	25 Nov 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_268866	A/Back-headed_Gull/Netherlands/9/2016	20 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_268800	A/Black-headed_Gull/Netherlands/17/2016	20 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_268799	A/Back-headed_Gull/Netherlands/8/2016	20 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_268929	A/Common_Eider/Netherlands/2/2016	20 Dec 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.
EPI_ISL_269693	A/Common_Pochard/Netherlands/1/2016	25 Nov 2016	Erasmus Medical Center	Erasmus Medical Center	Poen,MJ, Van Der Jeugd,HP, Vuong,O, Scheuer,RD, Fouchier,RAM et al.

Antibody detection

Serum samples were initially screened for the presence of clade 2.3.4.4 H5(N8)-specific (A/Chicken/Netherlands/EMC-3/2014 and A/Great Black-backed Gull/Netherlands/3/2016) and LPAI H5(N2)-specific (A/Mallard/Netherlands/3/1999) antibodies in a haemagglutination inhibition (HI) assay according to standard procedures [10,22]. Due to the generally high non-specific haemagglutination induced by wild bird sera in previous HI assays [10], all sera were pre-treated with 10% turkey red blood cells for 1 hour at 4 °C before analysis. Negative controls, based on incubation of serum without virus, were used to measure non-specific haemagglutination of each serum sample. Serum samples from experimentally inoculated ferrets [23] were used as positive controls. Serum samples that tested positive for either LPAI H5N2 or HPAI H5 clade 2.3.4.4-specific antibodies were further tested in an HI assay against HPAI viruses of the H5 clades 1 (A/Viet Nam/1194/2004), 2.1 (A/Indonesia/5/2005), 2.2 (A/Turkey/Turkey/1/2005), and 2.3.4 (A/Anhui/1/2005), and retested against the 2016 clade 2.3.4.4 virus (A/Great Black-backed gull/Netherlands/3/2016). The viruses used, except the 2016 HPAI H5N8 virus and the LPAI H5N2 virus, were recombinant viruses based on an A/PR/8/34 virus

backbone, containing the HA (without the multi-basic cleavage site) and NA of the representative H5 strains to enable this study within biosafety level 2 laboratories. Assays with the wild type 2016 HPAI H5N8 virus were performed simultaneously in biosafety level 3 conditions. Subsequently, samples were tested in a virus neutralisation (VN) assay as described previously [10], using titrated virus stocks of the same LPAI H5N2 and HPAI H5 clade 1, 2.1, 2.2, 2.3.4, and 2016 2.3.4.4 representatives. Sera were categorised as being either LPAI or HPAI biased or ambiguous, where a bias is defined as a cutoff of > 1 log2 differences in titre in HI assays [24].

Results

Study population

Here we report the data of 5,167 wild birds that were tested for the presence of avian influenza viruses between 8 November 2016 and 31 March 2017 in response to the re-introduction of HPAI H5N8 viruses in the Dutch wild bird population on 8 November 2016 [17]. In addition, we report on all data obtained in our routine active surveillance activities before the first evidence of re-introduction of HPAI H5N8 virus into the Netherlands, 1 February until 7 November 2016 (n = 5,523) (Table 2, Figure 1). All birds were caught alive and did not show clinical signs of disease. Also, samples were obtained from 36 birds belonging to 17 species that were sampled post mortem (Table 2). For antibody detection, serum samples from 459 birds of various species were analysed (Table 3). The majority of these samples were obtained between 13 November and 21 December 2016 (n = 367, 18 species) and on 8 February 2017 (n = 23 mallards *(Anas platyrhynchos)*). In addition, we included blood samples from Eurasian wigeons obtained on 1 March (n = 28) and 23 October 2016 (n = 41) that were not analysed previously (Table 3).

Virus detection, isolation and characterisation

There was no evidence for the presence of HPAI H5(N8) virus in any of the birds (n = 5,523) sampled during routine active surveillance between 1 February and 7 November 2016. In the subsequent period (between 8 November 2016 and 31 March 2017), samples from 145 birds (2.8%) tested positive for the presence of H5HA by RRT-PCR. The presence of HPAI H5 clade 2.3.4.4 virus was confirmed in samples of 57 birds (Table 2). Of these, 23 birds (17 mallards, 5 Eurasian wigeons and one common buzzard (*Buteo buteo*)) were caught and sampled alive without clinical signs, and from nine birds (Eurasian wigeons) fresh droppings were tested positive. (Table 4). In total, viruses were isolated in MDCK cells from 48 samples from 33 birds. All cultured viruses belonged to the HPAI H5N8 subtype. The last detection of HPAI

clade 2.3.4.4 virus in living birds was in mallards on 28 January 2017. Since then, no additional HPAI clade 2.3.4.4 H5 viruses have been detected in this study.

Table 2. Wild bird species sampled for virus detection in the Netherlands before and during the second wave of highly pathogenic avian influenza H5N8 virus in Europe and results of virological assays, February 2016–March 2017 (n = 10,726)

Order	Family	Species	1 February 2016 – 7 November 2016				8 November 2016–31 March 2017							
			Alive without clinical signs				Alive without clinical signs				Found dead			
			Number of birds Sampled	Number of birds AIV positive	Number of birds H5 positive	Pathotype	Number of birds sampled	Number of birds AIV positive	Number of birds H5 positive	Pathotype	Number of birds sampled	Number of birds AIV positive	Number of birds H5 positive	Pathotype
Anseri-formes	Ducks	Common eider *(Somateria mollissima)*	0	0	0	NA	0	0	0	NA	1	1	1	1x HPAI
		Common pochard *(Aythya ferina)*	0	0	0	NA	0	0	0	NA	1	1	1	n.i.
		Common shelduck *(Tadorna tadorna)*	2	0	0	NA	0	0	0	NA	0	0	0	NA
		Domestic duck *(Anas platyrhynchos domesticus)*	0	0	0	NA	1	0	0	NA	0	0	0	NA
		Egyptian goose *(Alopochen aegyptiaca)*	30	0	0	NA	17	0	0	NA	0	0	0	NA
		Eurasian teal *(Anas crecca)*	46	21	0	NA	42	5	1	1x LPAI	0	0	0	NA
		Eurasian wigeon *(Anas penelope)*	639	339	10	6x n.i., 4x LPAI	2,634	118	37	14x HPAI 23x n.i.	7	7	7	7x HPAI
		Gadwall *(Anas strepera)*	131	65	0	NA	11	1	1	1x n.i.	1	1	0	NA
		Garganey *(Anas querquedula)*	2	2	0	NA	0			NA	0	0	0	NA
		Greater scaup *(Aythya marila)*	1	0	0	NA	2	1	0	NA	0	0	0	NA
		Mallard *(Anas platyrhynchos)*	3,169	555	20	4x n.i., 16x LPAI	1,824	338	78	17x HPAI 5x LPAI 56x n.i.	3	3	2	2x HPAI
		Northern pintail *(Anas acuta)*	12	9	0	NA	6	0	0	NA	0	0	0	NA
		Northern shoveler *(Anas clypeata)*	14	4	0	NA	3	0	0	NA	0	0	0	NA

Order	Family	Species	1 February 2016 – 7 November 2016				8 November 2016–31 March 2017							
			Alive without clinical signs				Alive without clinical signs				Found dead			
			Number of birds Sampled	Number of birds AIV positive	Number of birds H5 positive	Pathotype	Number of birds sampled	Number of birds AIV positive	Number of birds H5 positive	Pathotype	Number of birds sampled	Number of birds AIV positive	Number of birds H5 positive	Pathotype
		Tufted duck (*Aythya fuligula*)	2	1	0	NA	2	0	0	NA	1	1	1	1x HPAI
	Geese	Barnacle goose (*Branta leucopsis*)	589	0	0	NA	0			NA	0	0	0	NA
		Bean goose (*Anser fabalis*)	0	0	0	NA	1	0	0	NA	0	0	0	NA
		Canada goose (*Branta canadensis*)	23	0	0	NA	3	0	0	NA	0	0	0	NA
		Great white-fronted goose (*Anser albifrons*)	27	0	0	NA	40	0	0	NA	0	0	0	NA
		Greylag goose (*Anser anser*)	310	3	1	1x LPAI	0			NA	0	0	0	NA
	Swans	Bewick's swan (*Cygnus columbianus bewickii*)	0	0	0	NA	92	3	0	NA	0	0	0	NA
		Mute swan (*Cygnus olor*)	0	0	0	NA	36	0	0	NA	0	0	0	NA
		Whooper swan (*Cygnus cygnus*)	0	0	0	NA	3	0	0	NA	0	0	0	NA
Charadriiformes	Gulls	Black-headed gull (*Chroicocephalus ridibundus*)	432	59	0	NA	287	2	0	NA	4	4	3	3x HPAI
		Caspian gull (*Larus cachinnans*)	0	0	0	NA	1	0	0	NA	1	1	0	NA
		Eurasian herring gull (*Larus argentatus*)	24	0	0	NA	20	1	0	NA	2	2	2	1x HPAI, 1x n.i.
		Great black-backed gull (*Larus marinus*)	0	0	0	NA	0	0	0	NA	8	8	5	5x HPAI
		Lesser black-backed gull (*Larus fuscus*)	66	0	0	NA	0	0	0	NA	1	1	1	1x HPAI
		Mew gull (*Larus canus*)	2	0	0	NA	13	0	0	NA	1	1	1	1x HPAI
		Yellow-legged gull (*Larus michahellis*)	0	0	0	NA	0	0	0	NA	1	0	0	NA
Gruiformes	Coots	Common coot (*Fulica atra*)	2	0	0	NA	100	0	0	NA	0	0	0	NA

Order	Family	Species	1 February 2016 – 7 November 2016				8 November 2016–31 March 2017							
			Alive without clinical signs				Alive without clinical signs				Found dead			
			Number of birds Sampled	Number of birds AIV positive	Number of birds H5 positive	Pathotype	Number of birds sampled	Number of birds AIV positive	Number of birds H5 positive	Pathotype	Number of birds sampled	Number of birds AIV positive	Number of birds H5 positive	Pathotype
		Common moorhen *(Gallinula chloropus)*	0	0	0	NA	4	0	0	NA	0	0	0	NA
		Water rail *(Rallus aquaticus)*	0	0	0	NA	20	0	0	NA	0	0	0	NA
Pelicani formes	Ardeidae	Grey heron *(Ardea cinerea)*	0	0	0	NA	3	0	0	NA	0	0	0	NA
Podici-pediformes	Grebes	Great crested grebe *(Podiceps cristatus)*	0	0	0	NA	0	0	0	NA	1	1	1	1x HPAI
Suliformes	Cormorants	Great cormorant *(Phalacrocorax carbo)*	0	0	0	NA	0	0	0	NA	1	0	0	NA
Passeriformes	Corvidae	Eurasian magpie *(Pica pica)*	0	0	0	NA	1	0	0	NA	0	0	0	NA
Falconiformes	Falcons	Peregrine falcon *(Falco peregrinus)*	0	0	0	NA	0	0	0	NA	1	1	1	1x HPAI
Accipitriformes	Accipitridae	Common buzzard *(Buteo buteo)*	0	0	0	NA	1	1	1	1x HPAI	1	1	1	1x HPAI
Total	NA	NA	5,523	1,058	31	NA	5,167	470	118	32x HPAI	36	34	27	25x HPAI

AIV: avian influenza virus; HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza; NA: not applicable; n.i.: not identifiable because of low virus load.

Virus sequencing and phylogenetic analysis

Full length HA and NA sequences of all 48 isolates were obtained by Sanger sequencing. Analysis of these 48 samples showed no differences between sequences obtained from cloacal and oropharyngeal swabs from the same bird, so only one sequence per bird was included in further analyses. In accordance with previous reports [11,15,25], our phylogenetic analysis (Figure 2) shows a clear distinction for both HA and NA between the 2014/15 group A HPAI H5N8 viruses and the 2016/17 group B viruses. Also, the Russian viruses from May 2016 were distinguishable from the ones that entered eastern European countries (Croatia and Czech Republic) and subsequently more western European counties like Germany and the Netherlands for both HA and NA. The subclade consisting only of Dutch duck and gull viruses might indicate more local virus evolution within the Netherlands. In

support, the viruses detected in live mallards in early 2017 appear as offspring from Eurasian wigeon viruses that were detected 3 weeks earlier and were highly similar to other Dutch viruses that caused mortality in other bird species (Figure 2). However, the number of sequences from other outbreaks in Europe at present is too limited to draw solid conclusions.

Table 3. Wild bird species sampled in the Netherlands for antibody detection in response to the second wave of highly pathogenic avian influenza (HPAI) H5N8 virus in Europe, 2016/2017 (n = 459) and those showing HPAI H5 clade 2.3.4.4-specific antibodies (n = 20) based on repeated haemagglutination inhibition assays (HI), March 2016–February 2017

Order	Family	Species	Number of individuals					
			1 March 2016		23 October 2016		13 November 2016 – 8 February 2017	
			Tested	H5 clade 2.3.4.4-specific antibodies	Tested	H5 clade 2.3.4.4-specific antibodies	Tested	H5 clade 2.3.4.4-specific antibodies
Anseriformes	Ducks	Egyptian goose (Alopochen aegyptiaca)	0	0	0	0	10	0
		Eurasian teal (Anas crecca)	0	0	0	0	22	0
		Eurasian wigeon (Anas penelope)	28	2	41	1	63	2
		Gadwall (Anas strepera)	0	0	0	0	5	0
		Mallard (Anas platyrhynchos)	0	0	0	0	72	11
		Northern pintail (Anas acuta)	0	0	0	0	6	0
		Tufted duck (Aythya fuligula)	0	0	0	0	1	0
	Swans	Bewick's swan (Cygnus columbianus bewickii)	0	0	0	0	20	0
		Mute swan (Cygnus olor)	0	0	0	0	24	3
		Whooper swan (Cygnus cygnus)	0	0	0	0	3	0
Charadriifor-mes	Gulls	Black-headed gull (Chroicocephalus ridibundus)	0	0	0	0	88	1
		Caspian gull (Larus cachinnans)	0	0	0	0	1	0
		Eurasian herring gull (Larus argentatus)	0	0	0	0	15	0
		Mew gull (Larus canus)	0	0	0	0	7	0
Gruiformes	Rails	Common coot (Fulica atra)	0	0	0	0	35	0
		Water rail (Rallus aquaticus)	0	0	0	0	16	0
Passerifor-mes	Corvidae	Eurasian magpie (Pica pica)	0	0	0	0	1	0
Pelicanifor-mes	Ardeidae	Grey heron (Ardea cinerea)	0	0	0	0	1	0
Total	NA	NA	28	2	41	1	390	17

NA: not applicable.

Full genome sequences were obtained for six isolates by Sanger sequencing. The six isolates shared 99.1–99.7% nt sequence identity across all of the eight genes in the genome. Basic local alignment search tool (BLAST, https://www.ncbi.nlm.nih.gov/blast/) search results for earlier detected viruses showed the highest identity (97.8–99.0%) with the HPAI H5N8 group B viruses for HA, NA and non-structural protein (NS). The remaining five gene segments (polymerases PB2, PB1 and PA, nucleoprotein (NP) and matrix protein (MP)) showed the highest identity with Eurasian LPAI viruses (Table 5). New reassortment events were observed for the PA and NP genes since the original detection of the HPAI H5N8 virus in Russia in May 2016.

Influenza A virus H5-specific antibody detection in wild birds

Seroreactivity of 459 wild bird sera was determined for different influenza H5 viruses. In a total of 29 sera, antibody titres directed to LPAI H5(N2) (A/Mallard/Netherlands/3/1999) or 2016 HPAI H5 clade 2.3.4.4 (A/Great Black-backed Gull/Netherlands/3/2016) or to both of these viruses were detected. There was good correspondence for high reacting sera (HI titre ≥ 40) between the HI antibody titres generated with the 2014 HPAI H5 clade 2.3.4.4 virus and the 2016 virus, suggesting that there has been limited antigenic drift of HPAI H5N8 viruses since 2014. In sera with lower HI titres, there was a strong bias to only react with the 2016 H5 clade 2.3.4.4 virus. When the HI assay for H5 clade 2.3.4.4-specific antibody positive sera was repeated, all but three titres were reproduced. Of the 10 sera showing antibody titres to both LPAI H5 and HPAI H5 clade 2.3.4.4 virus, one was LPAI-biased, two were HPAI-biased and seven showed ambiguous titres. Overall, 4.2% (18/431) of the sera obtained from October 2016 showed evidence of the presence of HPAI clade 2.3.4.4 H5-specific antibodies based on HI assays in duplo (Table 6) which was confirmed by VN assays in 14/18 samples from October 2016 onwards and 1/2 from 1 March 2016. The overall HPAI H5 antibody incidence between October and December 2016 was 2.0% (8/408). However, in mallards sampled on 8 February 2017 this was 43.5% (10/23) compared with 2.0% (1/49) in mallards sampled between October and December 2016. Comparing the 2016/17 winter with the same seasons in previous years, indicated that mallards and black-headed gulls (*Chroicocephalus ridibundus*) first tested positive for HPAI H5 clade 2.3.4.4-specific antibodies in the 2016/17 winter. In contrast, for Eurasian wigeons, common coots (*Fulica atra*) and mute swans (*Cygnus olor*) the detected incidence appeared to be lower in 2016/17 compared to the 2014/15 winter (Table 7). Taking into account all the bird species considered by the surveillance over the different winters, a preliminary incidence of HPAI H5 clade 2.3.4.4.-specific antibodies can be calculated as 0% before 2014, rising to 4.6% during the first outbreak of HPAI H5N8 virus, decreasing to 3.5% in the 2015/16 winter and rising to 4.2% in the 2016/17 winter (Table 7).

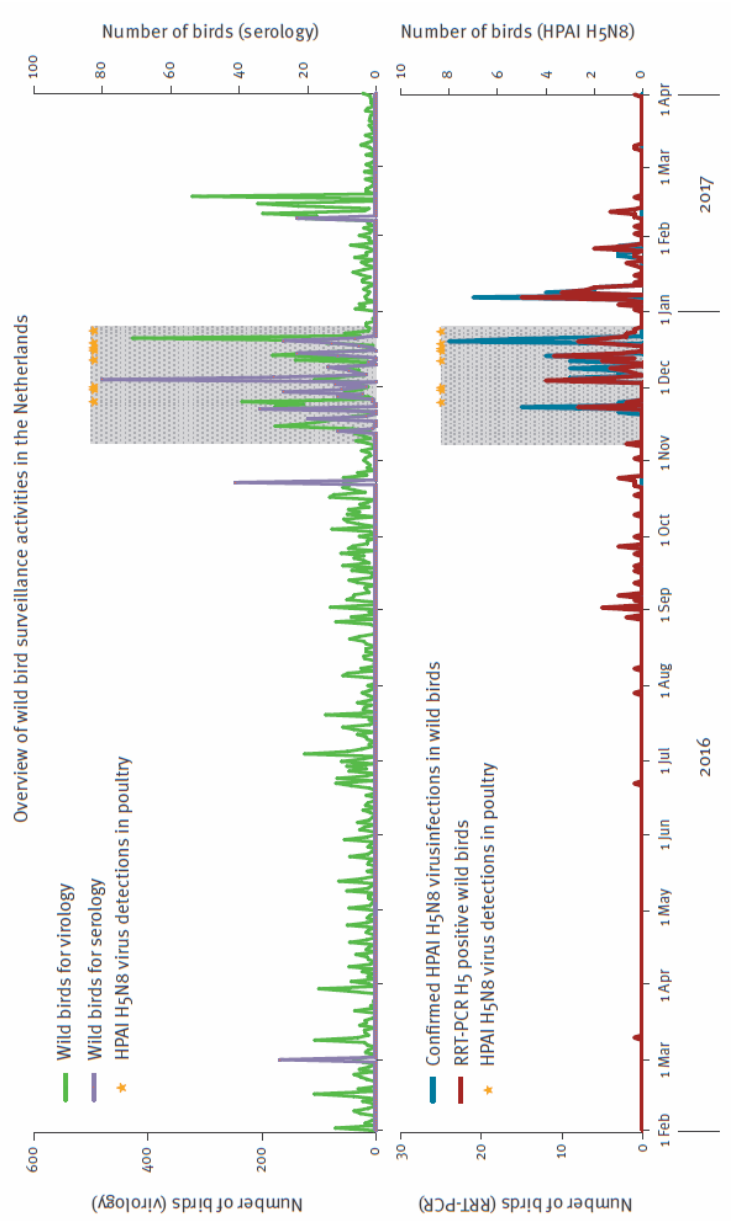

Figure 1. Overview of wild bird surveillance activities in the Netherlands between 1 February 2016 and 31 March 2017, with intensified surveillance from 13 November–31 December 2016 and 8–19 February 2017. RRT-PCR: real-time reverse-transcription PCR. Displayed over time are the number of birds tested for virus presence (green line), the number of birds positive for H5 in the RRT-PCR (dark red line) with the number of confirmed HPAI H5N8 cases (blue line), and the number of birds included for serology (purple line). In addition, the period with the largest wild-bird die-offs (light grey box) and the HPAI H5N8 detections in commercial poultry in the Netherlands (orange stars) are displayed.

Table 4. Overview of wild birds or wild birds' droppings, which were sampled in the Netherlands during active surveillance, and positive for highly pathogenic avian influenza H5N8 virus, 23 November 2016–28 January 2017 (n = 32 birds)

Species	Location	Status	Date	Number of animals
Eurasian wigeon	Echtenerburg	Dropping	23 November 2016	1
Eurasian wigeon	Warder	Live without clinical signs	04 December 2016	3
Common buzzard	Hippolytushoef	Live without clinical signs	07 December 2016	1
Eurasian wigeon	Oud Alblas	Live without clinical signs	08 December 2016	1
Eurasian wigeon	Oud Alblas	Live without clinical signs	12 December 2016	1
Eurasian wigeon	Nijkerk	Dropping	12 December 2016	3
Eurasian wigeon	Nijkerk	Dropping	14 December 2016	2
Eurasian wigeon	Nijkerk	Dropping	21 December 2016	3
Mallard	Oud Alblas	Live without clinical signs	07 January 2017	6
Mallard	Oud Alblas	Live without clinical signs	09 January 2017	4
Mallard	Oud Alblas	Live without clinical signs	10 January 2017	2
Mallard	Oud Alblas	Live without clinical signs	11 January 2017	2
Mallard	Oud Alblas	Live without clinical signs	24 January 2017	1
Mallard	Oud Alblas	Live without clinical signs	25 January 2017	1
Mallard	Oud Alblas	Live without clinical signs	28 January 2017	1

Table 5. Search results for sequences[a] with high similarity to the eight genes found in the full genomes of six highly pathogenic avian influenza H5N8 virus isolates from the Netherlands, 2016/17

Gene	BLAST result	Identity	Classification
PB2	[6/6] A/duck/Bangladesh/26920/2015(H3N6)	>98,7%	LPAI
PB1	[5/6] A/chicken/Hunan/S1267/2010(H4N6)	>97,7%	LPAI
	[1/6] A/duck/Mongolia/179/2015(H3N8)	97,5%	LPAI
PA	[6/6] A/duck/Mongolia/129/2015(H3N3)	>98,0%	LPAI
HA	[6/6] A/duck/Eastern China/S1109/2014(H5N8)	>98,7%	HPAI H5 clade 2.3.4.4
NP	[6/6] A/Mallard/Netherlands/15/2011(H6N8)	>99,2%	LPAI
NA	[6/6] A/duck/Eastern China/S1109/2014(H5N8)	>98,6%	HPAI H5 clade 2.3.4.4
MP	[6/6] A/duck/Mongolia/179/2015(H3N8)	>98,1%	LPAI
NS	[3/6] A/duck/Eastern China/S1109/2014(H5N8)	>98.8%	HPAI H5 clade 2.3.4.4
	[3/6] A/goose/Yangzhou/0420/2014(H5N8)	>97.9%	HPAI H5 clade 2.3.4.4

BLAST: basic local alignment search tool; HA: haemagglutinin; MP: matrix protein; NA: neuraminidase; NP: nucleoprotein; NS: non-structural protein; PA or PB: polymerases (PA, PB2, PB1).
[a] Searches were carried out using the National Center for Biotechnology Information (NCBI) nucleotide-BLAST.

Table 6. Details of the results on low pathogenic (LPAI) H5- and highly pathogenic avian influenza (HPAI) H5 clade 2.3.4.4-specific antibody positive sera using haemagglutination inhibition assays and the resulting HPAI/LPAI bias, Netherlands, 2016/17 (n = 29)

Sample ID	Species	Collection date	LPAI	HPAI H5 clade 2.3.4.4	Bias
C320-297	Black-headed gull	29 November 2016	<10	20	HPAI
320-312	Black-headed gull	05 December 2016	<10	20[a]	HPAI
309-9	Eurasian wigeon	01 March 2016	<10	20	HPAI
309-13	Eurasian wigeon	01 March 2016	<10	10	Ambiguous
309-23	Eurasian wigeon	01 March 2016	<10	20[a]	HPAI
C309-51	Eurasian wigeon	23 October 2016	40	40	Ambiguous
318-57	Eurasian wigeon	04 December 2016	<10	10[a]	Ambiguous
318-63	Eurasian wigeon	04 December 2016	40	640	HPAI
318-70	Eurasian wigeon	04 December 2016	20	<10	LPAI
318-75	Eurasian wigeon	04 December 2016	10	80	HPAI
320-55	Mallard	09 December 2016	320	40	LPAI
314-1813	Mallard	08 February 2017	<10	20	HPAI
314-1815	Mallard	08 February 2017	30	20	Ambiguous

Sample ID	Species	Collection date	LPAI	HPAI H5 clade 2.3.4.4	Bias
314-1817	Mallard	08 February 2017	40	20	Ambiguous
314-1819	Mallard	08 February 2017	<10	40	HPAI
314-1820	Mallard	08 February 2017	30	<10	LPAI
314-1821	Mallard	08 February 2017	<10	10	Ambiguous
314-1823	Mallard	08 February 2017	40	<10	LPAI
314-1824	Mallard	08 February 2017	<10	120	HPAI
314-1825	Mallard	08 February 2017	40	20	Ambiguous
314-1826	Mallard	08 February 2017	40	40	Ambiguous
314-1827	Mallard	08 February 2017	10	<10	Ambiguous
314-1832	Mallard	08 February 2017	10	20	Ambiguous
314-1835	Mallard	08 February 2017	30	20	Ambiguous
320-295	Mute swan	05 December 2016	<10	30	HPAI
320-641	Mute swan	07 December 2016	<10	30	HPAI
320-699	Mute swan	14 December 2016	30	<10	LPAI
320-729	Mute swan	15 December 2016	<10	20	HPAI
320-355	Whooper Swan	20 December 2016	80	<10	LPAI

HPAI H5 clade 2.3.4.4: highly pathogenic avian influenza A/Great-black backed gull/Netherlands/3/2016 (H5N8); LPAI: low pathogenic avian influenza A/Mallard/Netherlands/3/1999 (H5N2). [a]Titre could not be confirmed in a second haemagglutination inhibition HI assay.

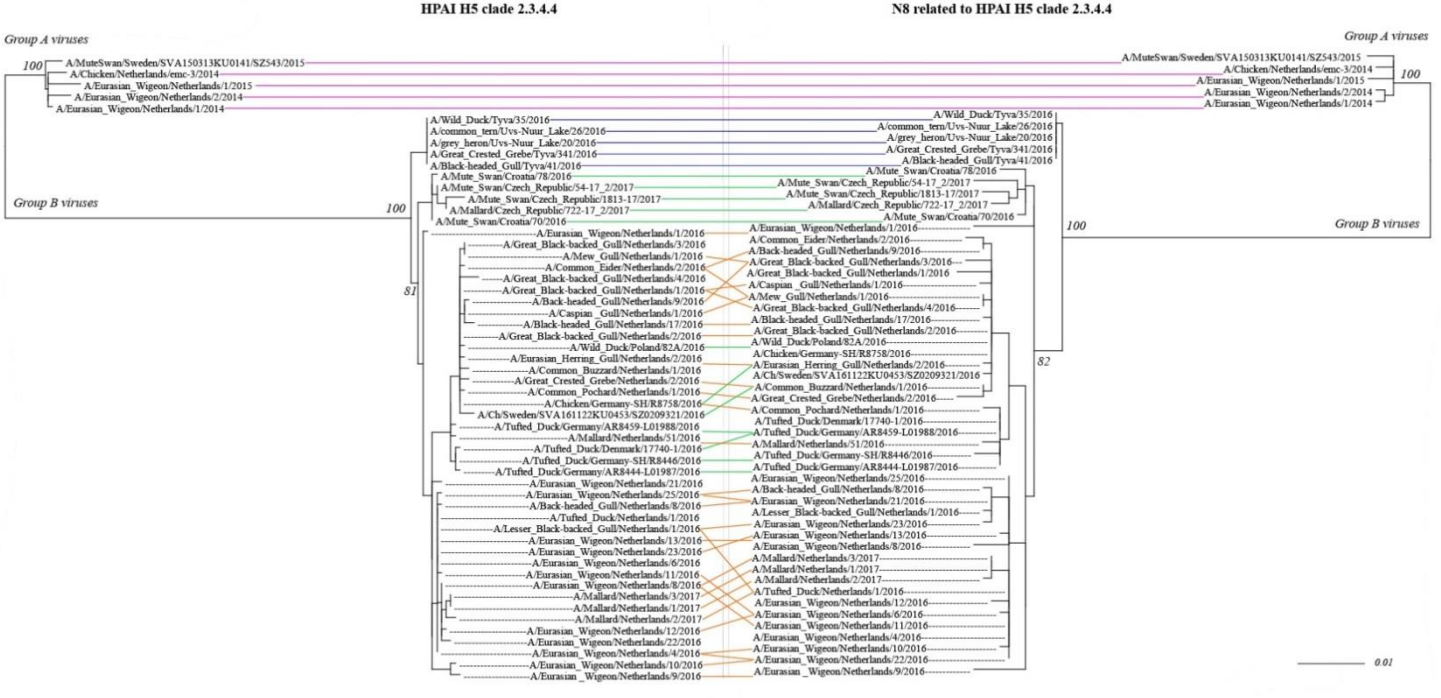
HPAI H5 clade 2.3.4.4
N8 related to HPAI H5 clade 2.3.4.4
Group A viruses
Group B viruses
100
81
82
0.01

Figure 2. Tanglegram of highly pathogenic avian influenza H5 clade 2.3.4.4 virus (left) and the accompanying N8 genes (right) based on 250 bootstraps Only bootstrap values above 70% are shown. The 2014/15 group A H5 viruses with their N8 genes (pink) are phylogenetically distinct from the 2016/17 group B viruses first detected in Russia (Tyva and Uvs-Nuur Lake) in May–June 2016 (blue) and later in other European countries (green) and the Netherlands (orange).

Table 7. Overview of highly pathogenic avian influenza H5 clade 2.3.4.4-specific antibody incidence in the Netherlands based on haemagglutination inhibition assays starting from the first wave of this virus in 2014/2015 up to February 2017

	2014/15[a]		2015/16[b]		2016/17[c]	
Species	Positive/ total	Percentage	Positive/ total	Percentage	Positive/ total	Percentage
Eurasian wigeon	12/78	15.4%	5/73	6.8%	3/104	2.9%
Lesser white-fronted goose	1/3	33.3%	0	U	0	U
Mute swan	29/88	33.0%	5/24	20.8%	3/24	12.5%
Common coot	1/84	1.2%	1/22	4.5%	0/35	0%
Black-headed gull	0/262	0.0%	0/31	U	1/88	1.1%
Mallard	0/93	0.0%	0/18	U	11/72	15.3%
Egyptian goose	0/62	0.0%	1/28	3.6%	0/10	0%
Total	43/940	4.6%	12/347	3.5%	18/431	4.2%

U: unknown. [a]Data previously published [10]. [b]Data (partly) previously published [10] and supplemented with Eurasian wigeon data from this study (n = 28) from 1 March 2016. [c]Data obtained in the current study from 23 October 2016 to 8 February 2017.

Discussion

Here, we report on our virological findings in wild birds during the second wave of European HPAI H5(N8) outbreaks in 2016/17 and further investigate the use of serology in addition to virology in an outbreak situation. In this study we detected HPAI H5N8 viruses in 57 birds of 12 species. Initially, HPAI H5N8 virus was detected in dead wild birds by passive surveillance in mainly tufted ducks and Eurasian wigeons, followed by scavengers [16]. After these die-offs, the virus was detected in live wild birds and shifted from being found mostly Eurasian wigeons early in the outbreak towards mallards later in the outbreak, despite the fact that both species were screened throughout time. Although the number of HPAI H5(N8) infected wild birds identified by passive surveillance in this study and others [16-18] was much higher because of the massive die-offs and subsequent mandatory testing, the high virus prevalence in mallards would have been missed in passive surveillance studies since

hardly any mallards were found dead and infected [16]. Likewise, the period of time of virus detection lasted longer in active surveillance compared with passive surveillance. Our results show that the mallard viruses from January 2017 were largely indistinguishable from the other HPAI H5N8 viruses, including those of tufted ducks, indicating that mallards might be more resistant to disease compared with other duck species, similarly to previous findings for HPAI H5N1 in mallards [26] and might therefore act as a reservoir species.

Results of analyses at the whole genome level indicated that the HA, NA and NS genes of Dutch H5N8 viruses were most closely related to 2014 HPAI H5N8 group B Eastern China viruses, while the other five genes were derived from Eurasian LPAI viruses. This genetic makeup is similar to viruses detected in Russia (May 2016) and Germany (autumn/winter 2016) [11,15]. Compared with the May 2016 Russian viruses, viruses in the Netherlands showed similar new reassortment events for the NP and PA genes as was described for the German viruses [15] (Table 5).

In contrast to the 2014/15 European emergence of HPAI H5N8, when a single lineage of HPAI spread across Europe, the chain of events during the 2016/17 HPAI H5 emergence shows more similarities to the 2014/15 situation in the United States (US), where the HPAI H5N8 group A viruses reassorted with local LPAI viruses causing massive and long lasting detection in both poultry and wild birds and local die-offs in wild birds [6]. While this manuscript was in preparation, detections of HPAIH5 clade 2.3.4.4 virus in Europe were still reported in Belgium, Luxembourg, the Netherlands and the United Kingdom [18], even though migrating birds had largely left their European wintering sites, suggesting that virus amplification was now occurring in local resident birds. This is a cause of concern, as establishment of HPAI viruses among wild birds is difficult to control and may give rise to a situation comparable to that in Asia with new outbreaks in wild birds and poultry not being caused by novel introductions of HPAI viruses from distant areas, but from within the local populations. It remains unclear, however, what drivers are responsible for the duration of virus circulation in a wild bird population, either long (US 2014/15 and Europe 2016/17) or short (Europe 2014/15), and based on current knowledge we cannot predict how the H5 situation among wild birds in Europe will evolve.

In case of introduction of new HPAI viruses, it would be highly beneficial to be able to target active surveillance to key species for virus detection to avoid excessive costs, sampling efforts, and inclusion of unnecessarily large numbers of animals. We therefore examined the use of experimental approaches for serology for the second time in an outbreak situation. To confirm serological data and to be able to determine the HPAI/LPAI and HPAI clade bias with some accuracy, we performed both HI and VN assays. Sera of 12 birds showed exclusive titres or a bias towards HPAI virus, seven to LPAI virus and seven remained ambiguous [24]. VN assays confirmed the presence of HPAI H5 clade 2.3.4.4-specific antibodies in 14/18 sera

from October 2016 onwards and 1/2 from 1 March 2016. High cross-reactivity patterns and low initial titres in both assays showed that specifying biases towards one of the different HPAI H5 clades is very difficult. Further optimisation and validation of the assays are required to provide rough estimates of the seropositivity in subsequent years. Preliminary comparisons between the winters using the same HI assay starting from the 2014/15 winter show an outbreak-related incidence of HPAI H5 clade 2.3.4.4.-specific antibodies of 0% before 2014, rising to 4.6% during the first outbreak of HPAI H5N8 virus, decreasing to 3.5% in the 2015/16 winter and rising to 4.2% in the 2016/17 winter. Despite a similar antibody incidence between both outbreak periods, an apparent decreasing antibody incidence in two species detected throughout all three screening periods (Eurasian wigeons and mute swans) can be observed. This might be a consequence of differences in timing in peak prevalence between the first wave in 2014, with a very limited number of wild birds detected with a HPAI H5N8 infection (i.e. local virus amplification) [27,28] but high antibody incidence, and the current second wave, with substantial local virus replication and lower incidence of antibodies. These data could suggest that virus amplification in wigeons in 2014/15 took place before arrival of these birds in the Netherlands, whereas in 2016/17 virus amplification primarily took place within the Netherlands resulting in associated die-offs [14,16]. Unfortunately, we were unable to collect sera from wigeons late in the season in 2016/17 to confirm increasing antibody incidence. However, the mallards that were tested later in the outbreak (February 2017) showed an increase in antibody incidence after a peak in virus detections a few weeks earlier compared with those tested earlier in the outbreak (November–December 2016).

Recently, clade 2.3.4.4 H5N6 viruses started to circulate in both poultry and wild birds in south-east Asian counties [29] after their original detection in China [30], resembling HPAI H5N8 dispersion of 2014. In contrast to HPAI H5N8 viruses, these H5N6 viruses have caused sporadic human infections, including fatalities [31]. Hence it will be important to monitor the movements of these viruses by intense monitoring of wild bird populations in the coming winter seasons. In terms of multiple intracontinental spread of HPAI H5 viruses, global outbreaks were preceded by detections on breeding sites in Russia (Uvs-Nuur Lake district) and China (Qinghai Lake) after their initial detections in south-east Asia [11,32-35]. Increasing global collaborations and performing annual targeted active surveillance in China and on Russian breeding sites, and in Europe as autumn migration starts, will be important to provide early warning signals of HPAI virus dissemination.

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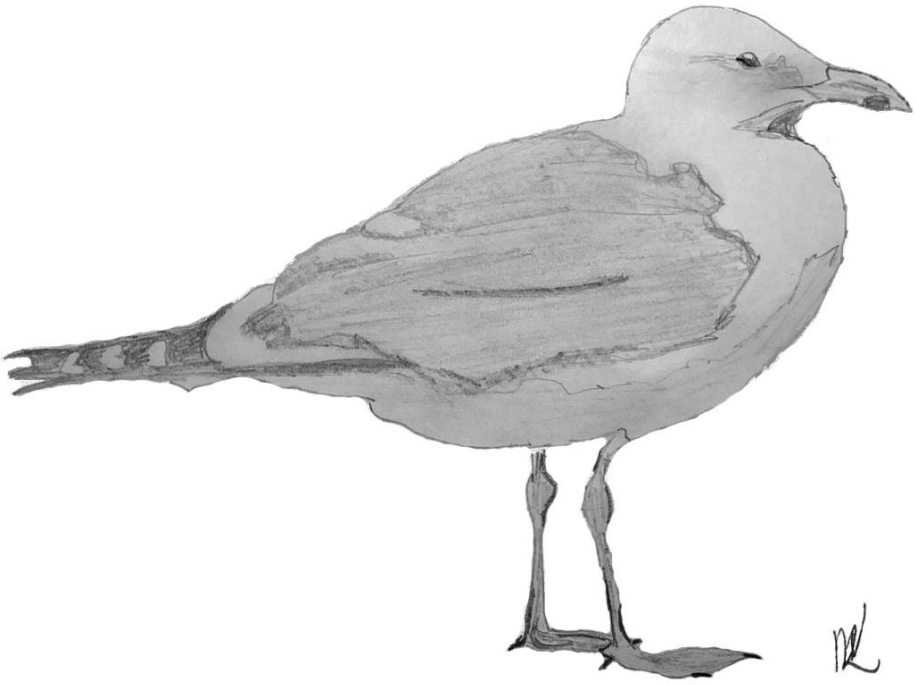
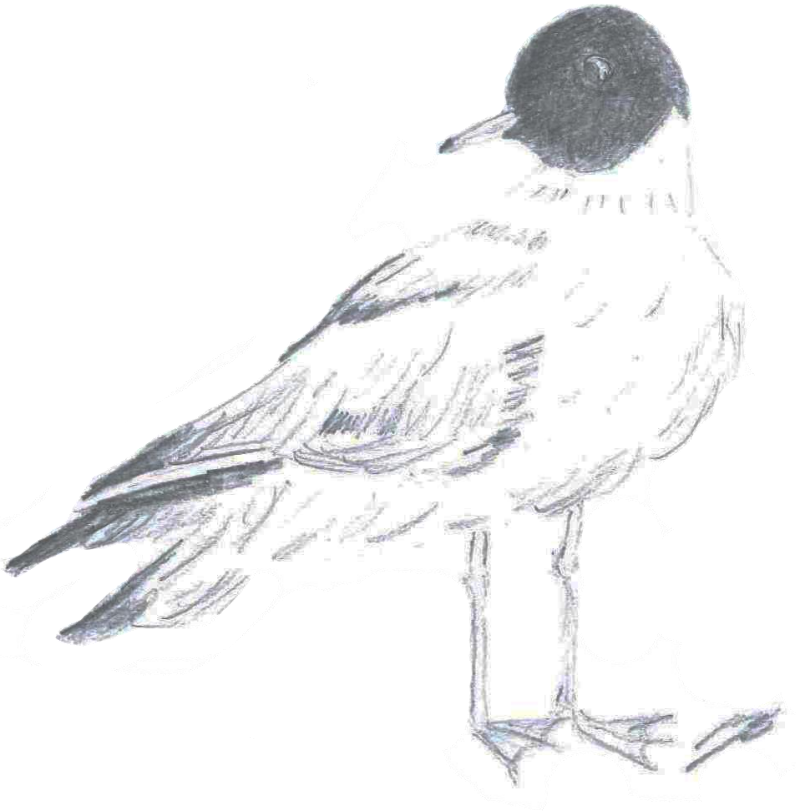


Mat

Chapter 3

Highly pathogenic avian influenza virus A/H5N6 in Europe



Marjolein

Chapter 3

Co-circulation of genetically distinct highly pathogenic avian influenza A clade 2.3.4.4 (H5N6) viruses in wild waterfowl and poultry in Europe and East Asia, 2017-18

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Abstract

Highly pathogenic avian influenza (HPAI) H5 clade 2.3.4.4 viruses were first introduced into Europe in late 2014 and re-introduced in late 2016, following detections in Asia and Russia. In contrast to the 2014-15 H5N8 wave, there was substantial local virus amplification in wild birds in Europe in 2016-17 and associated wild bird mortality, with evidence for occasional gene exchange with low pathogenic avian influenza (LPAI) viruses. Since December 2017, several European countries have again reported events or outbreaks with HPAI H5N6 reassortant viruses in both wild birds and poultry respectively. Previous phylogenetic studies have shown that the two earliest incursions of HPAI H5N8 viruses originated in Southeast Asia and subsequently spread to Europe. In contrast, this study indicates that recent HPAI H5N6 viruses evolved from the H5N8 2016-17 viruses during 2017 by reassortment of a European HPAI H5N8 virus and wild host reservoir LPAI viruses. The genetic and phenotypic differences between these outbreaks and the continuing detections of HPAI viruses in Europe are a cause of concern for both animal and human health. The current co-circulation of potentially zoonotic HPAI and LPAI virus strains in Asia warrants the determination of drivers responsible for the global spread of Asian lineage viruses and the potential threat they pose to public health.

Introduction

Highly pathogenic avian influenza (HPAI) viruses cause outbreaks of disease, often resulting in mortality in poultry and wild bird species. Since 2003, HPAI H5 viruses of the A/Goose/Guangdong/1/1996 (GsGd) lineage have been circulating enzootically in poultry in several countries in South and Southeast Asia, and Africa. Periodically, these HPAI H5 viruses have been introduced into wild birds with subsequent spread to other geographic areas, likely through bird migration (1, 2). Since late 2013, HPAI viruses with an H5 heamagglutinin (HA) from clade 2.3.4.4 viruses with different neuraminidase (NA) subtypes (e.g. H5N8, H5N6, H5N1) have been circulating in Southeast Asia. Clade 2.3.4.4 (H5N8) viruses of two distinct groups, commonly referred to as group A (Buan-like) and group B (Gochang-like), were first detected in China and South Korea in late 2013/early 2014 (3-5). Viruses belonging to group A emerged in late 2014 and spread to North America and Europe almost simultaneously. After their initial detection, clade 2.3.4.4 group B (H5N8) viruses were not detected in Southeast Asia until May 2016, when they were detected at Lake Uvs-Nur, Russia (6) and Qinghai lake, China (7). From May 2016, the clade 2.3.4.4 group B H5N8 viruses subsequently spread to most European countries causing numerous outbreaks in poultry (8) and massive die-offs in wild birds (9-11), and infected both poultry and wild birds in multiple African countries (8, 12, 13). Several reassortment events also occurred, leading to the emergence and detection of HPAI H5N5 in several European countries, Georgia and Israel between November 2016 and June 2017 (14) and HPAI H5N6 in Greece in February 2017 (12). HPAI H5N6 first emerged in poultry in early 2014, having previously been detected in an environmental sample in late 2013 and in a live duck, sampled in early 2014 in China. Genetic analysis showed this HPAI H5N6 virus to be a reassortant consisting of a clade 2.3.4.4 HA, an NA related to Chinese low pathogenic avian influenza (LPAI) H6N6 duck viruses, and an internal gene cassette closely related to 2011 HPAI H5 clade 2.3.2.1 viruses (15-17). The first viruses were later assigned to groups C and D. Currently, clade 2.3.4.4 group C (H5N6 viruses from China in 2013 (18), Laos and Vietnam in 2014 and Hong Kong in 2015, and H5N1 viruses from China and Vietnam in 2014) and group D (H5N6 viruses from China and Vietnam 2013-14, including human strains) viruses have also been classified as sub-groups (19, 20) although this nomenclature has not been formally adopted. HPAI H5N6 viruses belonging to clade 2.3.4.4 group B were first detected in late 2017 in Japan, South Korea (21, 22) and the Netherlands (23). From mid-December 2017, HPAI H5N6 group B viruses were also detected in wild birds in Switzerland, the United Kingdom, Germany, Sweden, Ireland, Denmark, Iran, Slovakia and Finland (Figure 1). In the Netherlands and Germany, the virus was also detected in poultry. In the same period, HPAI H5N6 viruses of clade 2.3.4.4 group B, along with clade 2.3.4.4 groups C and D have been detected in East Asian countries (22).

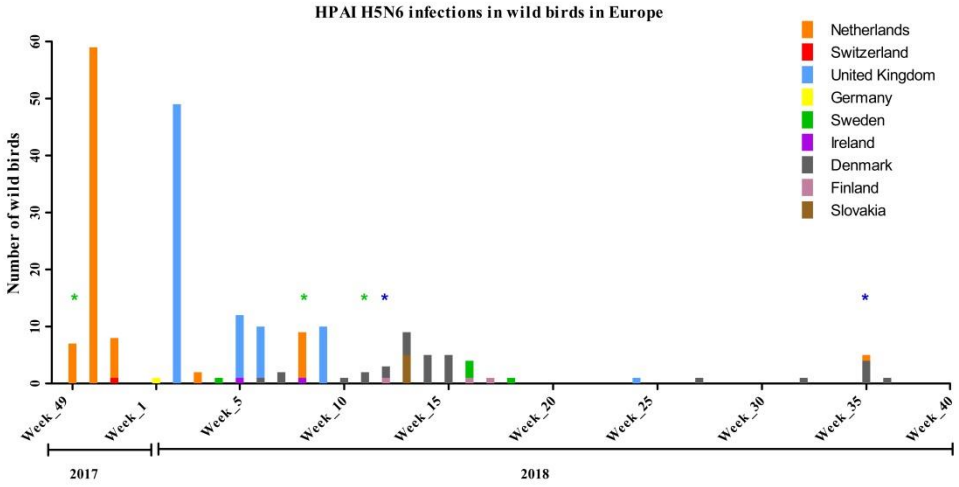

Figure 1. Overview of the number of wild birds reported to be infected with HPAI H5N6 based on the OIE Update on Avian influenza in animals (types H5 and H7) list 2017/2018 (12, 26) per week starting from the first detection on 7 December 2017. The colours represent the country of detection. The asterisks (*) indicate the detections of HPAI H5N6 viruses in commercial (green) and backyard (blue) poultry.

HPAI H5 viruses pose a significant threat to not only animal health, particularly to poultry, but also to human health owing to their zoonotic potential (24, 25). In April 2014, the first fatal human case of HPAI H5N6 virus infection was identified in China (26), with these viruses phylogenetically clustering in unofficially-defined clade 2.3.4.4 group C and 2.3.4.4 group D (19), and one case in group B (27). To date, (5 October 2018), a total of 21 H5N6 human cases have been reported in China with a high case fatality rate in diagnosed individuals (28, 29). Ferret studies showed that although Asian 'zoonotic' HPAI H5N6 viruses replicated to high titres in the respiratory tract, there was no evidence for airborne transmissibility of these viruses (30).

In this study, we attempt to determine the source of all eight influenza virus gene segments, and the estimates for the time to the most recent common ancestors (TMRCA) for the H5 HA and N6 NA gene segments to investigate when and where these viruses have evolved. We report the genetic relationships between the latest HPAI H5N6 viruses isolated from both European and Asian wild birds and poultry by using whole genome sequencing and characterize the emerging strains relative to other circulating viruses in the region.

Materials and methods

Wild bird surveillance and sequencing

Active surveillance programs in wild birds, i.e. sampling of living healthy birds, are limited and often project-based, and resulting data are often not publicly available. Here, continuous active surveillance activities of influenza virus circulation in wild birds are reported for the Netherlands and the Republic of Georgia. Wild birds of various species were caught and sampled for virus detection as described previously (31-33). Briefly, samples were tested for the presence of avian influenza A H5 viruses using a matrix gene specific and H5 HA gene specific real-time RT-PCR analysis followed by either Sanger sequencing, as described before (31) (primer sequences are available upon request), or by MinION sequencing (Oxford Nanopore technologies). For MinION sequencing, RNA was extracted using the QIAamp Viral RNA Mini Kit (52904, Qiagen, UK) and a multi segment RT-PCR amplification was performed using the Superscript III high-fidelity RT-PCR kit (12574-035, Invitrogen,USA) according to manufacturer's instructions using the Opti1 primer set with influenza-specific universal primers complementary to the conserved 12–13 nucleotides at the end of all 8 genomic segments: Opti1-F1 5' GTTACGCGCCAGCAAAAGCAGG, Opti1-F2 5'GTTACGCGCCAGCGAAAGCAGG and Opti1-R1 5'GTTACGCGCCAGTAGAAACAAGG. MinION sequencing was performed according to manufacturer's instructions using the ID Native barcoding genomic DNA kit (EXP-NBD103 and SQK-LSK108, Oxford Nanopore, UK). Raw sequence data were demultiplexed using Porechop (https://github.com/rrwick/Porechop), and a reference-based alignment was performed using CLC Genomics software, workbench 8 (CLC Bio), and the full genome Sanger sequence of A/Black-headed_Gull/Netherlands/29/2017 served as a reference (EPI_ISL_289714). Primers and adaptors were trimmed from the raw sequence data and the Phred score for alignment was set to 8 and minimum require coverage was set to 100 reads per position. Discrepancies in the sequences (insertions or deletions) compared to close reference strains occurred only in homopolymeric regions, and were manually checked and resolved by incorporating an "N" at these positions.

Strains of Interest

To better understand the newly emerging HPAI H5N6 viruses from the 2.3.4.4 group B lineage, viruses of the H5N6 subtype isolated in or after October 2017 were assigned as strains of interest (SOI). Depending on the continent of isolation, they were further classified as Asia-SOI or Europe-SOI (Table 1). Whole genome sequences for these viruses were obtained from public databases (GISAID and Genbank).

European countries and non-European collaborators were asked to contribute any additional HPAI H5N6 and recent HxN6 sequence data via FLU-LAB-NET (European Union) or personal communication. HxN6 viruses were not considered SOI but included in the general set to test if any SOI N6s were closely related to them. In addition, the Animal and Plant Quarantine Agency (APQA) from South Korea shared three whole genomes from HPAI H5N6 detections since November 2017 (GISAID accession numbers EPI_ISL_288436, EPI_ISL_288437, EPI_ISL_292349). Sequences of NA genes from recent (2016-2017) LPAI HxN6 viruses from wild birds from the Netherlands, Belgium, Hungary and Croatia were also obtained for the general set. Further, sequences of H5N6 viruses that were isolated from humans (HUM) in China in 2015, 2016 and 2017 (Table 1) were collected from GISAID (Supplemental Table 1) to test whether the genes of any of the emerging SOI were related to those zoonotic strains.

Table 1. Overview of our strains of interest (SOI) of highly pathogenic avian influenza viruses H5N6 divided in Asia-SOI, Europe-SOI and human H5N6 viruses. Viruses marked with an asterisk (*) were obtained and sequenced within the surveillance activities described in this manuscript.

Set	Strainname	Isolation date
Asia-SOI	A/mute_swan/Shimane/3211A001/2017	05 November 2017
	A/chicken/Vietnam/QuangBinh/BoTrach1113/2017	13 November 2017
	A/mallard/Korea/Jeju-H24/2017	17 November 2017
	A/duck/Korea/HD1/2017	17 November 2017
	A/spoonbill/Taiwan/DB645/2017	01 December 2017
	A/duck/Korea/H35/2017	10 December 2017
	A/Mallard/Korea/H17-1825/2017	22 December 2017
	A/Mandarin_duck/Korea/K17-1815/2017	22 December 2017
	A/Mandarin_duck/Korea/K17-1817/2017	22 December 2017
	A/Mandarin_duck/Korea/K17-1826/2017	22 December 2017
	A/Mandarin_duck/Korea/K17-1828/2017	22 December 2017
	A/Mandarin_duck/Korea/K17-1862/2017	23 December 2017
	A/Mandarin_duck/Korea/K17-1866/2017	23 December 2017
	A/Mandarin_duck/Korea/K17-1869/2017	23 December 2017
	A/Mandarin_duck/Korea/K17-1873/2017	23 December 2017
	A/Mandarin_duck/Korea/K17-1879/2017	23 December 2017
	A/Mandarin_duck/Korea/K17-1881/2017	23 December 2017

Set	Strainname	Isolation date
	A/Mandarin_duck/Korea/K17-1885/2017	23 December 2017
	A/Mandarin_duck/Korea/K17-1887/2017	23 December 2017
	A/Mandarin_duck/Korea/K17-1889/2017	23 December 2017
	A/Mandarin_duck/Korea/K17-1891/2017	23 December 2017
	A/Mandarin_duck/Korea/K17-1893/2017	23 December 2017
	A/Mandarin_duck/Korea/K17-1894/2017	23 December 2017
	A/Mandarin_duck/Korea/K17-1896/2017	23 December 2017
	A/Armenian_Gull/Republic_of_Georgia/4/2017*	27 December 2017
	A/Mandarin_duck/Korea/K18-3/2018	18 January 2018
	A/Mallard/Republic of Georgia/1/2018*	28 January 2018
	A/duck/Vietnam/QuangBinh/QN530206/2018	06 February 2018
	A/jungle_crow/Hyogo/2803E011/2018	03 March 2018
	A/jungle_crow/Hyogo/2803E022/2018	06 March 2018
Europe-SOI	A/Duck/Netherlands/17017236-001005/2017	07 December 2017
	A/Duck/Netherlands/17017237-001-005/2017	07 December 2017
	A/Tufted_Duck/Netherlands/17017367-007/2017	09 December 2017
	A/Mute_Swan/Netherlands/17017367-012/2017	09 December 2017
	A/Mute_Swan/Netherlands/17017377-001/2017	09 December 2017
	A/Great_Black-backed_Gull/Netherlands/1/2017*	18 December 2017
	A/Black-headed_gull/Netherlands/29/2017*	18 December 2017
	A/common_pochard/Germany-BY/AR09-L02421/2017	28 December 2017
	A/mute_swan/England/SA21_180652/2018	02 January 2018
	A/Canada_Goose/England/AV58_18OPpoolEP1/2018	05 January 2018
	A/pochard_duck/England/AVP_18_003254/2018	10 January 2018
	A/Great_Black-backed_Gull/Netherlands/1/2018*	23 January 2018
	A/Eurasian wigeon/Netherlands/1/2018*	07 February 2018
	A/White-tailed_Eagle/Denmark/3073-1w/2018	13 February 2018
	A/Chicken/Netherlands/EMC-1/2018	26 February 2018
	A/Domestic_duck/Netherlands/EMC-6/2018	13 March 2018
Human	A/Guangdong/ZQ874/2015	31 December 2015
	A/Shenzhen/1/2016	07 January 2016
	A/Anhui/33162/2016	28 April 2016
	A/Anhui/33163/2016	29 April 2016

Set	Strainname	Isolation date
	A/Hunan/55555/2016	18 November 2016
	A/Guangxi/55726/2016	24 November 2016
	A/Fujian-Sanyuan/21099/2017	25 December 2017

Reassortment analyses - Visualisation of phylogenetic incongruence

To study the evolution and gene reassortment events of HPAI H5N6 viruses, a phylogenetic incongruence analysis was performed by aligning maximum likelihood (ML) trees for all influenza virus gene segments, except NA. Publicly available sequences of all eight segments of avian influenza viruses isolated between 1 January 2007 and 30 September 2017 were downloaded from Genbank. This formed a set of 61,842 sequences including all segments from all strains namely - HA:8278, MP:6464, NA:7779, NP:7382, NS:6933, PA:8346, PB1:8410, PB2:8250. This set of sequences was supplemented with the additional H5N6 and HxN6 sequences contributed by collaborators.

Sequences were first subjected to a quality control step where all duplicate sequences, and sequences bearing duplicate IDs, were removed. They were then separated into individual sequence datasets for each segment (PB2, PB1, PA, HA, NP, NA, MP and NS). Python scripts obtained from https://github.com/ballesterus/Utensils.git were used to concatenate all segments from each strain. Only those viral strains which had sequences from all eight gene segments (4000 strains) were selected. This concatenated sequence data was down-sampled using CD-HIT-EST (Cluster Database at High Identity with Tolerance) (34, 35) to remove sequences with >95%, 90% and 80.0% sequence identity across the whole genome (all 8 segments). An 80% threshold was chosen as it provided the best compromise between retaining maximum possible diversity of sequences while retaining the ability to visualize. The resulting list of 141 strain names was used to extract sequences from those strains from each individual gene segment separately – resulting in the reduced whole-genome (WG) dataset for each segment.

Sequences of all segments from the strains of interest (SOI) were also subjected to the same down-sampling procedure separately before being added to their respective WG-segment dataset. However, they were down-sampled to 99.5% sequence identity (39 strains) to retain as much of the current diversity as possible. Sequences of all segments from all three strains that were isolated from humans (HUM) were also added, giving a total of 183 strains.

Final datasets were aligned using MAFFT v7.305b (36), and trimmed to only retain nucleotides from the starting ATG until the final STOP codon. We inferred Maximum Likelihood (ML) phylogenetic trees for each gene segment using IQ-TREE, 1.5.5 (37) and ModelFinder (38) and obtained branch supports with Shimodaira-Hasegawa (SH)-like approximate Likelihood Ratio Test (aLRT, 1,000 replicates) and standard non-parametric bootstrap (100 replicates).

BALTIC (backronymed adaptable lightweight tree import code) was used to compare the phylogenetic structure of the internal genes of the SOI. The phylogenetic position of each strain was traced, coloured according to the HA (Asia-SOI, Europe-SOI, H5N8 2016/17 clade, H5N6 Chinese, human H5N6 (HUM), other H5Nx, and LPAI) across unrooted ML trees for HA and all internal gene segments. Figures were generated by modifying scripts from a similar analysis (39) and editing in Adobe Illustrator. We selected a qualitative palette of colours using http://colorbrewer2.org/. The modified version of all scripts are available in a github repository (https://github.com/delfinut/phylogenetic-incongruence).

Analysis of the H5 and N6 genes

For the H5 analysis, 1,251 HA sequences of strains isolated between January 2016 and March 2018 were downloaded from GISAID (Supplemental Table 1). For the N6 tree, NA gene sequences of 1,680 strains of LPAI HxN6 viruses from Genbank, isolated between January 2007 and January 2018, were used. BLAST was used to find the closest related sequences to the HA and NA sequences from the SOI in the entire influenza virus resource database in FluDB (www.fludb.org). GISAID has the most up-to-date HPAI sequences – hence this database was used to acquire HPAI H5 HA sequences; Genbank has a better representation of HPAI and LPAI strains taken together, which is why this database was used for N6 NA sequences which may or may not be associated with HPAI HAs.

The individual H5 and N6 datasets described above were first subjected to a quality control step where all duplicate sequences and sequences bearing duplicate IDs were removed. These datasets were first analyzed with the SOI and HUM strains. Sequences were aligned using MAFFT v7.305b (36), and used in the FastTree program (40, 41) to generate an initial tree. Since both HA and NA of SOI viruses formed two monophyletic clades each (excluding all other strains), with robust support (>0.80), the downloaded sequences were then subjected to down sampling to a cut-off of 99.5% identity before further analysis. SOI HA and NA sequences were also down-sampled to 99.9% identity respectively. All sequences from taxa which were found outside of the fully supported (100%) cluster with SOI were discarded.

Ten further sequences were discarded after analysis of the H5 HA ML tree with tempest v1.5, which identified them as outliers and did not conform to clock-likeness as suggested by the authors in (42). Similarly, nine further sequences were removed as outliers after analysis of the N6 NA ML tree with tempest v1.5. The H5 HA dataset included representatives from the HPAI clade 2.3.4.4 group B (H5N8) 2016-17 clade, European and Asian HPAI clade 2.3.4.4 group B, C and D H5N6 viruses, other HPAI H5Nx viruses, as well as the separate lineage of the group C and D Chinese H5N6 strains, to form a new dataset of 153 sequences from which a final H5 ML tree was inferred using IQ-TREE. The N6 NA dataset included a total of 183 sequences from the H5N6 SOI as well as the most closely related sequences that were from LPAI viruses, primarily from wild birds. The final N6 ML tree was inferred from this dataset using IQ-TREE.

Bayesian phylogenetic trees were inferred using BEAST v1.10.1 (43) to determine the time of emergence of SOI and the viruses they likely arose from. Path sampling/ stepping stone sampling (PS/SS) (44) was used to select the appropriate site substitution and clock models. BEAST calculates log marginal likelihood estimates (MLE) for each run from which log Bayes factors (BF) (which indicate support for one model over another) were calculated as the difference between the log MLEs. SRD06, i.e., the standard HKY site model with estimated base frequencies and gamma site heterogeneity with 4 gamma categories and 2 codon partitions (1+2, 3) was chosen over GTR with the empirical base frequencies and gamma categories with no codon partition (> 180 log BF) (45). An uncorrelated relaxed lognormal clock was used to allow for rate variation along different branches with GMRF Bayesian Skyride population prior (chosen over constant prior with > 20 log BF) and random starting tree. All other priors were set to default. MCMC was set to 70,000,000 generations. Two separate runs were performed to ensure convergence between runs. Log files were analyzed in Tracer v1.7.1 to determine convergence, and to check that ESS values were beyond threshold (>200). Log and trees files from both runs were combined using Log Combiner v 1.10.1. Tree annotator v1.10.1 was used to generate a maximum credibility tree (MCC) using 10% burn in and median node heights. The MCC tree was then annotated to include posterior probability values and time scales and plotted in R v 3.5 using the ggtree package (46).

Results

Wild bird surveillance and sequencing

Continuous active surveillance activities resulted in the screening of 2,769 wild birds in the Netherlands and 2,190 in the Republic of Georgia between 9 December 2017

and 30 June 2018. HPAI H5 viruses were detected in 10 Eurasian Wigeons *(Anas penelope*, February 2018) in the Netherlands, and in a Mallard (*Anas platyrhynchos*, 28 January 2018) and an Armenian Gull (*Larus armenicus*, 27 December 2017) in the Republic of Georgia. In addition, during opportunistic sampling of a small number of dead birds (passive surveillance) the virus was detected in two Great Black-backed Gulls *(Larus marinus*, 18 December 2018 and 23 January 2018) and a Black-headed Gull (*Chroicocephalus ridibundus*, 18 December 2017) that were found dead in the Netherlands (Table 2). Full genome sequences were obtained from one of the HPAI H5N6 infected Dutch Eurasian Wigeons and the three dead gulls, and from both Georgian birds. All obtained sequences have been uploaded to GISAID with accession numbers EPI_ISL_289713, EPI_ISL_289714, EPI_ISL_302823, EPI_ISL_302824, EPI_ISL_303520, EPI_ISL_312376. During the mentioned surveillance period no additional LPAI HxN6 viruses were detected.

Table 2. Wild bird species sampled for virus detection during the 2017/18 outbreaks of highly pathogenic avian influenza H5N6 virus in the Netherlands (n= 2,816), and the Republic of Georgia (n=2,190), 9 December 2017 - 30 June 2018.

			Republic of Georgia				The Netherlands							
			Alive without clinical signs				Alive without clinical signs				Found dead			
			No. birds				No. birds				No. birds			
Order	Family	Species	sampled	AIV positive	H5 positive	Pathotype	sampled	AIV positive	H5 positive	Pathotype	sampled	AIV positive	H5 positive	Pathotype
Accipitriformes	Accipitridae	Hen harrier (*Circus cyaneus*)	0	0	0	NA	1	0	0	NA	0	0	0	NA
Anseriformes	Ducks	Common shelduck (*Tadorna tadorna*)	0	0	0	NA	2	0	0	NA	1	0	0	NA
		Eurasian teal (*Anas crecca*)	86	43	0	NA	236	54	2	2x LPAI	0	0	0	NA
		Eurasian wigeon (*Anas penelope*)	4	2	0	NA	332	64	32	10x HPAI 3x LPAI 19xn.i.	0	0	0	NA
		Gadwall (*Anas strepera*)	12	6	0	NA	114	33	2	1x LPAI 1x n.i.	0	0	0	NA
		Garganey *Spatula querquedula*)	32	16	0	NA	0	0	0	NA	0	0	0	NA
		Mallard (*Anas platyrhynchos*)	454	227	1	1x HPAI	2021	131	10	3x LPAI 7x n.i.	2	0	0	NA
		Northern pintail (*Anas acuta*)	22	11	0	NA	2	0	0	NA	1	1	0	NA
		Northern shoveler (*Spatula clypeata*)	0	0	0	NA	3	0	0	NA	0	0	0	NA
		Ruddy shelduck (*Tadorna ferruginea*)	2	1	0	NA	0	0	0	NA	0	0	0	NA
		Tufted duck (*Aythya fuligula*)	8	4	0	NA	12	0	0	NA	0	0	0	NA

Order	Family	Species	Republic of Georgia				The Netherlands							
			Alive without clinical signs				Alive without clinical signs				Found dead			
			No. birds				No. birds				No. birds			
			sampled	AIV positive	H5 positive	Pathotype	sampled	AIV positive	H5 positive	Pathotype	sampled	AIV positive	H5 positive	Pathotype
Anseriformes		Duck spp. (*Anas spp.*)	0	0	0	NA	21	1	0	NA	0	0	0	NA
	Geese	Greylag goose (*Anser anser*)	0	0	0	NA	0	0	0	NA	1	0	0	NA
		Pink-footed goose (*Anser brachyrhynchus*)	0	0	0	NA	1	0	0	NA	0	0	0	NA
	Swans	Bewick's swan (*Cygnus columbianus bewickii*)	0	0	0	NA	15	0	0	NA	0	0	0	NA
		Mute swan (*Cygnus olor*)	0	0	0	NA	0	0	0	NA	1	0	0	NA
Charadriiformes	Gulls	Armenian Gull (*Larus armenicus*)	234	117	1	1x HPAI	0	0	0	NA	0	0	0	NA
		Black-headed gull (*Chroicocephalus ridibundus*)	1,036	518	0	NA	0	0	0	NA	8	2	1	1x HPAI
		Eurasian herring gull (*Larus argentatus*)	0	0	0	NA	0	0	0	NA	25	0	0	NA
		Great black-backed gull (*Larus marinus*)	0	0	0	NA	0	0	0	NA	6	2	3	2x HPAI 1xn.i.
		Mediterranean Gull (*Ichthyaetus melanocephalus*)	2	1	0	NA	0	0	0	NA	0	0	0	NA
		Mew gull (*Larus canus*)	2	1	0	NA	1	0	0	NA	1	0	0	NA
		Slender-billed Gull (*Chroicocephalus genei*)	4	2	0	NA	0	0	0	NA	0	0	0	NA
		Yellow-legged Gull (*Larus michahellis*)	282	141	0	NA	0	0	0	NA	0	0	0	NA
	Waders	Common snipe (*Gallinago gallinago*)	0	0	0	NA	2	0	0	NA	0	0	0	NA
Gaviiformes		Northern lapwing (*Vanellus vanellus*)	0	0	0	NA	2	0	0	NA	0	0	0	NA
		Arctic Loon (*Gavia arctica*)	2	1	0	NA	0	0	0	NA	0	0	0	NA
Gruiformes	Rails	Common moorhen (*Gallinula chloropus*)	0	0	0	NA	2	0	0	NA	1	0	0	NA
		Water rail (*Rallus aquaticus*)	0	0	0	NA	2	0	0	NA	0	0	0	NA
Podicipediformes		Eared Grebe (*Podiceps nigricollis*)	4	2	0	NA	0	0	0	NA	0	0	0	NA
		Great crested grebe (*Podiceps cristatus*)	2	1	0	NA	0	0	0	NA	0	0	0	NA
Procellariiformes		Levantine Shearwater (*Puffinus yelkouan*)	2	1	0	NA	0	0	0	NA	0	0	0	NA
Total			2,190	1,095	2		2,769	283	46		47	5	4	

No.: number; NA: not applicable; AIV: Avian influenza virus; HPAI: highly pathogenic avian influenza virus; n.i: not identifiable

Phylogenetic analyses of the HA segment

Previously, H5 clades have been somewhat geographically restricted with only intermittent incursion of Asian lineage viruses into Europe. However, here phylogenetic clustering of the Europe-derived H5N6 SOI was observed with H5N6 strains derived from Asia, such as those isolated from ducks in South Korea, a Black-faced Spoonbill *(Platalea minor)* in Taiwan, and a Mute Swan *(Cygnus olor)* in Japan in November/December 2017 (Asia-SOI). These latest H5N6 strains were also phylogenetically similar to recent European/Russian HPAI H5N8 viruses rather than Asian-derived H5N6 viruses such as the Chinese H5N6s that have been associated with zoonotic infections (Supplemental figure 1A). The recent, and to date, only, human H5N6 group B strain (27) (A/Fujian-Sanyuan/21099/2017(H5N6)) clustered closer to, but was still distinct from the recent European H5N6 strains (Figure 2, Supplemental figure 1A).

With the exception of two Vietnamese viruses (A/duck/Vietnam/QuangBinh/QN530206/2018 and A/chicken/Vietnam/QuangBinh/BoTrach1113/2017, figure 2 marked with §) that clustered together with the Chinese HPAI H5N6 viruses, both the European-SOI and Asian-SOI sets of H5N6 viruses had an estimated common ancestor that circulated in early July 2016 (95% confidence interval April – September 2016). The H5N6 virus isolated from a single poultry outbreak in Greece during the 2016-17 HPAI H5N8 epizootic (A/chicken/Greece/39_2017a/2017) clustered with other 2016-17 European H5N8 viruses, suggesting this strain was not ancestral to the SOI (Figure 2, Supplemental figure 1A).

However, additional heterogeneity was observed within the SOI. Phylogenetically clustering within the Europe-SOI were two Asia-SOI strains; these two Asia-SOI strains were isolated in the Republic of Georgia. The Republic of Georgia is located in western Central Asia, on the eastern side of the Black Sea, and here, these viruses showed a closer phylogenetic relationship with European strains than with Asian strains. The Europe-SOI and the Georgian strains had a common ancestor that circulated in early September 2017 (95% confidence interval July – October 2017), suggesting that there were likely multiple genetically distinct H5 segments, whose subsequent diffusion within the wild bird population was not uniform across Eurasia. Similar finer grain heterogeneity was observed with the four recent Asian strains, isolated from South Korea in late 2017 and early 2018. These four South Korean strains were phylogenetically closer to the European-SOI but distinct from other

recent Asian H5N6 2.3.4.4 group B viruses, isolated from birds in Japan, South Korea and Taiwan in late 2017/early 2018. The Korean and European viruses shared a common ancestor that circulated in July 2017 (95% confidence interval May – September 2017). This suggests that potentially two separate HA reassortment events led to heterogenous H5N6 viruses circulating within Eurasia from July 2016 and co-circulating in South Korea in late 2017 (Figure 2).

Phylogenetic analyses of the NA segment

Since the H5N6 HA arose from the recent H5N8 strains, attempts were made to trace the reassortment event or events that potentially led to the emergence of these H5N6 viruses. All N6 NA segments derived from LPAI and HPAI viruses from 2007-January 2018 were collected and combined with the SOI to trace the origin of N6. The closest genetically related N6 to all HPAI H5N6 strains appeared to be LPAI H4N6 strains from the Republic of Georgia in 2016 (Figure 3, Supplemental figure 1B).

BEAST analysis estimated that a common ancestor for all N6 genes related to HPAI H5 viruses that circulated in early January 2016 (albeit with a large 95% confidence interval between April 2015 to October 2016). The N6 segments of the Korean and European/Georgian viruses seemed to have diverged right at the beginning of 2017. The most recent common ancestor for the N6 related to only the European/Georgian HPAI H5N6 viruses that circulated in April 2017 (95% confidence interval December 2016 – August 2017). The N6 from the Greek H5N6 strain from February 2017 phylogenetically clustered alone and diverged from its closest relatives, some of the Asia-SOI, in late summer 2016, indicating that this Greek virus was an unrelated reassortment event that did not continue to circulate and was not ancestral to the currently circulating N6 genes related to the HPAI H5 viruses (Figure 3).

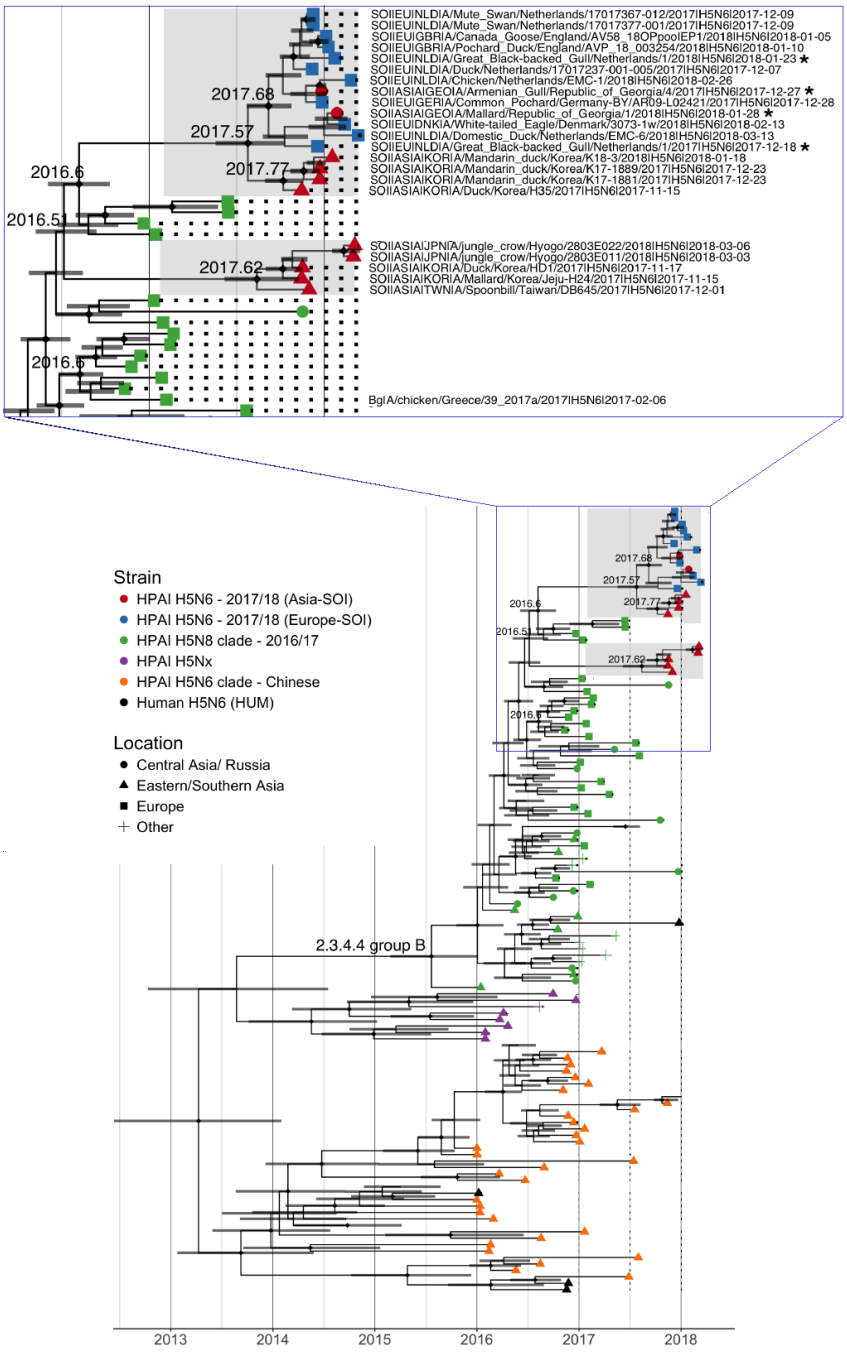

Figure 2. BEAST trees from viral sequences of HA (H5) gene sequences isolated from avian hosts between January 2016 and April 2018 with the addition of four H5N6 HA genes isolated from humans

and seven co-circulating HPAI H5N2/H5N8 viruses (purple). Tip symbols are coloured according to the HA origin displaying our European-SOI (blue), Asian-SOI (red), the Chinese HPAI H5N6 viruses (orange) with the Chinese human-derived viruses (black), the 2016-17 HPAI H5N8 viruses (green) and other HPAI H5Nx viruses (purple) . Tip symbols depict the location: Central Asia/Russia (●), Eastern/Southern Asia (▲), Europe (■) and other (+). Presence of node symbol (♦) indicates posterior probability > 0.85. The numbers above the nodes represent the time to most recent common ancestor (tMRCA), the grey bars display the accompanying 95% confidence interval. Viruses marked with an asterisk (*) were obtained and sequenced within the surveillance activities described in this manuscript. HPAI: highly pathogenic avian influenza. NB! Only a part of the tree is shown in detail for the purpose of printing it in this thesis. The original trees will be published online with the manuscript, or are available upon request.

Full genome phylogeny

BALTIC (backronymed adaptable lightweight tree import code) was used to compare the phylogenetic structure of the internal genes of the SOI compared to other HPAI H5 and LPAI viruses. Supplemental Figures 1A-H show the ML trees for all eight gene segments of the SOI together with a down-sampled set of all avian viruses isolated between 2007 and 2018. To visualise incongruence, the phylogenetic position of each sequence (coloured according to the origin of its HA) was traced across all seven trees (Figure 4). The eighth gene segment, NA, is excluded from this because not all viruses were N6 viruses, hence this tree would rather show the obvious genetic differences between different NA subtypes, than tracing the N6.

The HA of the recent European/Asian SOI clustered within the 2016/17 HPAI H5N8 cluster, and the NA segments were most closely related to LPAI N6s, as described above (Figures 2 and 3, Supplemental figure 1A and 1B). A/Mallard/Republic_of_Georgia/1/2018 (H5N6) showed a closer relationship to recent European viruses than to Asian viruses for all eight gene segments (Figure 4, Supplemental figure1). The MP, NP, NS and PB1 segments of recent Asian and European SOI were related to respective internal genes from 2016-17 clade 2.3.4.4 (H5N8) strains (Supplemental figure 1). In contrast, although A/Armenian_Gull/Republic_of_Georgia/4/2017 (H5N6) was closely related to the European H5N6 viruses for the HA and NA segments, all of its internal genes clustered with those of LPAI and other HPAI H5 viruses, indicating extensive reassortment (Figure 2-4, Supplemental figure 1).

For PA, SOI from Europe and the Republic of Georgia (except A/Great_Black-backed_Gull/Netherlands/1/2017) phylogenetically clustered with LPAI H7, H3 and H4

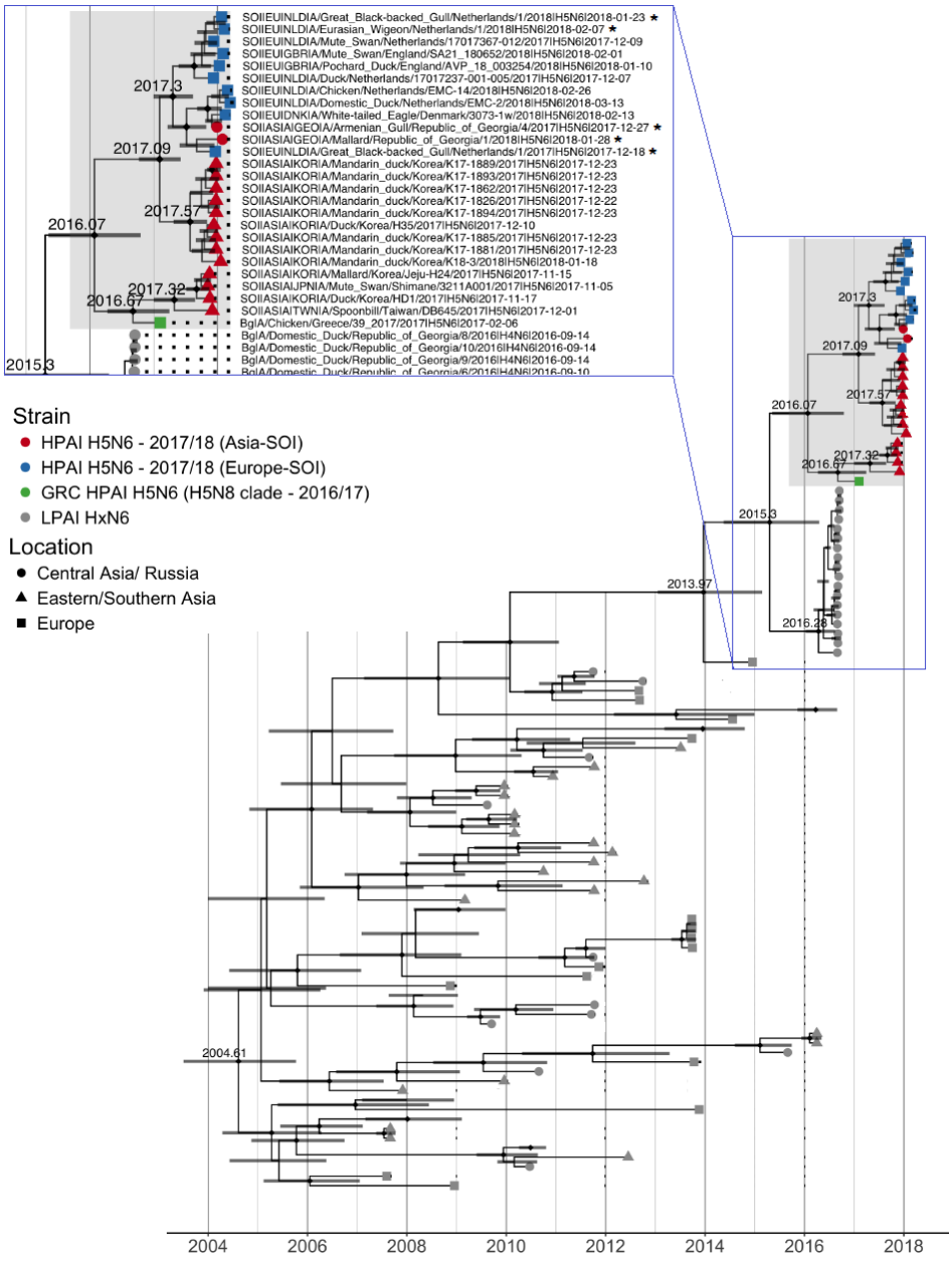

Figure 3. BEAST trees from viral sequences of NA (N6) gene sequences isolated from avian hosts between January 2007 and April 2018. Tip symbols are coloured according to the HA origin with recent Asian (red) and European (blue) HPAI H5N6 viruses, the early 2017 Greek (GRC) HPAI H5N6 (green), and (non-H5) HxN6 (grey) viruses. Symbols depict the location: Central Asia/Russia (●), Eastern/Southern Asia (▲) and Europe (■). Presence of node symbol (♦) indicates posterior probability > 0.85. The numbers above the nodes represent the time to most recent common

ancestor (tMRCA), the grey bars display the accompanying 95% confidence interval. Viruses marked with an asterisk (*) were obtained and sequenced within the surveillance activities described in this manuscript. HPAI: highly pathogenic avian influenza. NB! Only a part of the tree is shown in detail for the purpose of printing it in this thesis. The original trees will be published online with the manuscript, or are available upon request.

viruses from Bangladesh, the Netherlands and the Republic of Georgia from 2014 to 2016. Phylogenetically distinct from this PA group, the A/Great_Black-backed_Gull/Netherlands/1/2017 and all Asia-SOI (except the H5N6 virus from the Republic of Georgia) clustered together with 2016/17 Russian/European HPAI H5N8 viruses. These different PA clusters likely represent separate reassortment events. (Supplemental figure 1F).

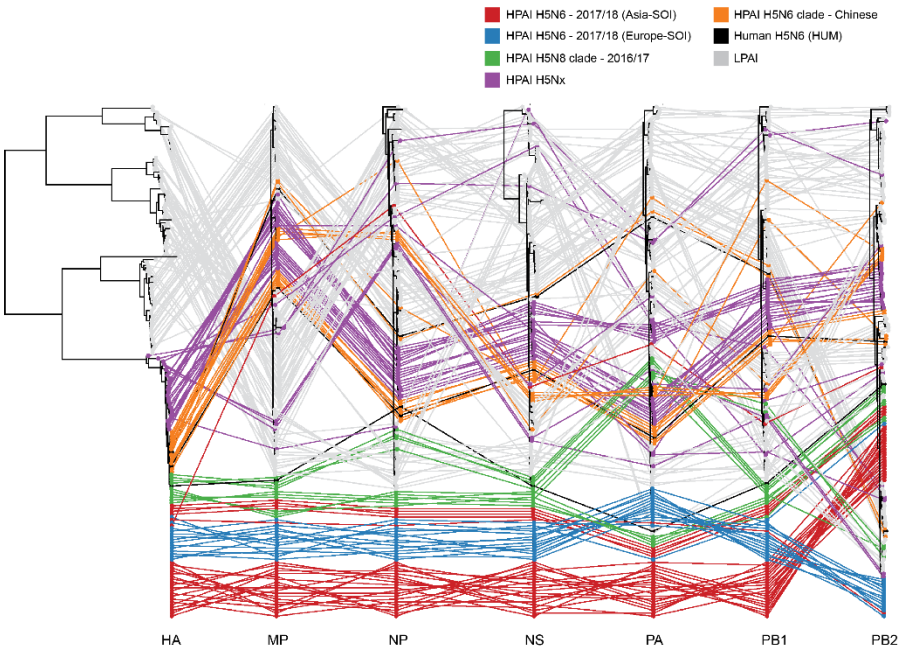

Figure 4. Phylogenetic incongruence analysis. Maximum likelihood trees for the HA segment and all internal genes MP, NP,NS,PA,PB1 and PB2 from equivalent strains were connected across the trees. Tips and connecting lines are coloured according to HA clade. HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza.

Phylogeny based on the PB2 gene resulted in similar clustering patterns as compared with PA. However, the closest-related PB2 genes to the Europe-SOI/Republic of Georgia cluster arose from viruses that circulated in 2014-15 in domestic birds in Europe. In contrast, PB2 segments of the Asia-SOI and A/Great_Black-backed_Gull/Netherlands/1/2017 clustered with PB2 segments from 2016/17 Russian/European H5N8 (Supplemental figure 1H). None of the H5N6 SOI gene segments were associated with respective segments found in or near the Chinese group C and D viruses (Figure 4). However, the recent group B human H5N6 virus (A/Fujian-Sanyuan/21099/2017(H5N6)) isolated in China in 2017 was more similar to HPAI H5N8 clade viruses with a PA segment that is closely related to both HPAI H5N8 clade and HPAI H5N6 European clade viruses.

Discussion

New reassortant HPAI clade 2.3.4.4 group B H5N6 viruses have been detected in both wild birds and poultry in several European and Asian countries from December 2017 onwards. Phylogenetic analyses of these viruses showed that although they were related to the 2016-17 European HPAI H5N8 viruses, they were not the result of continued circulation of the single HPAI clade 2.3.4.4 group B H5N6 virus that was detected in Greece in early 2017 during the 2016-17 clade 2.3.4.4 group B H5N8 European outbreak. Based on these results, we conclude that these European and some recent Asian HPAI H5N6 viruses are new reassortant viruses of HPAI H5 clade 2.3.4.4 group B viruses with LPAI virus segments likely derived from wild birds rather than from LPAI viruses in poultry or from introductions of the (zoonotic) Asian HPAI H5N6 viruses that have been frequently detected in Southeast Asia since 2014. The common ancestors of the HA and NA gene segments of the recent European HPAI H5N6 viruses were estimated to have circulated in early September 2017 (confidence interval: July - October) and April 2017 (confidence interval: December 2016 – August 2017), respectively. Previously published time estimates for the most recent common ancestors for these HPAI H5N6 viruses detected in the Netherlands (23) and Europe/Korea (47) were January – September 2016 and January – October 2016 for HA, and December 2014 – July 2016 and September 2015 (confidence interval: August 2014 – August 2016) for NA, respectively. These estimates are different from these new estimates and with much broader confidence intervals. This can be explained by the more extensive dataset in the present study, including both a representative set of circulating HPAI H5 viruses and a large number of LPAI viruses, that enabled us to narrow the time estimates of when reassortment events with other viruses may have occurred.

Although wild birds are generally not considered as a long-term maintenance reservoir for HPAI viruses, bird diversity, density, the aggregation of birds from different geographical areas and the high density of immune-naïve birds on breeding sites can all contribute to both temporary HPAI virus maintenance and to the potential generation of novel reassortants with LPAI gene segments. Previous phylogenetic studies have shown that both HPAI clade 2.3.4.4 H5N8 viruses that caused outbreaks in Europe in 2014-15 and 2016-17 originated in Southeast Asia and subsequently spread to Europe (1, 10), with diffusion mediated by wild birds (1). However, in contrast to previous epizootics where an HPAI H5 progenitor was derived from poultry and diffused in wild birds, it is more plausible that these recent H5N6 viruses evolved during 2017 by reassortment of a European HPAI H5N8 virus and wild host reservoir LPAI viruses, within the wild bird reservoir itself, given the more restricted gene pool in poultry. We therefore hypothesize that these reassortment events occurred either during 2017 on the Palearctic breeding grounds where birds from Europe and Asia gather when a large number of hatch-year birds entered the wild bird population, or just after breeding, when naïve hatch-year birds and adults dispersed from the breeding grounds to aggregate in large numbers, particularly to moult (48).

Previously, differences in mortality rates were found in HPAI H5 epizootics mediated by wild birds (14). Here, the combination of enzootic presence of LPAI viruses, a large immunologically heterogenous population, with possible pre-existing immunity to clade 2.3.4.4 group B viruses from the previous HPAI waves, and the start of migration might have enabled multiple novel reassortants to emerge and to disperse both east and west to the wintering grounds, resulting in co-circulation of reassortants of different genotypes in both Europe and East Asia. The timing of the first detection of the new H5N6 viruses was later (mid-December) (23) compared with both H5N8 outbreaks (early November (49) and late October (8) respectively) but the variation in detection was likely within normal ecological variation of wild bird movements and population dynamics. Possible ecological and population-based explanations for such outbreak timing variation include the presence of pre-existing immunity to clade 2.3.4.4 group B viruses possibly leading to less severe infections, the potential for partial immunity to facilitate co-infection and recovery rather than mortality, with reassortment and onwards transmission resulting from these altered population factors. Additionally partial or pre-existing immunity might lead to a relative reduction in disease burden and the number of infected birds, fewer infected wild birds without obvious increased mortality, differences in climatic factors between years that influenced the dispersal of wild birds (50) and thus relative prevalence, or the alteration of the structure and size of the wild bird population by the previous incursion (9) and the relative proportion of susceptible birds in the population differed across years.

Although some HPAI H5 viruses were associated with mortality in wild birds (9, 10, 51, 52), serological evidence and the detections of HPAI H5 viruses in clinically healthy birds (53-55) indicate that infections can be non-lethal, enabling the spread of these viruses over longer distances with flight. To the authors' knowledge all previous reports on HPAI H5N6 detections in Europe have been from either diseased or dead birds. Here, the detection of HPAI H5N6 virus from a clinically healthy Armenian Gull (December 2017) and a Mallard (January 2018) in the Republic of Georgia, and in clinically healthy Eurasian Wigeons in February 2017 in the Netherlands are reported. Wild birds are considered to be a temporary spill-over host for HPAI viruses that originate from poultry, and HPAI viruses are generally considered to be unable to become endemic in the wild bird population. The present results and those previously published (23, 47) indicate that the recent HPAI clade 2.3.4.4 group B H5N6 viruses were reassortants from several wild bird viruses, and time estimates for these reassortment events suggested that reassortment happened in wild birds without the involvement of poultry. In addition, HPAI H5N6 viruses have been detected in wild birds in Europe over the course of 2018, although there were no detections in commercial poultry after March 2018, nor in backyard poultry between March and September 2018 (56, 57). These viruses may indeed have adapted to wild birds by causing non-lethal infections, and thereby enabled themselves to be maintained in the wild bird population. To date the precise wild bird species involved in the long-distance dispersal of these viruses are still unknown, but with expanding genetic heterogeneity in both wild birds and poultry the risk of diffusion of HPAI variants among geographic areas is an ever-present threat.

Clade 2.3.4.4 group A and B H5N8 viruses have not been associated with human infections, and pathogenesis and transmission studies in ferrets showed that these viruses are not of current concern for human health (58-60). In contrast, clade 2.3.4.4 group B, C and D H5N6 viruses have been reported to cause human infections in China (26, 61), and show a high genetic variability (17). Based on the present phylogenetic results, all eight gene segments of the currently circulating European and most Asian H5N6 viruses, with the exception of some Chinese and Vietnamese viruses, clustered with either HPAI H5N8 or LPAI viruses, and showed a clear separation from the human clade 2.3.4.4 group C and D viruses. Recently the WHO reported a human infection with a clade 2.3.4.4 group B virus (A/Fujian-Sanyuan/21099/2017(H5N6)) (27) though this is to date, a single isolation. However, the evolutionary dynamics characterized in this study highlight the potential for rapid virus evolution, alterations in host susceptibility even within an H5 subclade and demonstrate the necessity to continually assess the risk of emerging variants to both animal and human health. It is also important to understand the underlying principles and drivers that enable global HPAI virus migration through both active

and passive, ecologically-targeted and longitudinal surveillance in wild birds and poultry.

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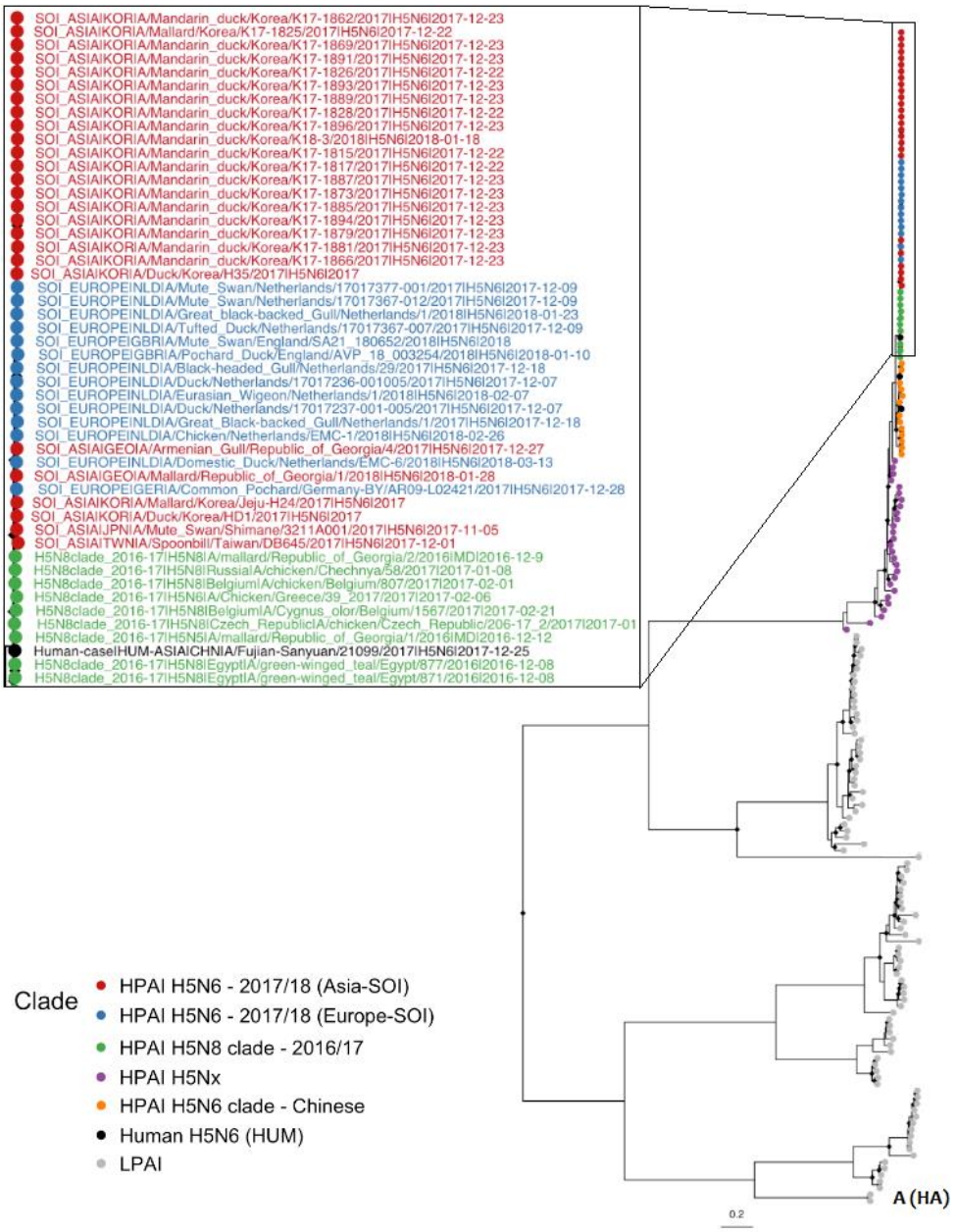
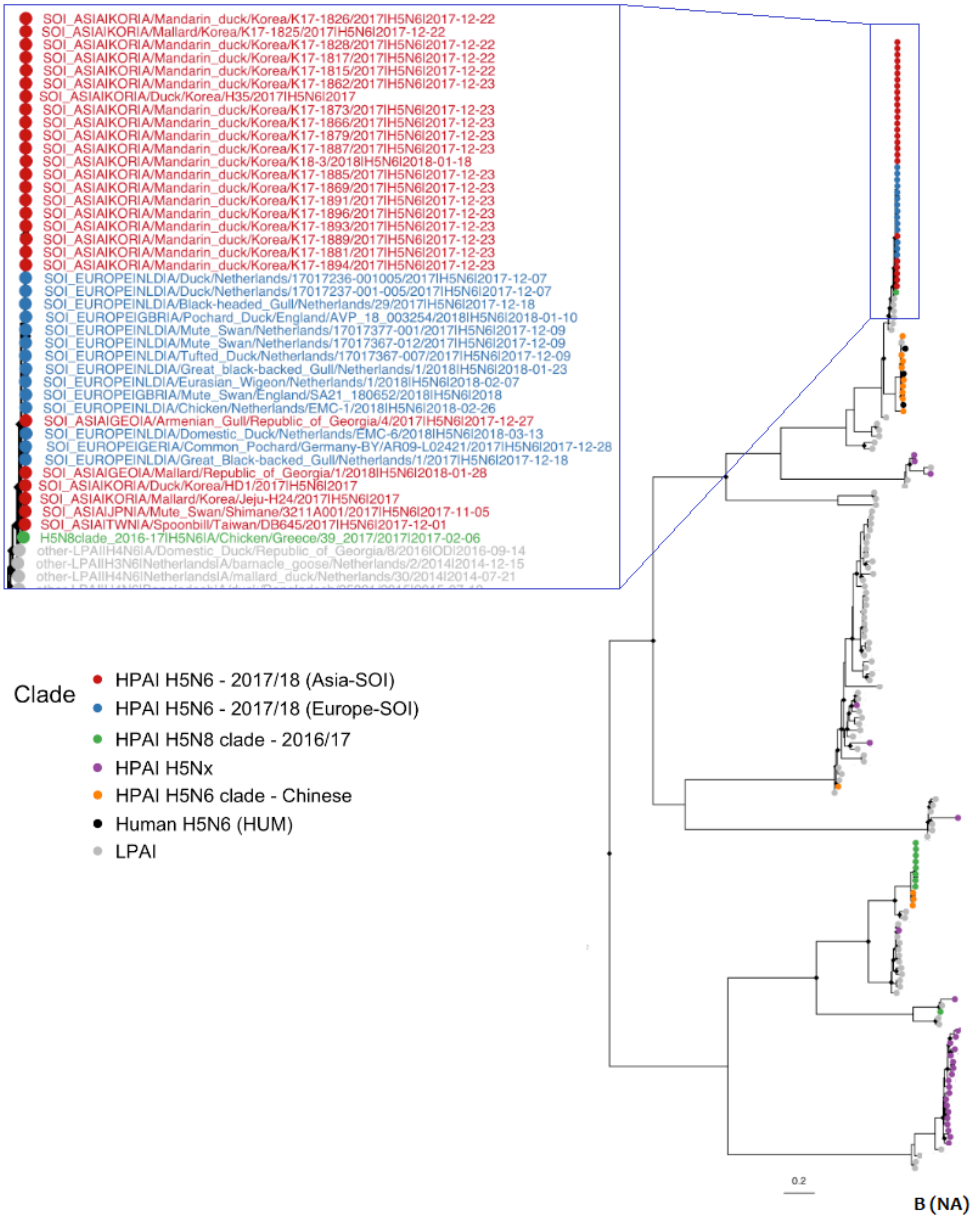
Supplemental material

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HPAI H5N6 - 2017/18 (Europe-SOI)
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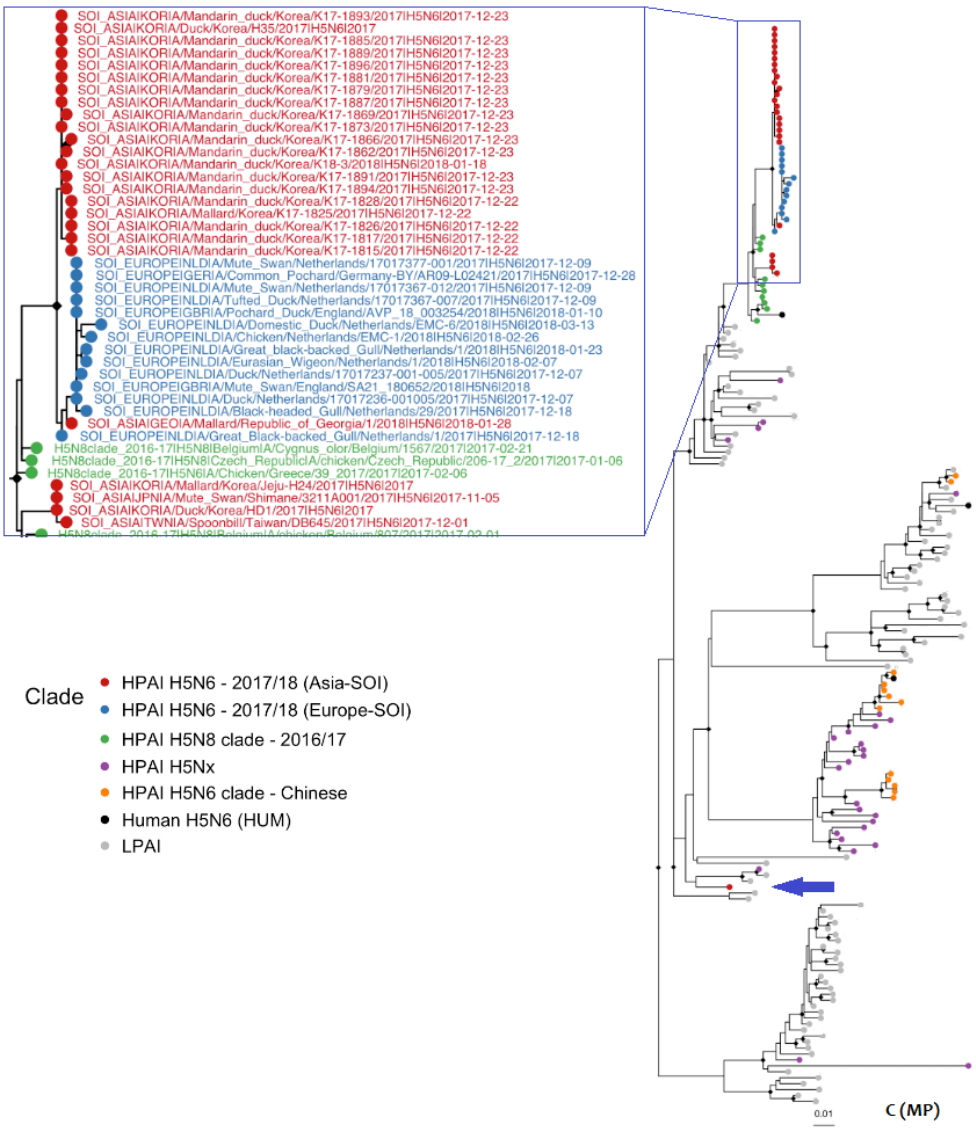
LPAI
0.2

B (NA)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SOI_ASIA|KOR|A/Mandarin_duck/Korea/K17-1879/2017|H5N6|2017-12-23
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SOI_ASIA|KOR|A/Mandarin_duck/Korea/K18-3/2018|H5N6|2018-01-18
SOI_ASIA|KOR|A/Mandarin_duck/Korea/K17-1891/2017|H5N6|2017-12-23
SOI_ASIA|KOR|A/Mandarin_duck/Korea/K17-1894/2017|H5N6|2017-12-23
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SOI_EUROPE|NLD|A/Mute_Swan/Netherlands/17017377-001/2017|H5N6|2017-12-09
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SOI_EUROPE|NLD|A/Chicken/Netherlands/EMC-1/2018|H5N6|2018-02-26
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SOI_ASIA|KOR|A/Duck/Korea/HD1/2017|H5N6|2017
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Clade
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HPAI H5N6 - 2017/18 (Europe-SOI)
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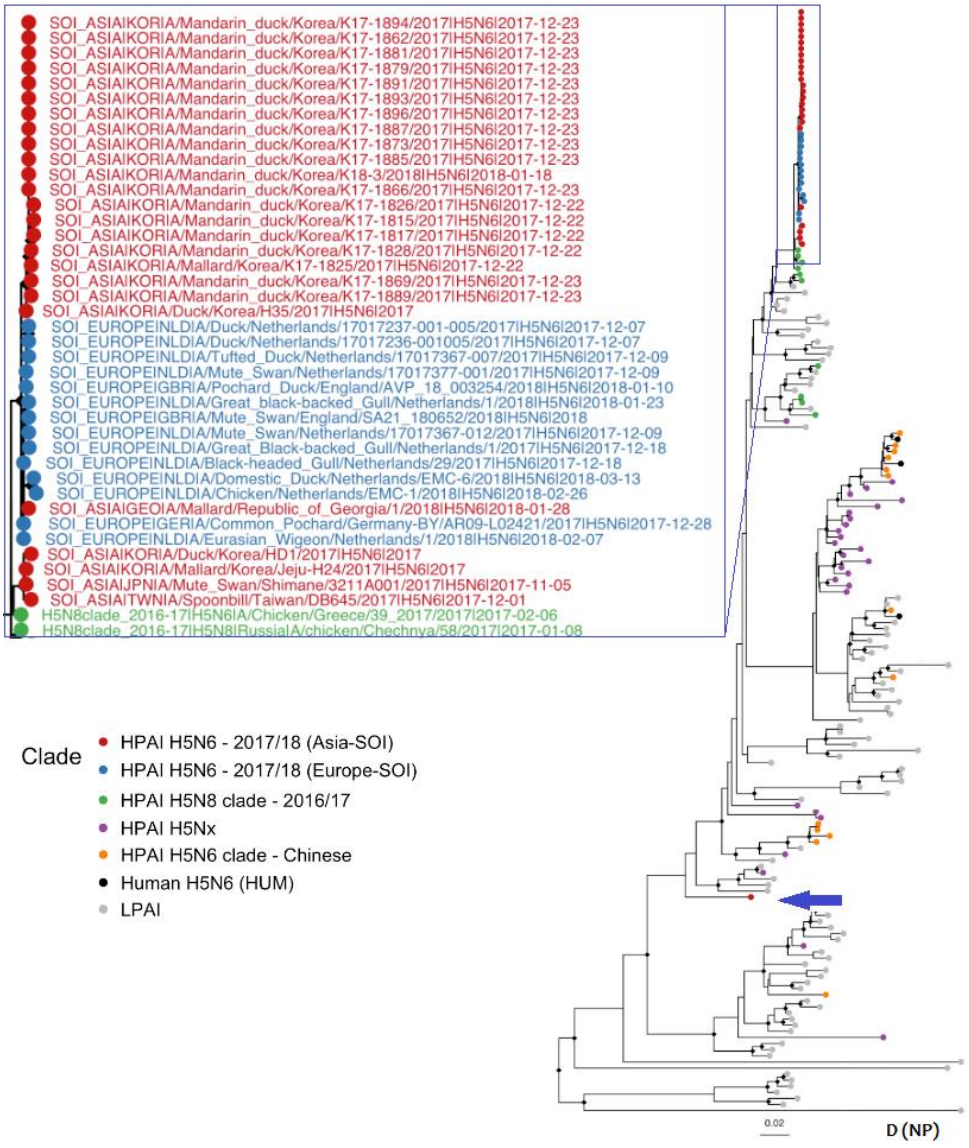
HPAI H5Nx
HPAI H5N6 clade - Chinese
Human H5N6 (HUM)
LPAI
0.01
C (MP)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Clade
HPAI H5N6 - 2017/18 (Asia-SOI)
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HPAI H5Nx
HPAI H5N6 clade - Chinese
Human H5N6 (HUM)
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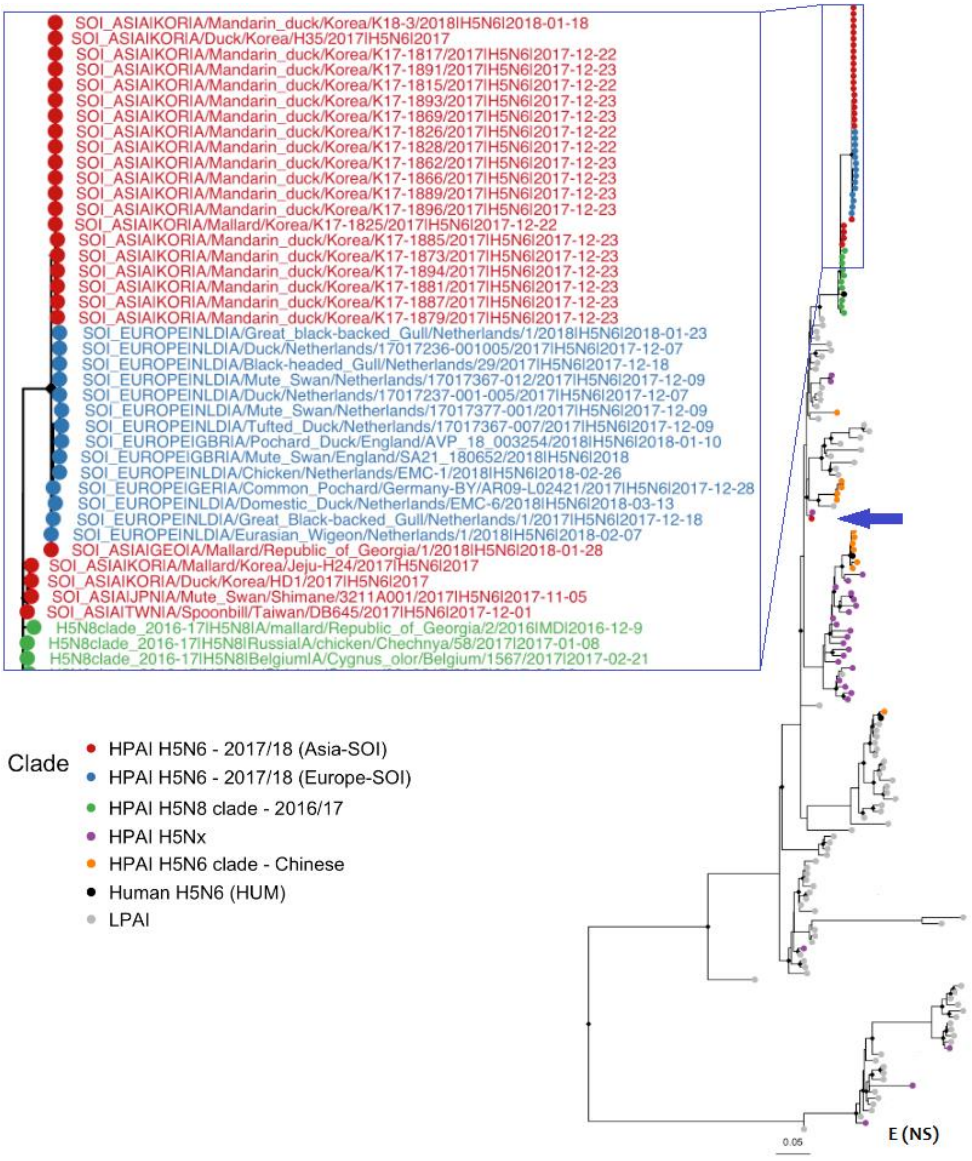
0.02
D (NP)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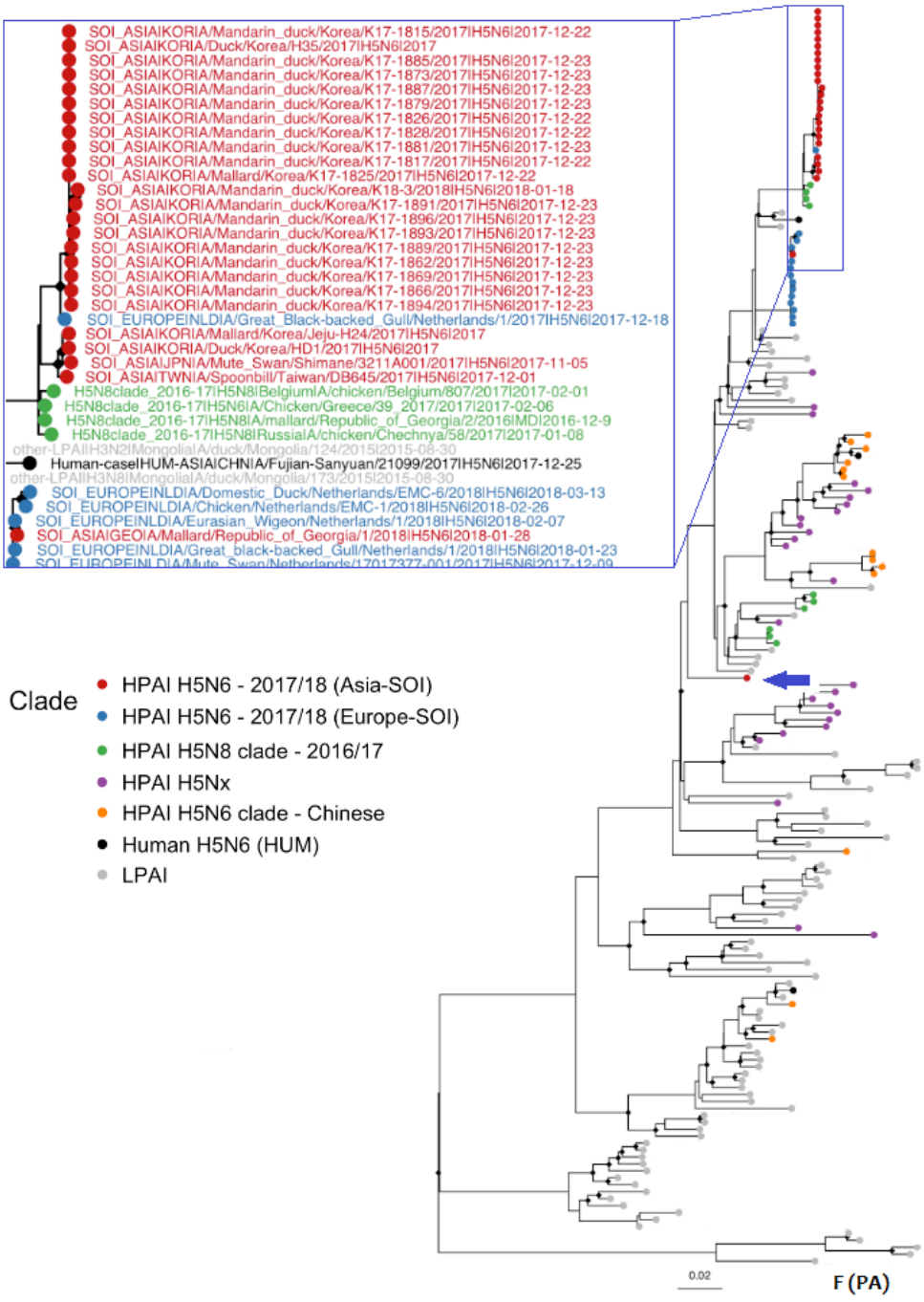
Clade
HPAI H5N6 - 2017/18 (Asia-SOI)
HPAI H5N6 - 2017/18 (Europe-SOI)
HPAI H5N8 clade - 2016/17
HPAI H5Nx
HPAI H5N6 clade - Chinese
Human H5N6 (HUM)
LPAI
0.05
E (NS)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SOI_ASIA|KOR|A/Mallard/Korea/K17-1825/2017|H5N6|2017-12-22
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SOI_ASIA|KOR|A/Mallard/Korea/Jeju-H24/2017|H5N6|2017
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Clade
HPAI H5N6 - 2017/18 (Asia-SOI)
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HPAI H5N6 clade - Chinese
Human H5N6 (HUM)
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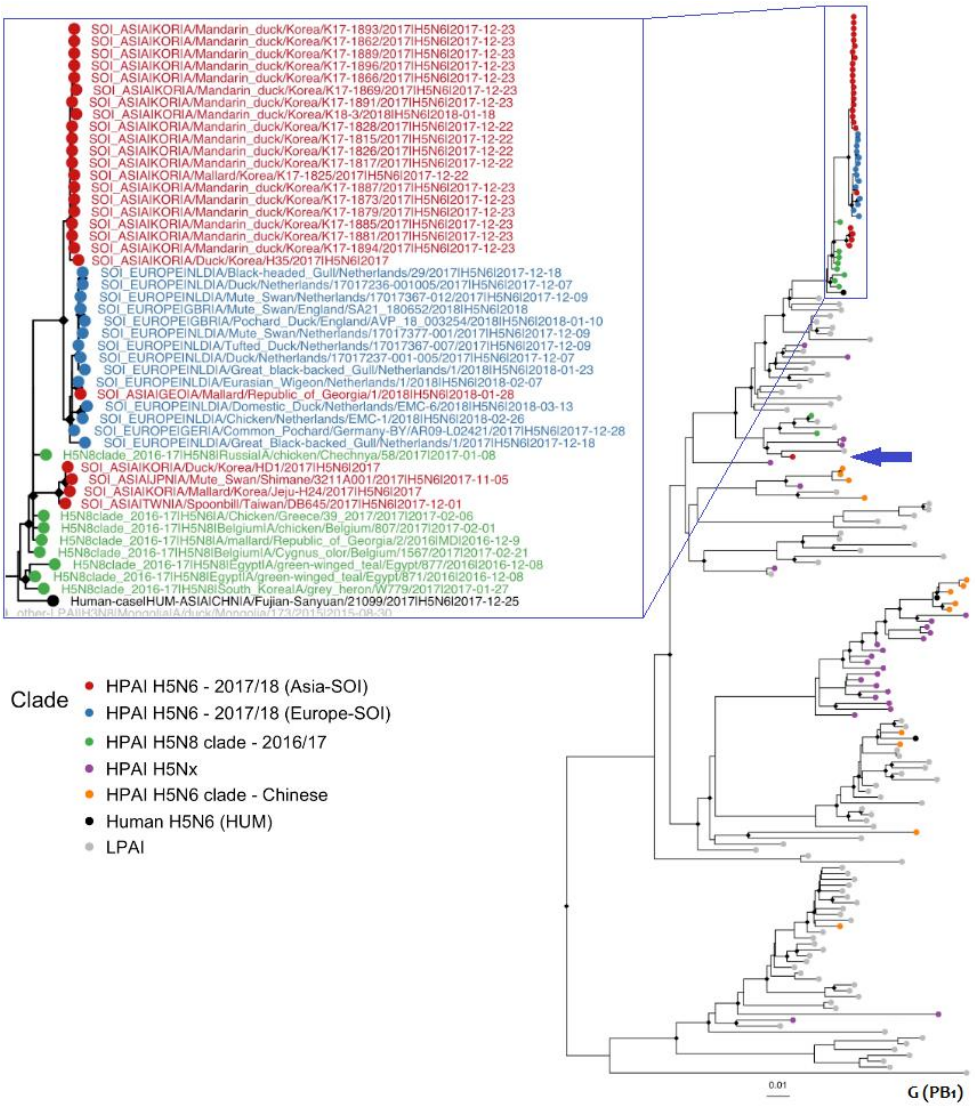
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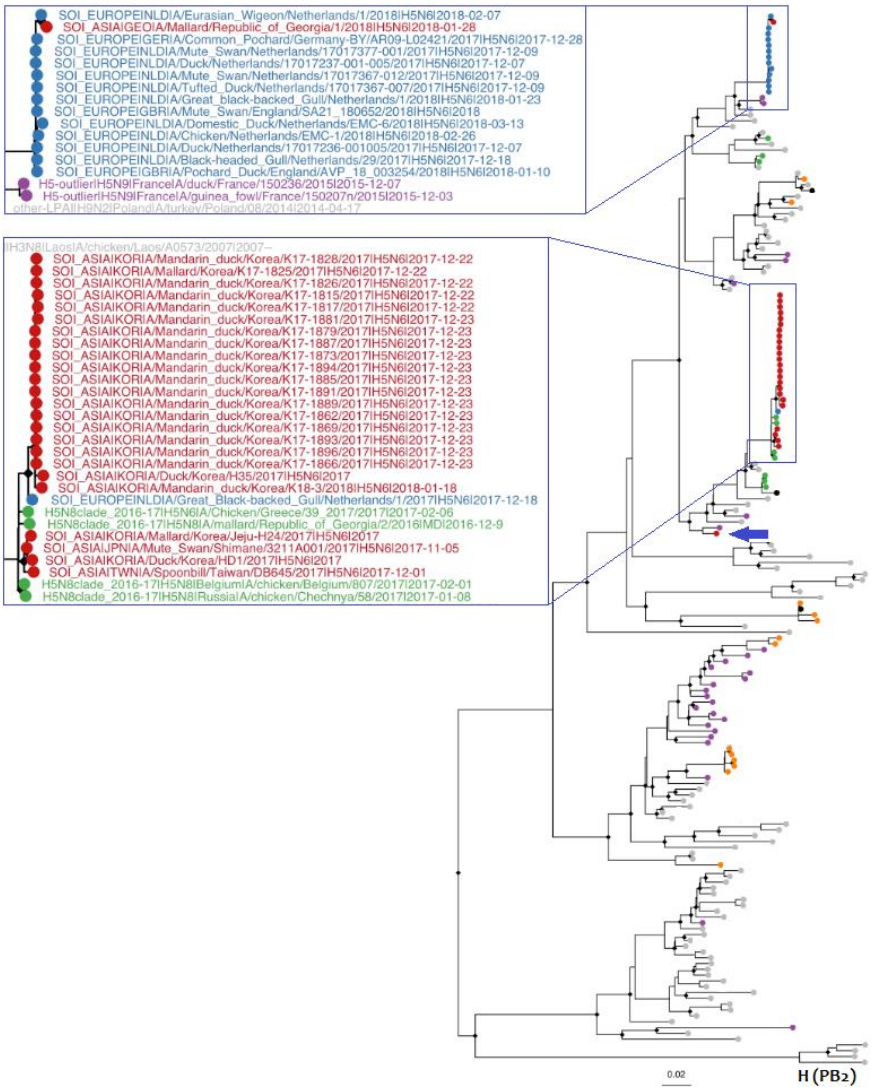
SOI_ASIA|KOR|A/Duck/Korea/H35/2017|H5N6|2017
SOI_EUROPE|NLD|A/Black-headed_Gull/Netherlands/29/2017|H5N6|2017-12-18
SOI_EUROPE|NLD|A/Duck/Netherlands/17017236-001005/2017|H5N6|2017-12-07
SOI_EUROPE|NLD|A/Mute_Swan/Netherlands/17017367-012/2017|H5N6|2017-12-09
SOI_EUROPE|GBR|A/Mute_Swan/England/SA21_180652/2018|H5N6|2018
SOI_EUROPE|GBR|A/Pochard_Duck/England/AVP_18_003254/2018|H5N6|2018-01-10
SOI_EUROPE|NLD|A/Mute_Swan/Netherlands/17017377-001/2017|H5N6|2017-12-09
SOI_EUROPE|NLD|A/Tufted_Duck/Netherlands/17017367-007/2017|H5N6|2017-12-09
SOI_EUROPE|NLD|A/Duck/Netherlands/17017237-001-005/2017|H5N6|2017-12-07
SOI_EUROPE|NLD|A/Great_black-backed_Gull/Netherlands/1/2018|H5N6|2018-01-23
SOI_EUROPE|NLD|A/Eurasian_Wigeon/Netherlands/1/2018|H5N6|2018-02-07
SOI_ASIA|GEO|A/Mallard/Republic_of_Georgia/1/2018|H5N6|2018-01-28
SOI_EUROPE|NLD|A/Domestic_Duck/Netherlands/EMC-6/2018|H5N6|2018-03-13
SOI_EUROPE|NLD|A/Chicken/Netherlands/EMC-1/2018|H5N6|2018-02-26
SOI_EUROPE|GER|A/Common_Pochard/Germany-BY/AR09-L02421/2017|H5N6|2017-12-28
SOI_EUROPE|NLD|A/Great_Black-backed_Gull/Netherlands/1/2017|H5N6|2017-12-18
H5N8clade_2016-17|H5N8|Russia|A/chicken/Chechnya/58/2017|2017-01-08
SOI_ASIA|KOR|A/Duck/Korea/HD1/2017|H5N6|2017
SOI_ASIA|JPN|A/Mute_Swan/Shimane/3211A001/2017|H5N6|2017-11-05
SOI_ASIA|KOR|A/Mallard/Korea/Jeju-H24/2017|H5N6|2017
SOI_ASIA|TWN|A/Spoonbill/Taiwan/DB645/2017|H5N6|2017-12-01
H5N8clade_2016-17|H5N6|A/Chicken/Greece/39_2017/2017|2017-02-06
H5N8clade_2016-17|H5N8|Belgium|A/chicken/Belgium/807/2017|2017-02-01
H5N8clade_2016-17|H5N8|A/mallard/Republic_of_Georgia/2/2016|MDI|2016-12-9
H5N8clade_2016-17|H5N8|Belgium|A/Cygnus_olor/Belgium/1567/2017|2017-02-21
H5N8clade_2016-17|H5N8|Egypt|A/green-winged_teal/Egypt/877/2016|2016-12-08
H5N8clade_2016-17|H5N8|Egypt|A/green-winged_teal/Egypt/871/2016|2016-12-08
H5N8clade_2016-17|H5N8|South_Korea|A/grey_heron/W779/2017|2017-01-27
Human-case|HUM-ASIA|CHN|A/Fujian-Sanyuan/21099/2017|H5N6|2017-12-25
Clade
HPAI H5N6 - 2017/18 (Asia-SOI)
HPAI H5N6 - 2017/18 (Europe-SOI)
HPAI H5N8 clade - 2016/17
HPAI H5Nx
HPAI H5N6 clade - Chinese
Human H5N6 (HUM)
LPAI
0.01
G (PB1)



Supplemental figure 1. Maximum likelihood tree for all 8 gene segments namely HA, NA, MP, NP, NS, PA, PB1, PB2 (A-H respectively). Tips are coloured according to HA clade: The Europe-SOI (blue), Asia-SOI (red), Chinese (orange) and human (black) HPAI H5N6 viruses, 2016-17 H5N8 viruses (green), other HPAI H5 viruses (purple) and LPAI viruses (grey). Node symbols (♦) are shown only where support value (alrt – approximate likelihood ratio test) is above 85%. The blue arrow in C-H point at A/Armenian_Gull/Republic_of_Georgia/1/ 18. NB! Only parts of the trees are shown in detail for the purpose of printing them in this thesis. The original trees will be published online with the manuscript, or are available upon request.

Supplemental Table 1. Acknowledgements of authors, and originating and submitting laboratories providing the sequences to Gisaid used for phylogenetic analysis. NB! Supplemental Table 1 is not shown due to the size. It will be published online with the manuscript.

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Chapter 4

The applicability of next-generation sequencing in outbreak situations

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Chapter 4

Comparison of sequencing methods and data processing pipelines for whole genome sequencing and minority single nucleotide variant (mSNV) analysis during an influenza A/H5N8 outbreak

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Abstract

As high-throughput sequencing technologies are becoming more widely adopted for analysing pathogens in disease outbreaks there needs to be assurance that the different sequencing technologies and approaches to data analysis will yield reliable and comparable results. Conversely, understanding where agreement cannot be achieved provides insight into the limitations of these approaches and also allows efforts to be focused on areas of the process that need improvement. This manuscript describes the next-generation sequencing of three closely related viruses, each analysed using different sequencing strategies, sequencing instruments and data processing pipelines. In order to determine the comparability and repeatability of consensus sequences and minority (sub-consensus) single nucleotide variants (mSNV) identification the biological samples, the sequence data from 3 sequencing platforms and the *.bam quality-trimmed alignment files of raw data of 3 influenza A/H5N8 viruses were shared. This analysis demonstrated that variation in the final result could be attributed to all stages in the process, but the most critical were the well-known homopolymer errors introduced by 454 sequencing, and the alignment processes in the different data processing pipelines which affected the consistency of mSNV detection. However, homopolymer errors aside, there was generally a good agreement between consensus sequences that were obtained for all combinations of sequencing platforms and data processing pipelines. Nevertheless, minority variant analysis needs a different level of careful standardization and awareness about the possible limitations, as shown in this study.

Introduction

Over the past decade, high-throughput sequencing technologies have evolved, providing faster, cheaper, and less laborious alternatives to obtain (whole genome) DNA and RNA sequences compared to traditional Sanger sequencing [1, 2]. The use of next-generation sequencing (NGS) technologies is continuously expanding and has revolutionized the field of genomics and molecular biology.

In many fields of infectious disease research, nucleotide changes in DNA or RNA sequences are used to monitor genetic adaptions indicative of evolution, the emergence of drug resistance, immune evasion or as a tool in epidemiological tracing [3]. In clinical settings, sequencing information is used to improve diagnostics and prognosis. NGS technologies play an increasingly important role in these processes as clinically or epidemiologically important nucleotide changes can be present in the minority of DNA or RNA sequences only, which might be missed with more traditional (consensus) sequencing methods which determine the most abundant sequence variants in a population. Minority Single Nucleotide Variants (mSNVs) are present in less than 50% of the sequenced virus population. These variants, initially occurring due to replication errors, can become fixed in the population when they have some sort of evolutionary advantage, for instance, mutations related to drug resistance. Furthermore, mSNVs can be also used for high-resolution molecular epidemiology, which becomes more and more important for outbreak assessment [4, 5]. Traditional Sanger sequencing for instance has been described to detect minority variants provided they are present in at least 10% of the analysed DNA or RNA strands within a sample [6, 7]. Hence, the use of traditional sequencing methods is usually restricted to obtaining consensus sequences or to determine heterozygosity in diploid organisms. In contrast, NGS technologies are able to detect low frequency mSNVs in sequence fragments or even whole genomes. Typically, NGS sensitivity for minority sequence variant identification is restricted to a level of variation of 0.1–1%, mainly due to sequencing related background errors [8-10], but sensitivity can be increased using sophisticated approaches like circle sequencing [11] or improved bioinformatic analysis workflows [10]. The reliability of mSNV analysis using NGS methods is influenced by many factors, like the quantity and quality of the input sample, the laboratory procedures, the type of sequencing platform and the software and settings used to analyse the raw sequence data.

Due to the technical improvements, NGS technologies have become more important as diagnostic tools to characterize pathogens in outbreak situations. However, the increasing use of these technologies to address new and important (outbreak related) research and surveillance questions emphasizes the need to determine the reproducibility of, and the important technical considerations affecting, outcomes obtained by different laboratories following different protocols. Given this, comparative studies focusing on different platforms and data analysis methods are

essential to cross-validate different methodologies and determine the reliability of newly obtained data. In addition, there is a growing need (as exemplified by the recent Ebola and Zika virus outbreaks) to share also comprehensive sequencing data as quickly as possible to help with source attribution and developing control strategies. However, the underlying technologies and methods used for NGS are still diverse and there is a strong demand for harmonization of laboratory procedures and approaches for a reliable and optimized analysis of the data.

This study is part of the European Union's HORIZON 2020 project "COMPARE" (http://www.compare-europe.eu/), aiming to improve the analytical tools for emerging zoonotic pathogens and its underpinning research. Here, both comparability and repeatability of NGS output data obtained from different sequence approaches were evaluated and demonstrated suitable sharing strategies for comprehensive NGS data sets. In November 2014, a newly emerging strain of highly pathogenic avian influenza (HPAI) virus was detected in several European countries [12, 13]. In the United Kingdom [14], Germany [15], and The Netherlands [16-18] this subtype was detected in commercial poultry farms within a few days of one another. In each of those countries, NGS was used to generate whole-genome sequences rapidly after detection, but as the laboratories in each country were working independently, different approaches were used for both sequencing and data analysis, and the data were shared as part of a wider study to determine the likely source of the outbreak [19]. It is important to determine whether the different analytical approaches have any impact on the outcome. Therefore, the aim of this study was to determine how comparable consensus and minority variant results were between laboratories performing their standard analyses, and whether discrepancies could be attributed to the SP, DPP or a combination of both. With the lack of a ground truth/gold standard, all datasets obtained were compared amongst each other. The hypothesis we test in this study is that outputs from NGS analysis of viruses will be comparable irrespective of laboratory, sequencing platform and data analysis platform.

Therefore, virus isolates obtained in each of the three countries (United Kingdom, Germany and the Netherlands) were shared between these three partners and subsequently sequenced and analysed in each of the three laboratories according to local procedures. In addition, the use of a specially designed data sharing platform, a COMPARE "Data Hub" at EMBL-EBI, Hinxton UK, was evaluated. This study presents genome coverage data, consensus sequences, the analysis of the comparability and repeatability of mSNV identifications of the different sequence platforms (SPs), and data processing pipelines (DPPs).

Our hypothesis was confirmed at the consensus sequence level, since consensus sequences could be reproduced independent of the combination of SP and DPP

used. However, the identification of minority variants appeared to be poorly reproducible, primarily due to the well-known errors in 454 sequencing, and due to differences induced by the alignment processes in the different DPPs. The interpretation of minority variant analysis thus needs a different level of careful standardization and awareness about the possible limitations as shown in this study.

Materials and Methods

Experimental design

Three avian influenza A virus isolates that were obtained from three different avian species during the 2014/15 outbreak of HPAI H5N8 virus in Europe were shared among three institutions in the United Kingdom (Animal Plant and Health Agency [APHA]), Germany (Friedrich-Loeffler-Institut [FLI]) and the Netherlands (Erasmus Medical Center [EMC]), later referred to as anonymized institutions I, II and III (Figure 1). All three institutions sequenced all three virus isolates according to their own standard procedures. Adaptors used in the sequencing processes were trimmed off before the raw sequence data files were shared. The sequence data files (*.fastq files), alignment files (*.bam files), sample metadata and experimental metadata were shared between the three laboratories and analysed in their own DPPs yielding sequence datasets for each virus (Table 1). Data sharing was facilitated via a "Data Hub" provided by the EMBL-EBI's European Nucleotide Archive (ENA) in the framework of the COMPARE collaborative project; all data were stored and subsequently published in ENA [20] (https://www.ebi.ac.uk/ena, for the accession numbers, see Table 1). ENA is an open repository for sequence and related data and a member of the International Nucleotide Sequence Database Collaboration (INSDC; http://www.insdc.org/) [21]. A full description of the COMPARE Data Hub system is provided in a preprint version of Amid et al. [22]. First, consensus sequences derived from a preliminary analysis were compared and one overarching consensus sequence was determined for each gene segment for each virus. This custom-made consensus was used by all three institutions as the reference genome for undertaking mSNV analysis. The resulting nine mSNV reports (originating from three whole-genome raw data sequences times three DPPs) were combined for all three viruses to check the reproducibility of mSNV identification when using different combinations of SP and DPP. The experimental design is summarized in figure 1.

Samples

All samples were obtained from outbreaks in commercial poultry holdings. Isolate A/duck/England/36254/2014 was obtained from pooled intestinal material from index case ducks (Anas platyrhynchos domesticus). Tissue homogenate material was

inoculated into embryonated chicken eggs and allantoic fluid was harvested at 1 day post-inoculation [14]. The Dutch isolate (A/chicken/Netherlands/EMC-3/2014) was obtained by passaging lung material of a dead commercial layer hen (Gallus gallus domesticus) in MDCK cells twice and harvesting the supernatant after approximately 40 hours post-inoculation [23]. The German isolate (A/turkey/Germany/AR2485/2014) originated from lung tissue of a commercially kept turkey (Meleagris gallopavo) and was passaged in embryonated chicken eggs [15]. (Table 1).

Table 1. Sample characteristics and accession details

	UKDD				DETU				NLCH			
Virus strain	A/duck/England/36254/2014				A/turkey/Germany/AR2485-L01478/2014				A/chicken/Netherlands/EMC-3/2014			
Isolation source	Pooled intestines				Lung tissue				Lung tissue			
Host Scientific Name	Anas platyrhynchos				Meleagris gallopavo				Gallus gallus domesticus			
Host Common Name	Domestic duck				Turkey				Chicken			
Collection Date	14 November 2014				04 November 2014				23 November 2014			
Collection Country	United Kingdom				Germany				Netherlands			
Collection Region	East Yorkshire				Mecklenburg-Western Pomerania				Ter Aar			
Influenza Test Method	MP gene RRT-PCR, H5 RRT-PCR				MP gene RRT-PCR, H5 RRT-PCR				MP gene RRT-PCR, H5 RRT-PCR			
Culture Status Sample	Egg passage 1				Egg passage 1				MDCK passage 2			
	Institution I	**Institution II**	**Institution III**		**Institution I**	**Institution II**	**Institution III**		**Institution I**	**Institution II**	**Institution III**	
Study Accession*	PRJEB9846	PRJEB12582	PRJEB9687		PRJEB9846	PRJEB12582	PRJEB9687		PRJEB9846	PRJEB12582	PRJEB9687	
Run Accession*	ERR972805	ERR1293054	ERR926712	ERR926713	ERR1435402	ERR1293053	ERR926714	ERR926715	ERR1435402	ERR1293055	ERR926717	ERR926718
DPP1 *.bam file run accession*	ERR3209376	ERR3209752	ERR9033756		ERR3209377	ERR3209753	ERR3093757		ERR3209375	ERR3209754	ERR3093758	
DPP2 *.bam file run accession*	ERR2992676	ERR2992677	ERR2992675		ERR2992679	ERR2992680	ERR2992678		ERR2992682	ERR2992683	ERR2992681	
DPP3 *.bam file run accession*	ERR2985803	ERR2985804	ERR2985802		ERR2985806	ERR2985807	ERR2985805		ERR2985809	ERR2985810	ERR2985808	
Experiment Accession 100k*	ERX319868615	ERX29868148	NA	NA	ERX319868616	ERX29868147	NA	NA	ERX319868617	ERX29868149	NA	NA
Run Accession 100k *	ERR3090788	ERR2984276	NA	NA	ERR3090789	ERR2984275	NA	NA	ERR3090790	ERR2984277	NA	NA

***** Using the Study Accession numbers in the European Nucleotide Archive all related data files can be accessed, or accessed directly from https://www.ebi.ac.uk/ena/data/view/accession, e.g.: https://www.ebi.ac.uk/ena/data/view/PRJEB9846 (Study Accession Institution I), https://www.ebi.ac.uk/ena/data/view/ERR972805 (Run Accession UKDD Institution I).

Sequencing

Institution I: SP1

RNA was extracted using a Qiagen QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturers' instructions except that carrier RNA was omitted from the AVL lysis buffer and the sample was eluted in 50µl RNAse-free water. RNA was then processed to double-stranded cDNA (cDNA Synthesis System, Roche) using random hexamers and purified using magnetic beads (AmpureXP, Beckman Coulter, USA). The double-stranded cDNA was diluted to 0.2 ng/µl and used to produce a sequencing library using the NexteraXT kit (Illumina, USA). Libraries were then sequenced in paired-end mode on an Illumina MiSeq (Illumina, USA), with run lengths varying from 2 x 75 bases (UKDD virus) to 2 x 150 bases (NLCH and DETU viruses) depending on whether time-constraints were implemented to provide a rapid response to an outbreak. Demultiplexing and removal of sequencing adapters was done by the MiSeq RTA software to generate raw fastq files. SP1 process included a limited 12-cycle PCR enrichment of the library. Post-hoc analysis showed that duplication levels were less than 0.02% of the total reads which were considered to have negligible impact on the results.

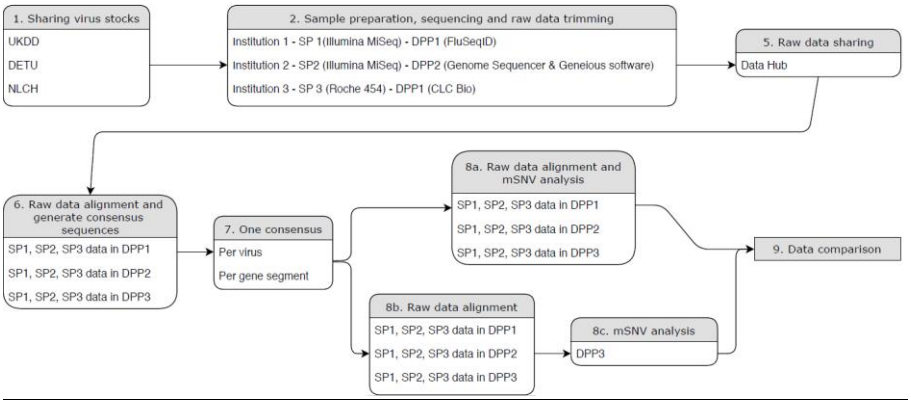

Figure 1. Flowchart of the experimental design. SP: sequence platform; DPP: data processing pipeline

Institution II: SP2

RNA was extracted using a combined approach with TRIzol (Thermo Fisher Scientific, USA) and an RNeasy Kit (Qiagen, Germany). Further concentration and cleaning was done with Agencourt RNAClean XP magnetic beads (Beckman Coulter, USA). RNA

was quantified using a Nanodrop UV spectrometer ND-1000 (Peqlab, Germany) and used as template for cDNA synthesis with a cDNA Synthesis System (Roche, Germany) with random hexamers. Fragmentation of the cDNA applying a target size of 300 bp was done with a Covaris M220 ultrasonicator. The sonicated cDNA was used for library preparation using Illumina indices (Illumina, USA) on a SPRI-TE library system (Beckman Coulter, USA) using a SPRIworks Fragment Library Cartridge II (for Roche FLX DNA sequencer; Beckman Coulter, USA) without automatic size selection. Subsequently, upper and lower size exclusion of the library was done with Ampure XP magnetic beads (Beckman Coulter, USA). The libraries were quality checked using High Sensitivity DNA Chips and reagents on a Bioanalyzer 2100 (Agilent Technologies, Germany) and quantified via qPCR with a Kapa Library Quantification Kit (Kapa Biosystems, USA) on a Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories, USA). SP2 did not amplify sample nor library. Sequencing was done on an Illumina MiSeq using MiSeq reagent kit v3 (Illumina, USA) resulting in paired end sequences with a read length of 300. Demultiplexed and adapter-trimmed reads were used to generate raw fastq files.

Institution III: SP3

RNA was extracted using the High Pure RNA isolation kit (Roche Diagnostics, Germany) according to manufacturer's instructions. RNA was converted to cDNA using the SuperScript III Reverse Transcriptase kit (Invitrogen, Thermo Fisher, USA) as described previously [24], and amplified by PCR using primers covering the full viral genome (Supplemental material). All 32 PCR fragments from approximately 400-600 nucleotides in length, were sequenced using the 454/Roche GS-FLX sequencing platform. The PCR fragments were pooled in equimolar ratio and purified using the MinElute PCR Purification kit (Qiagen, Germany) according to the manufacturer's instructions. Rapid Library preparation, Emulsion PCR and Next Generation 454-sequencing were performed according to instructions of the manufacturer (Roche Diagnostics, Germany). Protocols are described in the following manuals: Rapid Library Preparation Method Manual (Roche; May 2010), emPCR Amplification Method Manual –Lib-L (Roche; May 2010) and Sequencing Method Manual (Roche; May 2010). All three samples were sequenced in one run. Samples were pooled using MID adaptors to determine which sequences came from which sample, each sample was assigned two different MID's. Demultiplexing and basic trimming was done by CLC-bio software to generate raw fastq files (for detailed information, see supplemental material).

Data processing

Institution I: DPP1

In the FluSeqID script (https://github.com/ellisrichardj/FluSeqID) the following steps are run automatically: the mapping of raw sequence data to the host genome (BWA [25]), extracting reads that do not map to the host (Samtools [26]), assembling non-host reads (Velvet [27]), identification of the closest match for each genome segment (BLAST), mapping original data to the top reference segments (BWA), calling new consensus sequences (vcf2consensus.pl), performing further iterations of the last two steps to improve new consensus (IterMap), and finally outputting the genome consensus sequence. The data processing pipeline has in-build defaults for k-mer and coverage cut-off for de novo assembly, and the e-value cut-off for BLAST, which can be changed via command line options (see https://github.com/ellisrichardj/FluSeqID). For mSNV analysis, the reads were mapped to the unified consensus using BWA. Samtools was used to generate a pileup file which was then analysed using custom python and R scripts to determine the depth of coverage and basecalls at each position (available at https://github.com/ellisrichardj/MinorVar). In order to be included in the final output the minimum basecall quality was 20 and the minimum mapping quality was 50.

Institution II: DPP2

Raw sequence data were analysed and mapped using the Genome Sequencer software suite (v. 3.0; Roche, Mannheim, Germany) and the Geneious software suite (v. 9.0.5; Biomatters, Auckland, New Zealand). Raw reads were trimmed and subsets of each trimmed dataset were assembled de novo to generate reference sequences for each data set (Newbler Assembler of Genome Sequencer software suite v. 3.0; Roche, Mannheim, Germany). The trimmed raw influenza virus reads were mapped to the reference sequences (Newbler Mapper of Genome Sequencer software suite v. 3.0; Roche, Mannheim, Germany). The output assemblies were imported into the Geneious software suite (v. 9.0.5; Biomatters, Auckland, New Zealand) for further analysis and processing. Regions of low and high coverage and regions of low quality (minimum quality/phred score 20) were evaluated and if necessary, excluded from further analyses. Consensus sequences were generated and annotated. Trimmed raw reads of the datasets or subsets thereof were mapped to the consensus, mapping was fine-tuned and mSNVs were determined using generic SNP finder of the Geneious software suite, applying parameters of maximum p-value of 10-5 and filter for strand bias. The threshold for SNP identification was set at 1%, and variants were checked manually for accuracy.

Institution III: DPP3

Raw sequence data were analysed and mapped using the CLC Genomics software package, workbench 8 (CLC Bio). Reads obtained by 454 sequencing were sorted by MID adaptor and analysed using the parameters as shown in Supplemental material. In short, after sorting by MID, the sequence reads were trimmed at 30 nucleotides from the 3′ and 5′ ends to remove all primer sequences. Data from the shared Illumina sequence files had already been trimmed and were imported in CLC Bio without additional processing steps (see supplemental material for detailed CLC settings). Reads were initially aligned to the reference Sanger sequence of the HPAI virus A/chicken/Netherlands/EMC-3/2014 (H5N8). For the mSNV analysis the raw data were mapped to the custom-made consensus sequence per gene segment per sample. The threshold for mSNV identification was set at 1%, and registered minority variants were checked manually for accuracy (minimal quality/phred score 20). Those fastq files were shared with the other institutions.

Determining the influence of the DPP alignment steps versus DPPs mSNV identification methods

Data processing pipelines process raw data in several steps, roughly divided into trimming, aligning data to a reference sequence, and variant calling (the mSNV identification procedure). In order to determine to what extent the trimming and subsequent alignment processes contributed to the observed differences the nucleotide coverage results obtained by the three DPPs when aligning the same SP raw datasets were compared. To study the influence of the mSNV identification process, quality-trimmed alignment files that had been generated by each DPP and shared as *****.bam files were subjected to the mSNV identification process used in DPP3 to determine the differences in mSNV detection output when only the alignment processes differed. DPP3 mSNV detection parameters were set to the institutions default settings for mSNV identification using CLC-bio software and can be seen in the Supplemental material.

Data sharing

To test the applicability of real-time sequence data sharing within the COMPARE network, all raw sequence data used in this study were uploaded to and shared via a "Data Hub" in the environment of the European Nucleotide Archive (ENA). Each institution received its own study accession in which all raw sequence data files and metadata files were assigned with individual experimental accession numbers (Table 1). In addition to the sequence data, all trimmed alignment files (*****.bam) have been uploaded to the ENA. Using these hubs, sharing between institutions was facilitated

and immediate access to the data prior to the public release was possible to enable joint evaluation and comparison. All data files have been made publicly available via the ENA (https://www.ebi.ac.uk/ena).

mSNV analysis comparison

For the mSNV analyses the custom-made consensus for each virus isolate was used as a reference for mapping, thereby standardizing positions within the genome to make comparison between institutions easier. To avoid unnecessary increases in analytical time and memory, datasets were down-sampled to 100.000 reads per sample when needed. Each DPP produced a report on the identified mSNVs in a tabulated format. The analysis output files were filtered for mSNVs only, thereby ignoring detected nucleotide insertions and deletions (InDels). For the identification of mSNVs a minimum coverage threshold was applied. This minimum nucleotide coverage (i.e. number of reads per nucleotide after trimming) was determined using a basic sample size calculation method, $n = \log \beta / \log p'$. Here β represents the required power (e.g. for 95% chance of detecting something $\beta = 0.05$), and p' is 1 - the proportion of events that you want to detect. For a 95% certainty of detecting a variant at 1%, a minimum coverage of 298 reads per position is needed. For variants that occur in ≥5% of reads, the number of reads required is >58, and for variants that occur in ≥10% of the reads the minimum coverage is >28. For the mSNV identification literature commonly uses the arbitrary mSNV cut-off frequencies of ≥10%, ≥5% and ≥1%. However, it needs to be noted that these cut-off values are arbitrary. Therefore, where depth of coverage was sufficient, this study will report mSNV detected with a frequency of ≥1%.

Results

In order to determine the comparability and repeatability of consensus sequences and mSNV identification the biological samples, the sequence data from 3 SPs and the *.bam quality-trimmed alignment files of raw data of 3 influenza A/H5N8 viruses were shared. All data sets were subsequently analysed in 3 different DPPs. The resulting 9 mSNV reports per virus (3 SP data sets each analysed in 3 DPPs) were evaluated for comparability and the repeatability of mSNV identification using different combinations of SP and DPP.

Data sharing

Data sharing using the COMPARE "Data Hub" provided by ENA proved to be easy, quick and successful. The "Data Hub" enables the File Transport Protocol (FTP)

protected upload and download of large data files and facilitates sharing between collaborators with the possibility to evaluate and compare all data prior to their public release by generating and specifically sharing accession numbers using standard ENA procedures. The Data Hub used an influenza virus sample checklist. In addition, data sets are ultimately made publicly and through the INSDC network globally available and accessible in real-time as required without further upload to a different repository. Full details of the COMPARE Data Hub system are available in a submitted manuscript [22]. In summary, this process was suitable for quick data sharing in an outbreak scenario.

Designing custom-made consensus sequences

Each institution produced a consensus sequence for the 8 influenza gene segments (PB2, PB1, PA, HA, NP, NA, MP, NS) for each of the three viruses. The obtained consensus sequences were aligned using the BioEdit sequence alignment editor (version 7.2.0) [28]. For each of the 8 gene segments of the 3 viruses separately, 9 initial consensus sequences (3 SPs x 3 DPPs) were generated, resulting in 72 consensus sequences per virus. The custom-made consensus sequence per virus and per gene segment was 1) trimmed to a length represented by all 9 initial consensus sequences and 2) nucleotides had to be identical to at least 6/9 consensus sequences to be included. This resulted in a unique custom-made consensus for each gene segment for all three viruses.

Consensus sequences

When ignoring insertions and deletions in the homopolymer regions of the 454 data for most gene segments the identified consensus sequences did not differ between the different SP and DPP combinations used (Table 2). However, the number of insertions and deletions in homopolymer regions of the SP3 sequences were considerable in all 3 viruses. There was no clear difference in the number of insertions and deletions related to homopolymer regions between the different DPPs (20, 17 and 18 for DPP 1, 2 and 3 respectively). Nucleotide differences that were not related to homopolymer regions were only observed for sequences obtained in SP3 and SP2 data when processed in DPP1.

In summary, the homopolymer errors inherent in the 454 dataset caused problems for all DPPs, as expected. Consensus sequences generated by DPP1 from SP3 (454) data showed some unexpected differences, but it performed well with the SP1 data formats it was designed for and reasonably well with SP2 data. Overall, the consensus sequences can be reproduced by all DPPs using Illumina data but that the analysis of the 454 data from SP3 was more problematic, as it would require editing

of the sequences at homopolymer regions. Consensus sequences from this study can be found in the supplemental material.

Table 2. The differences in consensus sequences obtained from each SP/DPP combination, sorted per virus and per gene segment.

Virus	Segment	Start*	End	Number of InDels at homoplymer regions**	Other nucleotide differences***
NLCH	PB2	1	2280	2 (DPP1) 2 (DPP3)	C506A (SP3) G2101R (SP3)
	PB1	1	2277	1 (DPP1/DPP2/DPP3) 1 (DPP1/DPP2) 1 (DPP2/DPP3) 1 (DPP3)	A949W (SP3) 2272 ins AAG (SP2)
	PA	-6#	2190	1 (DPP1/DPP2) 2 (DPP1)	ND
	HA	7	1704	1 (DPP2/DPP3)	A427W (SP2)
	NP	1	1497	1 (DPP1)	C420Y (SP3)
	NA	4	1419	ND	ND
	MP	-5#	982	ND	ND
	NS	1	838	ND	ND
DETU	PB2	1	2287	1 (DPP1/DPP2/DPP3) 3 (DPP1)	2272 Del A (SP3)
	PB1	1	2277	1 (DPP1/DPP2/DPP3) 1 (DPP1) 1 (DPP2) 1 (DPP3)	T956C (SP3)
	PA	7	2189	1 (DPP1/DPP2)	ND
	HA	1	1728	1 (DPP2/DPP3)	ND
	NP	1	1497	2 (DPP3)	ND
	NA	1	1413	1 (DPP1)	778 ins CCA (SP3)
	MP	-1#	982	1 (DPP2)	ND
	NS	2	838	ND	ND
UKDD	PB2	1	2298	1 (DPP1/DPP2/DPP3) 1 (DPP3)	C504T (SP3) C506M (SP3)

	PB1	1	2277	1 (DPP1/DPP2/DPP3) 1 (DPP2/DPP3)	T951W (SP3)
	PA	1	2151	2 (DPP1) 1 (DPP2)	ND
	HA	1	1704	1 (DPP2/DPP3)	ND
	NP	1	1497	1 (DPP3)	T1003Y (SP2)
	NA	4	1420	ND	782 del TA (SP3)
	MP	-5#	982	1 (DPP2)	ND
	NS	-5#	849	ND	ND

The letter in brackets represents the DPP (column 5) or the SP (column 6) where the insertions/deletions or mutations were detected. InDel: insertions or deletion; SP: Sequence platform; DPP: Data processing pipeline; ND: not detected. * Start is counted from the ATG start codon; ** Exclusively identified in SP3 sequence data, InDels related to homopolymer regions; *** Exclusively identified in DPP1; # '-' indicates the number of nucleotides before the ATG start codon included in the consensus

mSNV analysis comparison

Nucleotide coverage and the influence of DPP-dependent alignment

The observed nucleotide coverages showed near to identical profiles for all three viruses. The coverage results obtained from the three different SPs and DPPs for the NLCH virus (Fig 2) and for the other two viruses (S1 Figure) were plotted. In general, lower nucleotide coverage was observed at the termini of each gene segment. The SP3 data showed more variation in nucleotide coverage within gene segments compared to SP1 and SP2 data, due to the sequencing of 32 PCR amplicons. The non-normalised number of raw sequence reads and influenza virus reads per virus per SP can be found in the S3 Table.

The differences in nucleotide coverage were visualized for the three different SP raw datasets analysed with the same DPP (Figure 2A). Overall, SP3 data (green lines) showed a lower coverage compared to SP1 (purple) and SP2 data (yellow). The overall coverage for SP1 and SP2 data was similar with small variations for different viruses and DPPs. The shorter read lengths in SP1 virus data did not appear to have influenced the overall nucleotide coverage substantially.

The differences in nucleotide coverage introduced by different alignment procedures were also assessed, by comparing the coverage results for each SP raw dataset analysed with the three different DPPs (Figure 2B). DPP2 (orange lines) generally retained the highest nucleotide coverage for data from the different SPs. However, DPP3 (grey lines) generally also retained high coverage for SP3 data, for

which it was optimized. The nucleotide coverage of SP3 data showed larger variation between the three different DPPs, leading to differences in nucleotide coverage up to 50% depending on the DPP, because DPP1 and DPP2 were not optimized for this SP. Data from SP2 were handled very similar by all three DPPs.

In conclusion, both the SP and the DPP influenced the number of reads per nucleotide position. SP3 showed the lowest output in number of reads compared to SP1 and SP2 Illumina data. The influence of the DPP depended highly on the data input, with best DPP performance for the SP dataset for which it was optimized.

The mSNV identification

The mSNV identification thresholds were set to ≥1% in all DPPs. Because of the high number of mSNVs identified, the comparison of these mSNVs started with a manually set arbitrary threshold of ≥10% that was subsequently decreased to ≥5%, and ≥1%. A mSNV position was identified when at least 1 of the SP/DPP combinations showed a variant that exceeded the frequency threshold, and when the coverage at that position exceeded the minimum number of reads needed to detect that variant with a 95% probability, as described previously. The presence of mSNV and coverage for all SP/DPP combinations were compared for each of the positions in which a mSNV had been detected in at least one of the combinations. The coverages indicated for those positions where no mSNVs were detected were derived from the alignment files and were not subjected to possible additional read filtering parameters in the mSNV identification process. The average quality (Q-score/phred score) was set to or exceeding 20.

Ten positions across the three virus genomes were identified with mSNVs occurring in ≥10% of reads. Three of the mSNVs (NLCH:PB2 G1879A , NLCH:PB2 G2101A and DETU:HA T963C) were detected in all SP/DPP combinations but with slightly different relative abundance. The other mSNVs were identified in only one (n=6) or two (n=1) of the SP/DPP combinations (Table 3).

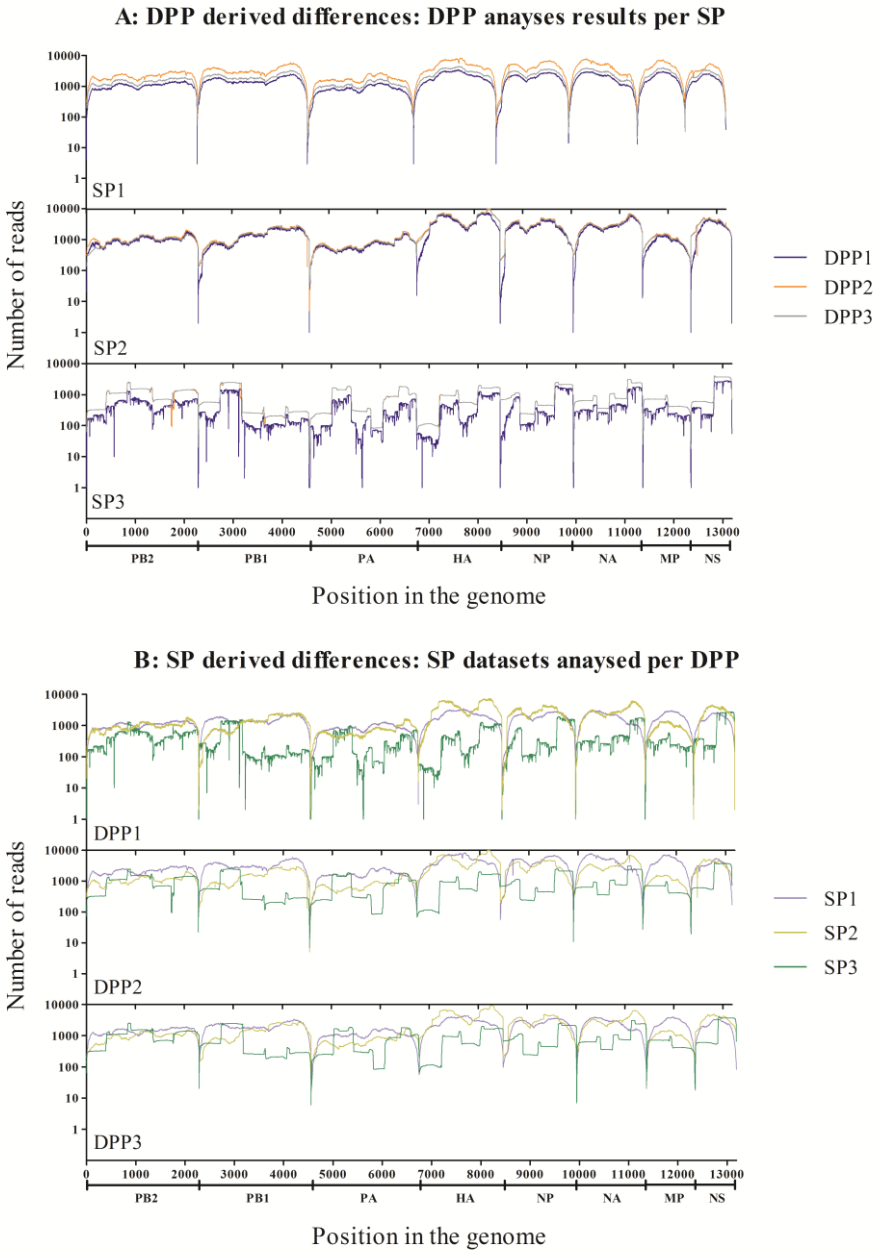

Fig 2: Nucleotide coverage. The non-normalised nucleotide coverage displayed as number of nucleotides per position for full genome sequences of the NLCH virus reads mapped to the NLCH reference sequences. Panel A shows the coverage results for the same SP dataset in the three different DPPs (DPP1: purple; DPP2: orange; DPP3 grey) for each of the SP datasets. Panel B shows the coverage when the same DPP is used to analyse data from the three different SPs (SP1: lilac; SP2:

yellow; SP3: green) for each of the DPPs. The X-axis represents the position in the genome, the Y-axis represents the number of sequence reads per position.

Table 3. The minority variants occurring in at least one of the sequence platform - data processing pipelines as a ≥5% variant.

Virus	Position	Sequence platform	Data processing pipeline					
			1		2		3	
			Minor variants	Percen tage	Minor variants	Percen tage	Minor variants	Percen tage
NLCH	PB2.1879 G→A	1	81/1301	6,20%	246/2716	9,10%	112/1203	9,30%
		2	47/956	4,90%	117/1137	10,30%	114/1064	10,70%
		3	49/530	9,20%	131/1341	9,80%	129/1338	9,60%
	PB2.2101 G→A	1	53/1118	4,70%	261/2704	9,70%	110/897	12,30%
		2	21/1578	1,30%	125/1875	6,70%	121/1463	8,30%
		3	13/542	2,40%	199/1433	13,90%	199/1435	13,90%
	PB2.2277 T→G	1	ND/479	<1%	86/1008	8,50%	33/190	17,40%
		2	ND/557	<1%	ND/623	<1%	ND/534	<1%
		3	ND/680	<1%	ND/1117	<1%	ND/1024	<1%
	PB1.87 A→G	1	ND/818	<1%	ND/1754	<1%	ND/1114	<1%
		2	25/230	10,90%	ND/376	<1%	ND/328	<1%
		3	ND/275	<1%	ND/537	<1%	ND/537	<1%
	PB1.2240 G→C	1	ND/664	<1%	54/1341	4,00%	38/418	9,10%
		2	ND/1231	<1%	ND/1271	<1%	ND/1233	<1%
		3	ND/161	<1%	ND/277	<1%	ND/276	<1%
	PB1.2268 A→G	1	ND/336	<1%	29/641	4,50%	11/176	6,30%
		2	ND/993	<1%	ND/1026	<1%	ND/1002	<1%
		3	ND/53	<1%	ND/159	<1%	ND/148	<1%
	HA.104 A→G	1	ND/733	<1%	ND/1761	<1%	ND/1151	<1%
		2	ND/437	<1%	ND/1370	<1%	ND/1156	<1%
		3	ND/1	<1%	ND/105	<1%	12/105	11,40%
	HA.1689 T→C	1	ND/390	<1%	ND/694	<1%	11/217	5,10%
		2	ND/2018	<1%	ND/4083	<1%	ND/3979	<1%
		3	ND/937	<1%	ND/1669	<1%	ND/1680	<1%

Virus	Position	Sequence platform	Data processing pipeline					
			1		2		3	
			Minor variants	Percentage	Minor variants	Percentage	Minor variants	Percentage
	NP.105 A→G	1	ND/182	<1%	ND/449	<1%	ND/343	<1%
		2	83/1507	5,50%	ND/1890	<1%	ND/1804	<1%
		3	ND/89	<1%	ND/704	<1%	ND/702	<1%
	NP.1239 A→T	1	32/2428	1,30%	279/5410	5,20%	ND/3092	<1%
		2	ND/2345	<1%	ND/2643	<1%	ND/2453	<1%
		3	ND/1711	<1%	ND/2111	<1%	ND/2117	<1%
	NP.1489 G→A	1	ND/182	<1%	26/336	7,70%	ND/172	<1%
		2	ND/436	<1%	ND/452	<1%	ND/444	<1%
		3	ND/1320	<1%	ND/1799	<1%	ND/1799	<1%
	NS.833 A→T	1	ND/187	<1%	ND/287	<1%	5/88	5,70%
		2	ND/1224	<1%	ND/1327	<1%	ND/1284	<1%
		3	ND/1367	<1%	ND/2430	<1%	ND/2333	<1%
DETU	PB2.1054 T→C	1	69/1369	5,00%	168/2637	6,40%	97/1304	7,40%
		2	60/1477	4,10%	115/1836	6,30%	99/1605	6,20%
		3	6/392	1,50%	94/2038	4,60%	48/1054	4,60%
	PB2.2257 A→C	1	ND/867	<1%	ND/1563	<1%	24/463	5,20%
		2	ND/531	<1%	ND/581	<1%	ND/378	<1%
		3	ND/893	<1%	ND/2286	<1%	ND/1346	<1%
	PB2.2277 T→G	1	ND/644	<1%	52/1150	4,50%	27/307	8,80%
		2	ND/418	<1%	ND/472	<1%	ND/284	<1%
		3	ND/1208	<1%	ND/1948	<1%	ND/1209	<1%
	PB1.14 C→T	1	ND/144	<1%	48/433	11,10%	ND/239	<1%
		2	ND/90	<1%	ND/355	<1%	ND/304	<1%
		3	ND/562	<1%	ND/792	<1%	ND/496	<1%
	PB1.23 T→G	1	ND/207	<1%	30/535	5,60%	ND/315	<1%
		2	ND/103	<1%	ND/365	<1%	ND/319	<1%
		3	ND/699	<1%	ND/950	<1%	ND/609	<1%
	PB1.87 A→G	1	ND/744	<1%	ND/1644	<1%	ND/1076	<1%
		2	49/365	13,40%	ND/677	<1%	ND/576	<1%
		3	ND/721	<1%	ND/1156	<1%	ND/793	<1%

Virus	Position	Sequence platform	Data processing pipeline					
			1		2		3	
			Minor variants	Percentage	Minor variants	Percentage	Minor variants	Percentage
	PB1.2240 G→C	1	ND/757	<1%	23/1517	1,50%	26/515	5,00%
		2	ND/944	<1%	ND/985	<1%	ND/806	<1%
		3	ND/274	<1%	ND/439	<1%	ND/253	<1%
	PB1.2268 A→G	1	5/470	1,10%	33/928	3,60%	22/278	7,90%
		2	ND/798	<1%	ND/829	<1%	ND/671	<1%
		3	ND/109	<1%	ND/259	<1%	ND/123	<1%
	PB1.2271 A→G	1	12/446	2,70%	59/901	6,50%	16/263	6,10%
		2	ND/729	<1%	47/810	5,80%	40/649	6,20%
		3	1/32	3,10%	ND/123	<1%	2/83	2,40%
	HA.867 C→T	1	59/1533	3,80%	206/3183	6,50%	104/1537	6,80%
		2	59/2031	2,90%	150/2525	5,90%	127/2253	5,60%
		3	11/180	6,10%	48/647	7,40%	28/385	7,30%
	HA.963 T→C	1	122/1401	8,70%	446/3071	14,50%	189/1419	13,30%
		2	90/1517	5,90%	318/2189	14,50%	247/1828	13,50%
		3	5/1969	7,20%	107/606	17,70%	47/293	16,00%
	NP.1491 C→A	1	ND/278	<1%	71/583	12,20%	ND/206	<1%
		2	ND/723	<1%	ND/769	<1%	ND/692	<1%
		3	ND/799	<1%	ND/2031	<1%	ND/1206	<1%
	NA.65 T→C	1	19/503	3,80%	52/1229	4,20%	16/467	3,40%
		2	20/662	3,00%	50/1104	4,50%	45/992	4,50%
		3	24/557	4,30%	53/1099	4,80%	37/727	5,10%
	NA.78 T→C	1	23/599	3,80%	57/1403	4,10%	20/557	3,60%
		2	21/692	3,00%	55/1147	4,80%	50/1033	4,80%
		3	23/580	4,00%	51/1124	4,50%	37/735	5,00%
	NA.89 T→C	1	23/713	3,20%	55/1670	3,30%	22/651	3,40%
		2	23/798	2,90%	56/1261	4,40%	50/1134	4,40%
		3	24/580	4,10%	55/1196	4,60%	40/775	5,20%
	NA.117 T→C	1	37/908	4,10%	87/2140	4,10%	36/818	4,40%
		2	28/1102	2,50%	67/1631	4,10%	ND/1459	<1%
		3	22/531	4,10%	57/1276	4,50%	42/812	5,20%

Virus	Position	Sequence platform	Data processing pipeline					
			1		2		3	
			Minor variants	Percentage	Minor variants	Percentage	Minor variants	Percentage
	NA.126 T→C	1	37/983	3,80%	83/2294	3,60%	36/876	4,10%
		2	31/1126	2,80%	72/1676	4,30%	65/1502	4,30%
		3	26/519	5,00%	62/1395	4,40%	43/812	5,30%
UKDD	PB2.2277 T→G	1	ND/415	<1%	28/507	5,50%	ND/475	<1%
		2	ND/589	<1%	ND/620	<1%	ND/601	<1%
		3	ND/1140	<1%	ND/1996	<1%	ND/2065	<1%
	PB1.87 A→G	1	ND/387	<1%	ND/440	<1%	ND/439	<1%
		2	26/327	8,00%	32/395	8,10%	ND/351	<1%
		3	ND/617	<1%	ND/1133	<1%	ND/1136	<1%
	PB1.728 C→A	1	ND/750	<1%	ND/832	<1%	ND/836	<1%
		2	ND/776	<1%	52/928	5,60%	ND/829	<1%
		3	ND/2459	<1%	ND/4290	<1%	ND/4293	<1%
	PB1.730 C→T	1	ND/742	<1%	ND/824	<1%	ND/826	<1%
		2	ND/767	<1%	57/1008	5,70%	ND/832	<1%
		3	ND/2339	<1%	ND//4286	<1%	ND/4289	<1%
	PB1.883 G→C	1	ND/942	<1%	ND/997	<1%	ND/997	<1%
		2	ND/1689	<1%	ND/1865	<1%	ND/1760	<1%
		3	ND/2479	<1%	47/690	6,80%	ND/3681	<1%
	PA.49 G→C	1	ND/103	<1%	6/117	5,10%	ND/115	<1%
		2	ND/337	<1%	ND/435	<1%	ND/392	<1%
		3	ND/111	<1%	ND/207	<1%	ND/204	<1%
	PA.82 C→T	1	ND/155	<1%	ND/180	<1%	ND/177	<1%
		2	ND/695	<1%	ND/809	<1%	ND/745	<1%
		3	ND/64	<1%	ND/247	<1%	30/248	12,10%
	NS.811 G→T	1	ND/221	<1%	17/270	6,30%	ND/249	<1%
		2	ND/2452	<1%	ND/2725	<1%	ND/2557	<1%
		3	ND/3117	<1%	ND/4125	<1%	ND/4139	<1%

Colours display the variant frequency with ≥10% (black), 5-10% (dark grey) and <5% (light grey). ND: not detected

Thirty-seven positions were identified with mSNVs occurring in ≥5% of reads. Of those, the same mSNV was identified in all SP/DPP combinations for 9 positions (24,3%), in seven or eight of the SP/DPP combinations for 2 positions (5,4%) and in at least two SP/DPP combinations for 19 positions (51.4%), although not always in a frequency of ≥5%. However, for 18 positions (48.6%) the mSNV was not reproduced at a ≥1% frequency in any of the other SP/DPP combinations (Table 3). Focussing on the separate SP data analysed in the 3 DPPs, most of the identified positions with ≥5% mSNVs in at least 1 SP/DPP combination were identified in SP1 data (47%) followed by SP2 (29%) and SP3 (24%) data.

Looking at the ≥5% mSNV reproducibility per SP dataset in all three DPPs within these thirty-seven positions, forty-eight SP datasets showed a ≥5% mSNV in at least one of the DPP outputs. Additionally, for eleven positions, all in the DETU virus, the variant was reproduced by all DPPs, however at a <5% frequency (for instance SP3 data at PB2.1054, and SP1 and SP2 data at NA.65) In 53% (31/59) of cases the same mSNVs from 1 SP dataset was reproduced in all three DPP's in at least a ≥1% frequency, in 31% (18/59) of cases the variant was only detected in 1 DPP even though coverage in the other DPPs was theoretically high enough to detect variants at a 1% level.

Lowering the threshold value to a mSNV frequency of ≥1% resulted in a large increase in the number of positions identified with mSNVs. To investigate the reproducibility of these mSNVs, the data for all 3 viruses was combined per SP in the three DPPs (influence of DPP), and per DPP analysing data from the three SPs (influence of SP) (Figure 3). The reproducibility of ≥1% variants using one SP dataset in all three DPPs was 10.3%, 8.9% and 23.3% for SP1, SP2 and SP3 sequences, respectively. The reproducibility of ≥1% variants using raw data of a virus sequenced in three different SPs was 15.1%, 6.5% and 15.1% for DPP1, DPP2 and DPP3 respectively. Most ≥1% variants were not reproduced by any of the other DPPs processing the same SP data (~75%) for SP1 and SP2 data. This was less for SP3 data but this might be due to the fact that many positions identified in SP3 data did not meet the minimum coverage criteria and were therefore discarded.

For brevity, the detailed results for the HA gene segment of the DETU virus are shown in Table 4. This virus segment was chosen because it showed the best reproducibility of results for ≥5% minority variants in all SP/DPP combinations. In the DETU HA segment, 33 positions containing a mSNV occurring in ≥1% of reads with sufficient coverage (≥298 reads) were identified. Only 3 of these positions (9%) were identified in all SP/DPP combinations. The majority of the positions (25/33, 76%) were only identified in one of the nine SP/DPP combinations. However, it needs to be noted that the SP3 data coverage was insufficient in all three DPPs to detect ≥1% variants for 11 of those positions (Table 4).

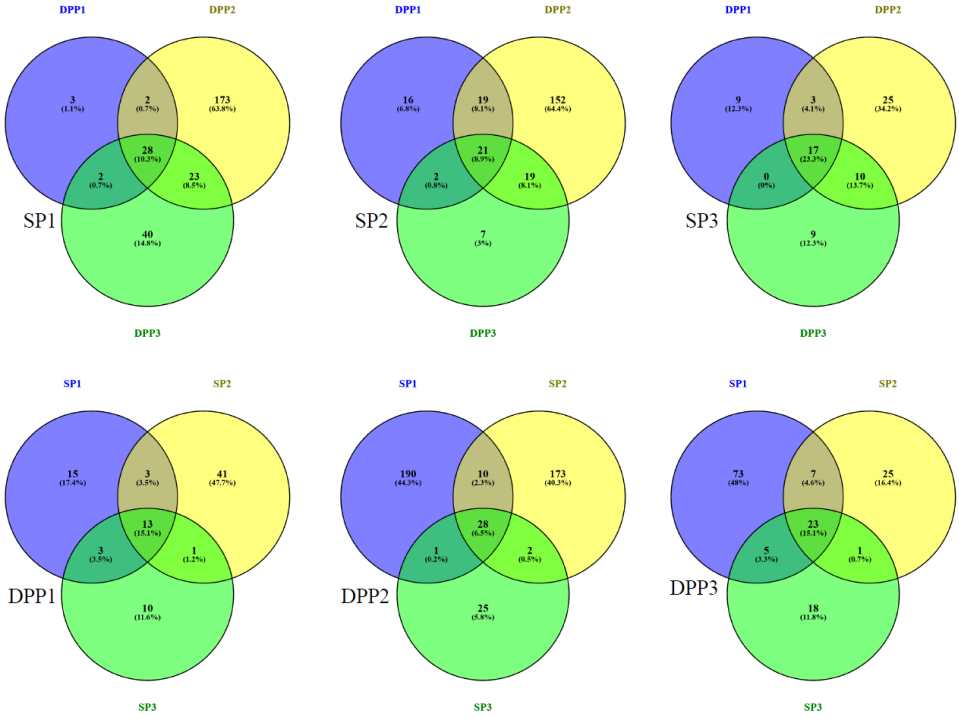

Figure 3. The reproducibility of ≥1% variants with sufficient coverage for all sequence data combined. Each figure shows the number of ≥1% variants detected per sequence platform (SP, top row) and data processing pipeline (DPP, bottom row) for SP1/DPP1 (left column), SP2/DPP2 (middle column), and SP3/DPP3 (right column). The colours represent the different DPPs and SPs respectively, in which the >1% variants were detected: SP1/DPP1 (purple), SP2/DPP2 (yellow) and SP3/DPP3 (green). Positions with ≥1% variants that were identified in more than one of the SPs or DPPs respectively are displayed in the overlapping coloured areas, the centre part representing the number of ≥1% variants that were detected with all three DPPs (top row) or SPs (bottom row). The total number of positions with ≥1% variants detected was 271in SP1, 236 in SP2, 73 in SP3, and 86 in DPP1, 429 in SP2, 152 in SP3. This figure was produced using Venny 2.1.

Table 4. The minority variants occurring in at least one of the sequence platform - data processing pipelines as a ≥1% variant in the HA segment of the DETU sample with a minimum coverage of 298 reads at that position.

Position	Sequence platform	Data processing pipeline					
		1		2		3	
		Minor variants	Percentage	Minor variants	Percentage	Minor variants	Percentage
HA:170 T→A	1	ND/935	<1%	ND/2191	<1%	ND/1348	<1%
	2	ND/300	<1%	11/693	1,59%	ND/551	<1%
	3	ND/82	<1%	ND/245	<1%	ND/210	<1%

Position	Sequence platform	Data processing pipeline					
		1		2		3	
		Minor variants	Percentage	Minor variants	Percentage	Minor variants	Percentage
HA.170 T→C	1	ND/935	<1%	ND/2191	<1%	ND/1348	<1%
	2	ND/300	<1%	18/693	2,60%	ND/551	<1%
	3	ND/82	<1%	ND/245	<1%	ND/210	<1%
HA.171 C→A	1	ND/931	<1%	ND/2184	<1%	ND/1339	<1%
	2	ND/323	<1%	12/698	1,72%	ND/558	<1%
	3	ND/82	<1%	ND/245	<1%	ND/210	<1%
HA.194 C→A	1	ND/991	<1%	ND/2397	<1%	ND/1455	<1%
	2	ND/353	<1%	22/701	3,14%	ND/553	<1%
	3	ND/58	<1%	ND/250	<1%	ND/212	<1%
HA.195 C→A	1	ND/995	<1%	ND/2390	<1%	ND/1464	<1%
	2	ND/356	<1%	20/701	2,85%	ND/553	<1%
	3	ND/55	<1%	ND/250	<1%	ND/212	<1%
HA.268 C→T	1	ND/1140	<1%	ND/2580	<1%	ND/1626	<1%
	2	ND/1293	<1%	25/1563	1,60%	ND/1338	<1%
	3	ND/88	<1%	ND/252	<1%	ND/212	<1%
HA.272 A→T	1	ND/1156	<1%	ND/2593	<1%	ND/1639	<1%
	2	17/1424	1,19%	20/1563	1,28%	ND/1404	<1%
	3	ND/81	<1%	ND/253	<1%	ND/213	<1%
HA.407 G→T	1	ND/1144	<1%	ND/2364	<1%	ND/1553	<1%
	2	ND/1773	<1%	31/2121	1,46%	ND/1855	<1%
	3	ND/74	<1%	ND/237	<1%	ND/212	<1%
HA.407 G→A	1	ND/1144	<1%	27/2364	1,14%	ND/1553	<1%
	2	ND/1773	<1%	ND/2121	<1%	ND/1856	<1%
	3	ND/74	<1%	ND/237	<1%	ND/212	<1%
HA.418 A→G	1	ND/1111	<1%	ND/2319	<1%	ND/1492	<1%
	2	29/2195	1,32%	38/2513	1,51%	ND/2197	<1%
	3	ND/69	<1%	ND/237	<1%	ND/212	<1%
HA.453 T→G	1	ND/1339	<1%	29/2736	1,06%	ND/1811	<1%
	2	ND/2342	<1%	ND/2695	<1%	ND/2384	<1%
	3	ND/91	<1%	ND/193	<1%	ND/179	<1%

Position	Sequence platform	Data processing pipeline					
		1		2		3	
		Minor variants	Percentage	Minor variants	Percentage	Minor variants	Percentage
HA.560 A→G	1	43/1587	2,71%	113/3385	3,34%	55/1517	3,63%
	2	56/2397	2,34%	145/2912	4,98%	113/2495	4,53%
	3	21/884	2,38%	72/1754	4,10%	43/1245	3,45%
HA.715 C→T	1	ND/1663	<1%	62/3832	1,62%	24/1582	1,52%
	2	26/2283	1,14%	55/2722	2,02%	50/2420	2,07%
	3	ND/531	<1%	20/1883	1,06%	15/1245	1,20%
HA.867 C→T	1	59/1533	3,85%	206/3183	6,47%	104/1537	6,77%
	2	59/2031	2,90%	150/2525	5,94%	127/2253	5,64%
	3	11/180	6,11%	48/647	7,42%	28/385	7,27%
HA.963 T→C	1	122/1401	8,71%	446/3071	14,52%	189/1419	13,32%
	2	90/1517	5,93%	318/2189	14,53%	247/1828	13,51%
	3	5/69	7,25%	107/606	17,66%	47/293	16,04%
HA.1000 A→C	1	ND/1409	<1%	48/2962	1,62%	ND/1873	<1%
	2	ND/1629	<1%	ND/1919	<1%	ND/1645	<1%
	3	ND/84	<1%	ND/614	<1%	ND/293	<1%
HA.1177 G→A	1	ND/1222	<1%	ND/2224	<1%	ND/1597	<1%
	2	ND/1652	<1%	34/1901	1,79%	ND/1724	<1%
	3	ND/289	<1%	ND/549	<1%	ND/270	<1%
HA.1183 A→G	1	ND/1210	<1%	ND/2226	<1%	ND/1589	<1%
	2	ND/1770	<1%	ND/1892	<1%	ND/1723	<1%
	3	ND/280	<1%	6/547	1,10%	ND/268	<1%
HA.1199 T→G	1	ND/1182	<1%	ND/2124	<1%	ND/1518	<1%
	2	ND/1615	<1%	27/1899	1,42%	ND/1732	<1%
	3	ND/296	<1%	ND/545	<1%	ND/266	<1%
HA.1263 A→G	1	16/963	1,66%	57/1841	3,10%	26/954	2,73%
	2	26/1924	1,35%	56/2207	2,54%	41/1967	2,08%
	3	ND/1161	<1%	63/2226	2,83%	33/1350	2,44%
HA.1430 A→G	1	ND/1311	<1%	ND/2870	<1%	ND/1827	<1%
	2	ND/1498	<1%	36/1924	1,87%	ND/1659	<1%
	3	ND/955	<1%	ND/2391	<1%	ND/1452	<1%

Position	Sequence platform	Data processing pipeline					
		1		2		3	
		Minor variants	Percentage	Minor variants	Percentage	Minor variants	Percentage
HA.1455 C→T	1	ND/1333	<1%	ND/2753	<1%	14/1233	1,14%
	2	ND/1846	<1%	ND/2242	<1%	ND/1895	<1%
	3	ND/1093	<1%	ND/2373	<1%	ND/1449	<1%
HA.1543 A→G	1	25/1209	2,07%	94/2757	3,41%	37/1142	3,24%
	2	ND/1660	<1%	56/1857	3,02%	41/1585	2,59%
	3	ND/1182	<1%	ND/3324	<1%	ND/1972	<1%
HA.1624 C→A	1	ND/998	<1%	ND/2174	<1%	ND/1478	<1%
	2	ND/1173	<1%	25/1291	1,94%	ND/1120	<1%
	3	ND/2218	<1%	ND/3654	<1%	ND/2244	<1%
HA.1634 C→A	1	ND/930	<1%	ND/2032	<1%	ND/1388	<1%
	2	ND/1091	<1%	16/1218	1,31%	ND/1048	<1%
	3	ND/2616	<1%	ND/3704	<1%	ND/2269	<1%
HA.1638 C→A	1	ND/932	<1%	ND/1991	<1%	ND/1368	<1%
	2	ND/1083	<1%	15/1180	1,27%	ND/1010	<1%
	3	ND/2600	<1%	ND/3709	<1%	ND/2276	<1%
HA.1643 T→A	1	ND/875	<1%	ND/1892	<1%	ND/1291	<1%
	2	ND/1028	<1%	13/1110	1,17%	ND/944	<1%
	3	ND/2612	<1%	ND/3703	<1%	ND/2278	<1%
HA.1643 T→G	1	ND/875	<1%	ND/1892	<1%	ND/1291	<1%
	2	ND/1028	<1%	12/1110	1,08%	ND/944	<1%
	3	ND/2612	<1%	ND/3703	<1%	ND/2278	<1%
HA.1691 G→A	1	ND/596	<1%	ND/1110	<1%	7/404	1,73%
	2	ND/767	<1%	ND/873	<1%	ND/696	<1%
	3	ND/2499	<1%	ND/3575	<1%	ND/2222	<1%
HA.1693 A→T	1	ND/582	<1%	ND/1081	<1%	7/391	1,79%
	2	ND/751	<1%	ND/864	<1%	ND/690	<1%
	3	ND/2310	<1%	ND/3569	<1%	ND/2219	<1%
HA.1695 T→C	1	ND/555	<1%	ND/1030	<1%	7/366	1,91%
	2	ND/779	<1%	ND/3557	<1%	ND/688	<1%
	3	ND/1767	<1%	ND/3557	<1%	ND/2220	<1%

Position	Sequence platform	Data processing pipeline					
		1		2		3	
		Minor variants	Percentage	Minor variants	Percentage	Minor variants	Percentage
HA.1698 C→T	1	ND/537	<1%	ND/977	<1%	ND/601	<1%
	2	ND/758	<1%	11/852	1,29%	ND/681	<1%
	3	ND/2260	<1%	ND/3520	<1%	ND/2113	<1%
HA.1705 A→G	1	ND/492	<1%	ND/883	<1%	ND/528	<1%
	2	ND/733	<1%	11/832	1,32%	ND/660	<1%
	3	ND/1709	<1%	ND/3300	<1%	ND/2016	<1%

Positions with ≥1% variants are marked in black, positions with a too low coverage (<298 reads/position) to detect ≥1% variants are marked in light grey. Numbers are displayed as [number of variants]/[number of reads on that position]. ND: not detected.

Determining the influence of the minor variant detection method

To isolate the effect of just the mSNV identification step in the DPP, independent of quality-trimmed alignment files (*****.bam files) of the raw data (subdivided per virus, per SP and per DPP) were shared and subjected to the mSNV detection process used in DPP3 and compared to the original outcomes from DPP1 and DPP2 (Table 5). In the majority of positions, the different mSNV identification processes did not influence the results, as 84% (119/142) of the mSNVs were identified regardless of the mSNV identification process. Twenty-three mSNVs that were not reproduced by DPP3 mSNV identification analysis, were reproduced when the 'Direction and position Filters' in DPP3 were ignored (Table 5, marked with # of ##). These parameters filter out mSNVs when the set criteria for the read direction (variant must occur in both forward and reverse reads), relative read direction (statistical approach of forward/reverse balance) and read position (removal of systemic errors) are not met. This indicates different DPPs deal differently with quality parameters, and data could be excluded or included based on the DPP used. In addition, 9 additional mSNVs were identified in the *****.bam files compared to the original mSNV outputs. It needs to be noted that the coverage of SP data analysed by DPP1 for positions identified with mSNVs was considerably lower compared to the coverage at that position in the input *****.bam files, suggesting additional quality filtering in the mSNV detection step of DPP1. However, the influence on mSNV identification was limited most likely due to the initial high nucleotide coverage.

To better visualise the differences in coverages and allele counts a graphical display of the data for four positions showing mSNVs in different frequencies for each

SP/DPP combination is included in the supplemental material (S2 figure) In general, SNVs were rarely missed due to low coverage.

Table 5. The reproducibility of positions with at least one ≥5% variant when alignment files from the respective DPPs are all uploaded into DPP3 for only the mSNV identification process versus when the mSNV identifications are fully performed by the respective DPPs.

Virus	Position	Sequence platform	Data Processing pipeline						Bam file generating processing pipeline					
			1		2		3				2		3	
			Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)
NLCH	PB2.1879 G→A	1	81/ 1301	6,2	246/ 2716	9,1	112/ 1203	9,3	132/ 1375	9,6	246/ 2716	9,1	121/ 1301	9,3
		2	47/ 956	4,9	117/ 1137	10,3	114/ 1064	10,7	119/ 1122	10,6	117/ 1137	10,3	114/ 1064	10, 7
		3	49/ 530	9,2	131/ 1341	9,8	129/ 1338	9,6	54/ 542	10,0	131/ 1341	9,8	129/ 1338	9,6
	PB2.2101 G→A	1	53/ 1118	4,7	261/ 2704	9,7	110/ 897	12,3	138/ 1180	11,7	261/ 2704	9,7	121/ 1086	11,1
		2	21/ 1578	1,3	125/ 1875	6,7	121/ 1463	8,3	ND/ 1856##	<1	ND/ 1850#	<1	121/ 1463	8,3
		3	13/ 542	2,4	199/ 1433	13,9	199/ 1435	13,9	87/ 625	13,9	199/ 1433	13,9	199/ 1435	13, 9
	PB2.2277 T→G	1	ND/ 479	<1	86/ 1008	8,5	33/ 190	17,4	ND/ 849	<1	ND/ 1008##	<1	37/ 281	13, 2
		2	ND/ 557	<1	ND/ 623	<1	ND/ 534	<1	ND/ 619	<1	ND/ 623	<1	ND/ 534	<1
		3	ND/ 680	<1	ND/ 1117	<1	ND/ 1024	<1	ND/ 708	<1	ND/ 1117	<1	ND/ 1027	<1
	PB1.87 A→G	1	ND/ 818	<1	ND/ 1754	<1	ND/ 1114	<1	ND/ 1264	<1	ND/ 1753	<1	ND/ 1114	<1
		2	25/ 230	10,9	ND/ 376	<1	ND/ 328	<1	ND/ 368##	<1	ND/ 376	<1	ND/ 328	<1
		3	ND/ 275	<1	ND/ 537	<1	ND/ 537	<1	ND/ 278	<1	ND/ 537	<1	ND/ 537	<1
	PB1.2240 G→C	1	ND/ 664	<1	54/ 1341	4,0	38/ 418	9,1	ND/ 1004	<1	ND/ 1341#	<1	46/ 486	9,5
		2	ND/ 1231	<1	ND/ 1271	<1	ND/ 1233	<1	ND/ 1277	<1	ND/ 1271	<1	ND/ 1235	<1
		3	ND/ 161	<1	ND/ 277	<1	ND/ 276	<1	ND/ 163	<1	ND/ 277	<1	ND/ 276	<1
	PB1.2268 A→G	1	ND/ 336	<1	29/ 641	4,50	11/ 176	6,3	15/ 322	4,7	37/ 641	5,8	13/ 213	6,1
		2	ND/ 993	<1	ND/ 1026	<1	ND/ 1002	<1	ND/ 1025	<1	ND/ 1026	<1	ND/ 1002	<1
		3	ND/ 53	<1	ND/ 159	<1	ND/ 148	<1	ND/ 90	<	ND/ 159	<1	ND/ 151	<1
	PA.2167 T→G	1	ND/ 141	<1	ND/ 288	<1	ND/ 154	<1	ND/ 235	<1	21/ 288	7,3	ND/ 154	<1
		2	ND/ 757	<1	ND/ 807	<1	ND/ 773	<1	ND/ 812	<1	ND/ 807	<1	ND/ 733	<1
		3	ND/ 704	<1	ND/ 1070	<1	ND/ 1077	<1	ND/ 714	<1	ND/ 1070	<1	ND/ 1078	<1
	HA.104 A→G	1	ND/ 733	<1	ND/ 1761	<1	ND/ 1151	<1	ND/ 1175	<1	ND/ 1761	<1	ND/ 1135	<1
		2	ND/ 437	<1	ND/ 1370	<1	ND/ 1156	<1	ND/ 1326	<1	ND/ 1369	<1	ND/ 1142	<1
		3	ND/ 1	<1	ND/ 105	<1	12/ 105	11,4	ND/ 6	<1	ND/ 105	<1	12/ 105	11,4

Virus	Position	Sequence platform	Data Processing pipeline						Bam file generating processing pipeline					
			1		2		3				2		3	
			Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)
	HA.1689 T→C	1	ND/ 390	<1	ND/ 694	<1	11/ 217	5,1	ND/ 610	<1	ND/ 694	<1	13/ 260	5,0
		2	ND/ 2018	<1	ND/ 4083	<1	ND/ 3979	<1	ND/ 4045	<1	ND/ 4081	<1	ND/ 3979	<1
		3	ND/ 937	<1	ND/ 1669	<1	ND/ 1680	<1	ND/ 1106	<1	ND/ 1669	<1	ND/ 1680	<1
	NA.3 T→C	1	ND/ 32	<1	ND/ 105	<1	ND/ 49	<1	ND/ 92	<1	7/ 105	6,7	ND/ 49	<1
		2	ND/ 6	<1	ND/ 313	<1	ND/ 297	<1	ND/ 305	<1	ND/ 313	<1	ND/ 297	<1
		3	ND/ 2	<1	ND/ 25	<1	ND/ 25	<1	ND/ 6	<1	ND/ 25	<1	ND/ 25	<1
	NP.105 A→G	1	ND/ 182	<1	ND/ 449	<1	ND/ 343	<1	ND/ 374	<1	6/ 449	1,3	ND/ 343	<1
		2	83/ 1507	5,5	ND/ 1890	<1	ND/ 1804	<1	ND/ 1866##	<1	ND/ 1890	<1	ND/ 1805	<1
		3	ND/ 89	<1	ND/ 704	<1	ND/ 702	<1	ND/ 246	<1	ND/ 704	<1	ND/ 703	<1
	NP.1239 A→T	1	32/ 2428	1,3	279/ 5410	5,2	ND/ 3092	<1	ND/ 3372##	<1	ND/ 5410#	<1	ND/ 3092	<1
		2	ND/ 2345	<1	ND/ 2643	<1	ND/ 2453	<1	ND/ 2626	<1	ND/ 2643	<1	ND/ 2453	<1
		3	ND/ 1711	<1	ND/ 2111	<1	ND/ 2117	<1	ND/ 1712	<1	ND/ 2111	<1	ND/ 2117	<1
	NP.1489 G→A	1	ND/ 182	<1	26/ 336	7,7	ND/ 172	<1	ND/ 242	<1	26/ 376	6,9	ND/ 172	<1
		2	ND/ 436	<1	ND/ 452	<1	ND/ 444	<1	ND/ 451	<1	ND/ 451	<1	ND/ 444	<1
		3	ND/ 1320	<1	ND/ 1799	<1	ND/ 1799	<1	ND/ 1325	<1	ND/ 1799	<1	ND/ 1799	<1
	NS.827 C→T	1	ND/ 249	<1	19/ 419	4,5	ND/ 205	<1	ND/ 365	<1	21/ 412	5,3	ND/ 205	<1
		2	ND/ 1316	<1	ND/ 1423	<1	ND/ 1375	<1	ND/ 1427	<1	ND/ 1422	<1	ND/ 1375	<1
		3	ND/ 2091	<1	ND/ 2901	<1	ND/ 2757	<1	ND/ 2293	<1	ND/ 2898	<1	ND/ 2929	<1
	NS829 G→T	1	ND/ 221	<1	19/ 380	5,0	ND/ 179	<1	ND/ 328	<1	19/ 376	5,4	ND/ 179	<1
		2	ND/ 1302	<1	ND/ 1391	<1	ND/ 1341	<1	ND/ 1388	<1	ND/ 1389	<1	ND/ 1341	<1
		3	ND/ 2117	<1	ND/ 2852	<1	ND/ 2727	<1	ND/ 2279	<1	ND/ 2852	<1	ND/ 2880	<1
	NS.833 A→T	1	ND/ 187	<1	ND/ 287	<1	5/ 88	5,7	ND/ 259	<1	11/ 257	4,3	5/ 96	5,2
		2	ND/ 1224	<1	ND/ 1327	<1	ND/ 1284	<1	ND/ 1314	<1	ND/ 1322	<1	ND/ 1284	<1
		3	ND/ 1367	<1	ND/ 2430	<1	ND/ 2333	<1	ND/ 1779	<1	ND/ 2430	<1	ND/ 2360	<1
DETU	PB2.900 A→G	1	38/ 1335	2,9	136/ 2740	5,0	61/ 1231	5,0	68/ 1328	5,1	136/ 2740	5,0	65/ 1322	4,9
		2	35/ 1645	2,1	77/ 1800	4,3	66/ 1629	4,1	70/ 1775	4,0	77/ 1800	4,3	66/ 1629	4,1
		3	30/ 861	3,5	86/ 2308	3,7	47/ 1245	3,8	ND/ 1001##	<1	ND/ 2308#	<1	47/ 1245	3,8
	PB2.1054 T→C	1	69/ 1369	5,0	168/ 2637	6,4	97/ 1304	7,4	105/ 1393	7,5	168/ 2637	6,4	100/ 1376	7,3
		2	60/ 1477	4,1	115/ 1836	6,3	99/ 1605	6,2	113/ 1810	6,2	115/ 1836	6,3	99/ 1605	6,2
		3	6/ 392	1,5	94/ 2038	4,6	48/ 1054	4,6	32/ 524	6,1	94/ 2038	4,6	48/ 1054	4,6

Virus	Position	Sequence platform	Data Processing pipeline						Bam file generating processing pipeline					
			1		2		3				2		3	
			Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)
	PB2.2257 A→C	1	ND/ 867	<1	ND/ 1563	<1	24/ 463	5,2	ND/ 1447	<1	ND/ 1562	<1	26/ 472	5,5
		2	ND/ 531	<1	ND/ 581	<1	ND/ 378	<1	ND/ 588	<1	ND/ 580	<1	ND/ 378	<1
		3	ND/ 893	<1	ND/ 2286	<1	ND/ 1346	<1	ND/ 1341	<1	ND/ 2185	<1	ND/ 1347	<1
	PB2.2277 T→G	1	ND/ 644	<1	52/ 1150	4,5	27/ 307	8,8	ND/ 1062	<1	ND/ 1150#	<1	28/ 381	7,4
		2	ND/ 418	<1	ND/ 472	<1	ND/ 284	<1	ND/ 474	<1	ND/ 472	<1	ND/ 284	<1
		3	ND/ 1208	<1	ND/ 1948	<1	ND/ 1209	<1	ND/ 1251	<1	ND/ 1948	<1	ND/ 1214	<1
	PB1.14 C→T	1	ND/ 144	<1	48/ 433	11,1	ND/ 239	<1	ND/ 362	<1	48/ 433	11,1	ND/ 239	<1
		2	ND/ 90	<1	ND/ 355	<1	ND/ 304	<1	ND/ 345	<1	ND/ 351	<1	ND/ 304	<1
		3	ND/ 562	<1	ND/ 792	<1	ND/ 496	<1	ND/ 633	<1	ND/ 655	<1	ND/ 504	<1
	PB1.23 T→G	1	ND/ 207	<1	30/ 535	5,6	ND/ 315	<1	ND/ 470	<1	30/ 535	5,6	ND/ 315	<1
		2	ND/ 103	<1	ND/ 365	<1	ND/ 319	<1	ND/ 365	<1	4/ 365	2,0	ND/ 319	<1
		3	ND/ 699	<1	ND/ 950	<1	ND/ 609	<1	ND/ 702	<1	ND/ 950	<1	ND/ 609	<1
	PB1.87 A→G	1	ND/ 744	<1	ND/ 1644	<1	ND/ 1076	<1	ND/ 1218	<1	ND/ 1644	<1	ND/ 1076	<1
		2	49/ 365	13,4	ND/ 677	<1	ND/ 576	<1	13/ 638	2,0	ND/ 674	<1	ND/ 576	<1
		3	ND/ 721	<1	ND/ 1156	<1	ND/ 793	<1	ND/ 731	<1	ND/ 1156	<1	ND/ 793	<1
	PB1.2240 G→C	1	ND/ 757	<1	23/ 1517	1,5	26/ 515	5,0	ND/ 1266	<1	ND/ 1515#	<1	28/ 631	4,4
		2	ND/ 944	<1	ND/ 985	<1	ND/ 806	<1	ND/ 994	<1	ND/ 984	<1	ND/ 806	<1
		3	ND/ 274	<1	ND/ 439	<1	ND/ 253	<1	ND/ 301	<1	ND/ 439	<1	ND/ 253	<1
	PB1.2268 A→G	1	5/ 470	1,1	33/ 928	3,6	22/ 278	7,9	28/ 420	6,7	ND/ 928##	<1	23/ 354	6,5
		2	ND/ 798	<1	ND/ 829	<1	ND/ 671	<1	ND/ 839	<1	ND/ 829	<1	ND/ 671	<1
		3	ND/ 109	<1	ND/ 259	<1	ND/ 123	<1	ND/ 193	<1	ND/ 259	<1	ND/ 126	<1
	PB1.2271 A→G	1	12/ 446	2,7	59/ 901	6,5	16/ 263	6,1	29/ 413	7,0	59/ 901	6,6	21/ 336	6,3
		2	ND/ 729	<1	47/ 810	5,8	40/ 649	6,2	43/ 750	5,7	47/ 810	5,8	40/ 649	6,2
		3	1/ 32	3,1	ND/ 123	<1	2/ 83	2,4	5/ 75	6,7	5/ 124	4,0	1/ 83	2,4
	HA.867 C→T	1	59/ 1533	3,8	206/ 3183	6,5	104/ 1537	6,8	112/ 1584	7,1	206/ 3183	6,5	109/ 1573	6,9
		2	59/ 2031	2,9	150/ 2525	5,9	127/ 2253	5,6	144/ 2502	5,8	150/ 2525	5,9	127/ 2253	5,6
		3	11/ 180	6,1	48/ 647	7,4	28/ 385	7,3	13/ 182	7,1	48/ 647	7,4	28/ 385	7,3
	HA.963 T→C	1	122/ 1401	8,7	446/ 3071	14,5	189/ 1419	13,3	200/ 1468	13,6	446/ 3071	14,5	193/ 1455	13, 3
		2	90/ 1517	5,9	318/ 2189	14,5	247/ 1828	13,5	308/ 2165	14,2	318/ 2189	14,5	247/ 1828	13, 5
		3	5/ 69	7,2	107/ 606	17,7	47/ 293	16,0	12/ 81	14,8	107/ 606	17,7	47/ 293	16, 0

Virus	Position	Sequence platform	Data Processing pipeline						Bam file generating processing pipeline					
			1		2		3				2		3	
			Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)
	NP.1491 C→A	1	ND/ 278	<1	71/ 583	12,2	ND/ 206	<1	ND/ 390	<1	ND/ 579#	<1	ND/ 206	<1
		2	ND/ 723	<1	ND/ 769	<1	ND/ 692	<1	ND/ 766	<1	ND/ 769	<1	ND/ 692	<1
		3	ND/ 799	<1	ND/ 2031	<1	ND/ 1206	<1	ND/ 858	<1	ND/ 2031	<1	ND/ 1206	<1
	NA.65 T→C	1	19/ 503	3,8	52/ 1229	4,2	16/ 467	3,4	22/ 535	4,1	52/ 1229	4,2	20/ 540	3,7
		2	20/ 662	3,0	50/ 1104	4,5	45/ 992	4,5	52/ 1063	4,9	50/ 1104	4,5	45/ 992	4,5
		3	24/ 557	4,3	53/ 1099	4,8	37/ 727	5,1	28/ 584	4,8	53/ 1099	4,8	37/ 727	5,1
	NA.78 T→C	1	23/ 599	3,8	57/ 1403	4,1	20/ 557	3,6	23/ 622	3,7	57/ 1403	4,1	24/ 638	3,8
		2	21/ 692	3,0	55/ 1147	4,8	50/ 1033	4,8	54/ 1109	4,9	55/ 1147	4,8	50/ 1033	4,8
		3	23/ 580	4,0	51/ 1124	4,5	37/ 735	5,0	27/ 585	4,6	ND/ 1124#	<1	37/ 735	5,0
	NA.89 T→C	1	23/ 713	3,2	55/ 1670	3,3	22/ 651	3,4	26/ 731	3,6	55/ 1670	3,3	26/ 751	3,5
		2	23/ 798	2,9	56/ 1261	4,4	50/ 1134	4,4	54/ 1224	4,4	56/ 1261	4,4	50/ 1134	4,4
		3	24/ 580	4,1	55/ 1196	4,6	40/ 775	5,2	28/ 587	4,8	55/ 1196	4,6	40/ 775	5,2
	NA.117 T→C	1	37/ 908	4,1	87/ 2140	4,1	36/ 818	4,4	40/ 914	4,4	87/ 2140	4,7	43/ 922	4,7
		2	28/ 1102	2,5	67/ 1631	4,1	ND/ 1459	<1	70/ 1586	4,4	67/ 1631	4,1	ND/ 1459	<1
		3	22/ 531	4,1	57/ 1276	4,5	42/ 812	5,2	28/ 544	5,2	ND/ 1276#	<1	42/ 812	5,2
	NA.126 T→C	1	37/ 983	3,8	83/ 2294	3,6	36/ 876	4,1	39/ 973	4,0	83/ 2294	3,6	43/ 981	4,4
		2	31/ 1126	2,8	72/ 1676	4,3	65/ 1502	4,3	75/ 1616	4,6	72/ 1676	4,3	65/ 1502	4,3
		3	26/ 519	5,0	62/ 1395	4,4	43/ 812	5,3	30/ 537	5,6	62/ 1395	4,4	43/ 812	5,3
UKDD	PB2.2277 T→G	1	ND/ 415	<1	28/ 507	5,5	ND/ 475	<1	ND/ 503	<1	ND/ 507#	<1	ND/ 475	<1
		2	ND/ 589	<1	ND/ 620	<1	ND/ 601	<1	ND/ 627	<1	ND/ 620	<1	ND/ 601	<1
		3	ND/ 1140	<1	ND/ 1996	<1	ND/ 2065	<1	ND/ 1186	<1	ND/ 1996	<1	ND/ 2071	<1
	PB2.2278 T→G	1	ND/ 367	<1	ND/ 471	<1	ND/ 464##	<1	ND/ 465	<1	ND/ 471	<1	17/ 268	6,3
		2	ND/ 581	<1	ND/ 613	<1	ND/ 581	<1	ND/ 621	<1	ND/ 588	<1	ND/ 581	<1
		3	ND/ 1141	<1	ND/ 1985	<1	ND/ 1993	<1	ND/ 1184	<1	ND/ 1975	<1	ND/ 2004	<1
	PB1.87 A→G	1	ND/ 387	<1	ND/ 440	<1	ND/ 439	<1	ND/ 451	<1	ND/ 417	<1	ND/ 439	<1
		2	26/ 327	8,0	32/ 395	8,1	ND/ 351	<1	33/ 385	8,6	ND/ 395#	<1	ND/ 351	<1
		3	ND/ 617	<1	ND/ 1133	<1	ND/ 1136	<1	ND/ 622	<1	ND/ 1133	<1	ND/ 1136	<1
	PB1.728 C→A	1	ND/ 750	<1	ND/ 832	<1	ND/ 836	<1	ND/ 853	<1	ND/ 832	<1	ND/ 836	<1
		2	ND/ 776	<1	52/ 928	5,6	ND/ 829	<1	ND/ 888	<1	ND/ 912##	<1	ND/ 829	<1
		3	ND/ 2459	<1	ND/ 4290	<1	ND/ 4293	<1	ND/ 2471	<1	ND/ 4287	<1	ND/ 4292	<1

Virus	Position	Sequence platform	Data Processing pipeline						Bam file generating processing pipeline					
			1		2		3				2		3	
			Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)	Minor variants	Percentage (%)
	PB1.730 C→T	1	ND/ 742	<1	ND/ 824	<1	ND/ 826	<1	ND/ 844	<1	ND/ 824	<1	ND/ 826	<1
		2	ND/ 767	<1	57/ 1008	5,7	ND/ 832	<1	ND/ 893	<1	ND/ 1008#	<1	ND/ 832	<1
		3	ND/ 2339	<1	ND/ 4286	<1	ND/ 4289	<1	ND/ 2464	<1	ND/ 4285	<1	ND/ 4284	<1
	PB1.883 G→C	1	ND/ 942	<1	ND/ 997	<1	ND/ 997	<1	ND/ 1016	<1	ND/ 997	<1	ND/ 997	<1
		2	ND/ 1689	<1	ND/ 1856	<1	ND/ 1760	<1	ND/ 1867	<1	ND/ 1856	<1	ND/ 1760	<1
		3	ND/ 2479	<1	47/ 690	6,8	ND/ 3681	<1	ND/ 2635	<1	ND/ 690##	<1	ND/ 3697	<1
	PA.49 G→C	1	ND/ 103	<1	6/ 117	5,1	ND/ 115	<1	ND/ 113	<1	ND/ 117#	<1	ND/ 115	<1
		2	ND/ 337	<1	ND/ 435	<1	ND/ 392	<1	ND/ 441	<1	ND/ 434	<1	ND/ 392	<1
		3	ND/ 111	<1	ND/ 207	<1	ND/ 204	<1	ND/ 113	<1	ND/ 206	<1	ND/ 206	<1
	PA.82 C→T	1	ND/ 155	<1	ND/ 180	<1	ND/ 177	<1	ND/ 179	<1	ND/ 180	<1	ND/ 177	<1
		2	ND/ 695	<1	ND/ 809	<1	ND/ 745	<1	ND/ 797	<1	ND/ 809	<1	ND/ 745	<1
		3	ND/ 64	<1	ND/ 247	<1	30/ 248	12,1	ND/ 74	<1	ND/ 247	<1	30/ 248	12,1
	NS.811 G→T	1	ND/ 221	<1	17/ 270	6,3	ND/ 249	<1	ND/ 261	<1	ND/ 270#	<1	ND/ 249	<1
		2	ND/ 2452	<1	ND/ 2725	<1	ND/ 2557	<1	ND/ 2742	<1	ND/ 2725	<1	ND/ 2557	<1
		3	ND/ 3117	<1	ND/ 4125	<1	ND/ 4139	<1	ND/ 3188	<1	ND/ 4124	<1	ND/ 4142	<1

Locations containing mSNV detections in the DPP3 mSNV analysis of the bam files but not in the original DPPs are marked in dank grey; Locations containing ≥1% mSNVs that could be reproduced by deleting DPP3s default 'Direction and position filters' are marked in black, with those exactly reproduced (#) and those approximately reproduced but with different coverages and/or variants (##).

Discussion

NGS data are used for different applications. Although sequence technologies and the accompanying analysis tools are subjected to rapid development, a lot of follow-up research is based on initial findings. Accuracy and repeatability are key values for proper scientific research but the impact of NGS results also reaches beyond science to clinical settings where important clinical management and treatment decisions are based on such results. In this study the comparability and repeatability of NGS data analyses were analysed using identical input material per virus but different laboratory workflows from nucleic acid extraction and sequencing to data analysis. In addition, the COMPARE "Data Hub" platform was tested for the purpose of

sharing large raw datafiles between institutions in an outbreak situation. Using this platform, raw sequence data files up to the size of 8 Gigabytes, alignment files and metadata files of three influenza A/H5N8 viruses were successfully shared in real-time among 3 institutions to allow independent sequencing and analysis procedures, including mSNV identification, to be performed. The Data Hub is available for all institutions.

The aim of this study was to determine how comparable consensus and minority variant results were between laboratories performing their standard analyses, and whether discrepancies could be attributed to the SP, DPP or a combination of both. With the lack of a ground truth/gold standard, all data obtained were compared amongst each other. Importantly, reliable consensus sequences were generated independently of the SP/DPP combination used, although the well-known artefactual InDels in homopolymer regions in SP3 (Roche 454 genome sequencer) sequence data required manual editing. Such consensus sequences routinely form the basis for a detailed characterization of the influenza strain in an outbreak situation, as they are used for the prediction of pathogenicity and pandemic potential of influenza strains.

In contrast to the reproducible generation of consensus genome sequences, the hypothesis that minority variants could be identified reproducibly has to be rejected. The observed differences were mainly attributed to the alignment processes in the different DPPs. The interpretation of minority variant analysis thus needs a different level of careful standardization and awareness about the possible limitations as shown in this study. Reproducibility of mSNV results appeared to be influenced by both the different SPs and DPPs. There was limited reproducibility of mSNV identification data, even for relative high frequency mSNVs. As expected, the reproducibility was best (30%) for mSNVs occurring in high frequency (≥10%), and least for the low frequent (≥1%) mSNVs (8.9% to 23.3%). Also, the number of positions with 1-5% mSNVs (with sufficient coverage) was much higher (223 in SP1 data, 236 in SP2 data, and 74 in SP3 data) than the number of positions with >5-10% mSNVs (n=27) or >10% mSNVs (n=10).

The set-up of this study allowed many variables to influence the final result. The differences from first laboratory procedures and sample preparations up to the final analysis methods can all have contributed to the observed differences in mSNV identification. At this level, especially with lacking an NGS gold standard, it becomes difficult to determine which identified mSNVs are 'true variants' and which could be due to systematic errors introduced by RNA isolation methods, amplification, sequencing or manipulated by data processing pipeline settings. Unsurprisingly, the results of this study imply that the choice of SP influences the final output, but the results from this study also indicate that the DPP, especially the alignment process,

influences coverage and thereby mSNV frequencies. Although the aim of this study was to explicitly compare the three institutions own standard workflows, some parameters (like the phred score and detection limit) were synchronized between the different DPPs. Moreover, the data from each SP were re-processed in each DPP. However, all DPPs use different underlying algorithms and interpret the set parameters differently which might all contribute to the observed differences. These results are partly in line with previous research that showed the need of NGS result validation and concluded that only those mSNVs with a coverage >100 and a frequency of >40% could be identified by NGS methods without secondary confirmation [29], however, this conclusion was based on using the same sample preparation method within a single laboratory. Another recent study sets the cut-off for intrahost virus diversity at 3% with input of at least 1000 RNA copies and a read depth of at least 400x at each genome position for Illumina sequencing [30].

Although some studies have been published on SP error rates [31-34] and PCR amplification induced variants [35-38], a gold standard system for mSNV analysis is lacking. In addition, the DPPs can alter the data due to elimination or inclusion of certain sequences based on the set quality parameters. Allowing too many low-quality reads or being too stringent on the data will influence the coverage per position and might also influence the accuracy of the mSNV identification rate, especially when the coverage is low [39, 40]. Although a low comparability of mSNVs identified in the different SP and DPP combinations was observed, it can be concluded that 454 (SP3) sequencing has approximately the same accuracy as Illumina (SP1 and 2) sequencing based on the number and percentage of reproducibility of mSNVs when ignoring InDel errors in homopolymer regions. Although, Roche 454 sequencing machines are no longer in production, it added value to include 454 sequencing as an alternative sequence platform to Illumina. In addition, because Roche 454 was the first commercially successful next generation sequencing system, it was used in research that served as a fundament for follow-up studies [41]. A comparison of Illumina with newer third or fourth generation sequencing platforms (e.g. Nanopore or Pac Bio) would be interesting in the future. In addition, it would be interesting to compare mSNV results of SPs outputting small sequence reads (like Illumina, 454 and Ion Torrent) to new sequencing techniques that output full-length sequence data (e.g. Nanopore [42]). The latter might be less vulnerable to quality trimming parameters compared to small reads and might provide a more consistent nucleotide coverage over complete gene segment. However, the overall error rate remains higher than the shorter read technologies and recent work concludes that it is not currently suitable for the detection of minor variants [30].

For mSNV analyses by different labs, very stringent SP/DPP protocols need to be evaluated, for instance by cross-validating results. To allow a better comparison it would be recommended to create some kind of gold standard by for instance evaluating parameters based on sequencing of technical replicates, and controlled mixes of clones. The mSNV analysis can be valuable for epidemiological tracing, to monitor early evolutionary events, or drug resistance, possibly host adaptation, but this would require reproducibility of study outcomes within and between laboratories. As this is currently not that case, more understanding of biases and errors generated by sample processing (enrichment procedures), sequencing strategy (amplicons, shotgun), sequencing chemistry (each of which have their own internal error rates) and the approach to data processing and analysis is needed. Understanding the parameters and thresholds in the software can be difficult and a systematic study using a pipeline where the effect of changing each of these parameters both individually and in combination is required to determine the optimal settings for minor variant analysis.

As alternate high-throughput sequencing technologies arise there will be a need to understand inherent error profiles and how those are handled in data processing approaches. Cross-validation should be supported by international proficiency tests on NGS techniques including mSNV analyses that would be instrumental in validation of results and may foster the trust in NGS-based diagnostics.

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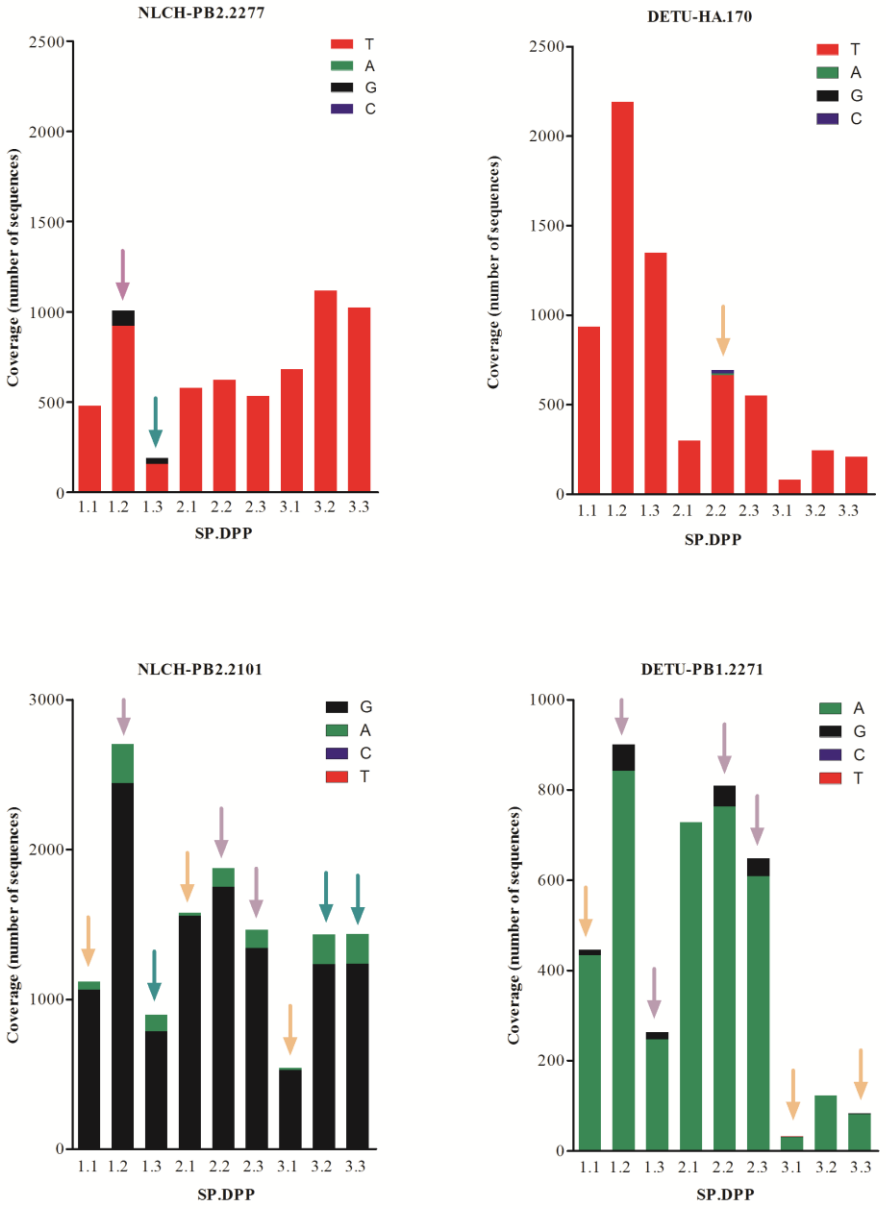
Supplemental Material

S1 Table. PCR primers used in SP3 to cover the influenza A H5N8 gene segments
Due to the size, this table was excluded from this thesis. This table will be published online with the manuscript or is available upon request
S2 Table. SP/DPP overarching consensus sequences
Due to the size, this table was excluded from this thesis. This table will be published online with the manuscript or is available upon request

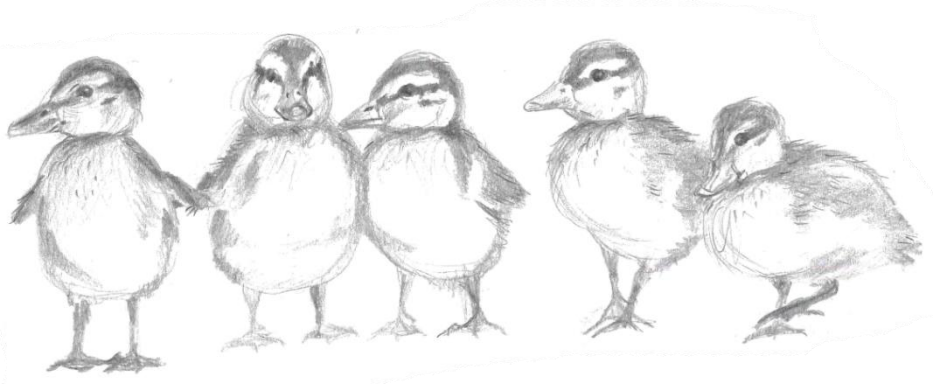
S3 Table. Number of raw sequences and influenza virus reads per SP per virus.
Due to the size, this file was excluded from this thesis. This file will be published online with the manuscript or is available upon request

S1 File. DPP3 Sequence analysis protocol
Due to the size, this file was excluded from this thesis. This file will be published online with the manuscript or is available upon request

S1 Figure. Nucleotide coverage. The non-normalised nucleotide coverage displayed as number of nucleotides per position for full genome sequences of the UKDD and DETU virus reads mapped to the corresponding reference sequences.
Due to the size, this file was excluded from this thesis. This file will be published online with the manuscript or is available upon request



S2 Figure. Graphical display of the coverage and allele counts for four positions, showing mSNVs in different frequencies for each SP/DPP combination. Arrows indicate the approximate percentages in which the mSNVs were detected; 1-5% (pink), 5-10% (purple) and >10% (green)



Josan

Chapter 5

Summarising discussion

Marjolein

Chapter 5

Summarising discussion

Avian influenza (AI) viruses are pathogens that occasionally jump to humans and non-avian species, and as a consequence represent pathogens of major concern for both animal and human health. Most AI viruses are low pathogenic avian influenza (LPAI) viruses, but two subtypes, H5 and H7, occasionally mutate to highly pathogenic avian influenza (HPAI) viruses in poultry ***[Chapter 1]***. HPAI viruses are associated with variable signs of disease in wild birds and high mortality rates in poultry. HPAI H5 viruses of the GSGD-lineage were first detected in China in 1996 and have circulated continuously in poultry since with frequent spill-overs to wild birds and subsequent virus emergence elsewhere in the world. These GSGD-lineage H5 viruses are subdivided in 10 different clades (clade 0-9) and multiple subclades ***[Chapter 1]***. This thesis describes research efforts to determine the role of wild birds in three successive outbreaks of HPAI H5 viruses in Europe since 2014 ***[Chapter 2.1, 2.2 and 3]***. In addition, we studied the applicability of serological assays to determine HPAI H5 host-species based on H5-specific antibody presence ***[Chapter 2.1 and 2.2]***. Lastly, we studied the applicability of next-generation sequencing (NGS) data for epidemiological studies in outbreak situations by evaluating the repeatability and comparability of NGS results from HPAI H5N8 viruses ***[Chapter 4]***. Based on the newly gained perspectives from this research, a number of proposals are provided to further improve surveillance efforts and related scientific output.

The role of wild birds in HPAI H5 outbreaks 2005-2018

Europe has been confronted with several outbreaks of Asian HPAI H5 viruses since 2005. The first outbreak was caused by a clade 2.2 H5N1 virus in 2005/06, followed by the detection of clade 2.3.2.1c H5N1 viruses in Eastern Europe in 2010. In 2014, clade 2.3.4.4 group A H5N8 viruses caused outbreaks across Europe, with a second introduction of clade 2.3.2.1c H5N1 virus in Eastern Europe in early 2015. Multiple outbreaks in Europe were caused by clade 2.3.4.4. group B H5N8 viruses in 2016/17 and clade 2.3.4.4 group B H5N6 viruses in 2017/18. Below we discuss our virological and serological findings from the three HPAI H5 clade 2.3.4.4 outbreaks and compare these to published data on previous HPAI H5 incursions, especially regarding the involvement and potential role of wild birds in these outbreaks.

2005/06: HPAI Clade 2.2 H5N1 viruses

The 2005/06 outbreak of HPAI H5 clade 2.2 viruses was preceded by virus detections in poultry and wild birds in Southeast Asia in late 2003/early 2004, with occasional detections in humans in China. From there, the virus was transported to Qinghai Lake, China where it was isolated from migratory birds during the spring of 2005 [1, 2]. By July 2005, these H5N1 viruses had spread to Russia and Kazakhstan [3, 4]. In October, the H5N1 virus was reported from Turkey, Romania [5] and Croatia [6], and from Ukraine in December 2005. Starting early 2006, the virus rapidly spread to central Europe, infecting poultry and wild birds, mainly mute swans (41% of dead birds) and tufted ducks (5% of birds) [7]. During the same period, related H5N1 viruses were also reported in wild birds and poultry in the Middle East and Africa.

2010: HPAI Clade 2.3.2.1c H5N1 viruses

In March 2010, viruses of clade 2.3.2.1c were detected in poultry and wild birds in Romania and Bulgaria, respectively [8]. These viruses had preceding detections in wild birds in Japan in 2008 and spread further into Qinghai Lake, China in the spring of 2009 [9], to Mongolia in the summer [10] and to Russia by October 2009 [11]. Phylogenetic studies showed that the European virus lineage was directly related to the clade 2.3.2.1 viruses detected at Qinghai Lake, China [8].

2014/15: HPAI clade 2.3.4.4 H5N8 group A viruses

HPAI GSGD-lineage H5 viruses of clade 2.3.4 were first identified in poultry in China in 2005 [12], and subsequently evolved into different subclades [13-15]. These viruses reassorted with other HPAI H5N1 viruses and Eurasian LPAI viruses, leading to the circulation of different subclades such as 2.3.4.4 in Southeast Asia since 2008 [15], although this subclade was not officially recognised until 2015 [16].

Group A (Buan-like) viruses were first identified in China and South Korea in early 2014 [13, 17]. Migratory birds were suspected to have transported these viruses from Eastern China to South Korea, leading to virus detections in poultry and wild birds near Donglim Lake, a wintering site for migratory birds in South Korea [18]. In late 2014, several outbreaks of group A viruses were reported by South Korea, China and Japan [19]. Meanwhile, group A H5N8 virus had also been identified in a Eurasian wigeon *(Anas penelope)*, a long-distance migrant, in Eastern Siberia in September 2014 [20]. Group A viruses further spread to Europe and North America in November and December 2014, respectively [21, 22]. In North America, these group A H5N8 viruses reassorted with local LPAI viruses leading to new reassortant viruses of the subtypes H5N1, H5N2, and H5N8 that caused major problems in the poultry industry until they disappeared in June 2015 [23]. The first detection of group A viruses in

Europe was in a German turkey farm on 4 November 2014, with 10 subsequent detections in poultry in Germany [24], the Netherlands [25], the United Kingdom [26], Italy [27], and lastly in Hungary on 23 February 2015 [27]. In response to these outbreaks, wild bird surveillance activities in the Netherlands were intensified, leading to the detection of HPAI H5N8 virus in droppings of three Eurasian wigeons in November 2014 [28] and February 2015 ***[Chapter 2.1]***. Considering the number of poultry farms infected, the number of detections in wild birds was very limited without any substantial mortality events observed. Also, from other European countries the number of detections in wild birds was limited to a hunted common teal *(Anas crecca)* [29], several dead mallards *(Anas platyrhynchos)* and a sick gull in Germany *(Larus spp.)* [19, 30-32]), and two dead mute swans *(Cygnus olor)* in Sweden [27]. A previous serological study had demonstrated that haemagglutination inhibition (HI) assays could be used to discriminate HPAI-exposed birds from LPAI-exposed birds based on H5-specific antibodies [33]. Serological testing specific for HPAI H5 clade 2.3.4.4 antibodies was first performed and validated with wild bird sera collected between November 2014 and May 2015 in the Netherlands ***[Chapter 2.1]***. These HI assays showed an overall HPAI H5 clade 2.3.4.4-specific antibody incidence of 4.6% (43/940 birds of 29 species). Antibodies were detected in 12 Eurasian wigeons, 29 mute swans, 1 lesser white-fronted goose *(Anser erythropus)* and 1 common coot *(Fulicia atra)* ***[Chapter 2.1]***. Confirmatory testing with a virus neutralisation (VN) assay confirmed antibodies in all species except the lesser white-fronted goose. The serological results were in agreement with results from virological studies showing the presence of virus in Eurasian wigeons in Russia [20] and the Netherlands [28] ***[Chapter 2.2]***, mute swans in Sweden [27], and a common coot in South Korea [34]. Although discrepancies between HI and VN assays were observed, and low titres could not always be detected by confirmatory testing by a second laboratory, serology showed promise as a cost-effective tool to identify HPAI H5 virus exposed host species ***[Chapter 2.1]***. The low number of virus-positive birds and the antibody incidence of 4.6% was strongly indicative for non-lethal or possibly even subclinical HPAI H5 infections in these four species. The limited active surveillance activities performed in Europe in 2014/15 and the possibility of non-lethal infections might explain this low number of wild birds found infected given that most countries in Europe relied on passive surveillance, if any at all. In response to this outbreak, a global phylogeny study [21] confirmed the previously published hypothesis [35] that wild birds were most likely responsible for this global dispersal of HPAI H5 clade 2.3.4.4 viruses.

2014/15: HPAI clade 2.3.2.1c H5N1 viruses

During 2014/15, a different clade 2.3.2.1c H5N1 virus was detected in captive wild birds in Dubai [36] after it had been detected in Russia in the spring of 2014 [37]. This virus

from Dubai detected in December 2014 clustered with viruses detected shortly thereafter in West African countries and Bulgaria and Romania in early 2015 [36, 37]. This virus seems to have been dispersed by wild migratory birds from Asia, although the early introduction into Dubai may have been caused by falconry activities [36, 38, 39].

2016/17: HPAI clade 2.3.4.4 H5N8 group B and reassortant viruses

Simultaneously with group A viruses, group B (Gochang-like) viruses were first identified as H5N8 viruses in China in 2013/14 [40] and in South Korea in 2014 [17]. In China, several group B viruses that had reassorted with Eurasian LPAI viruses were reported in domestic ducks and wild waterbirds during 2013/14 [13, 40-42]. Hereafter, the virus was not detected until it reappeared at Qinghai Lake, China [43] and Uvs-Nuur Lake, Russia, both in May 2016 [44]. Starting in October 2016, group B H5N8 viruses spread further to Europe, Africa [45], the Middle East [46], and became more widespread in Asia [47, 48]. In Europe, the deaths of many wild birds, mainly tufted ducks *(Aythya fuligula)*, great crested grebes *(Podiceps cristatus)* and Eurasian wigeons [49-51] ***[Chapter 2.2]*** preceded a number of virus outbreaks in poultry [52, 53]. Later, a relative high number of birds of prey were found dead as well [51]. Despite the large number of wild birds that died during this outbreak, the virus was also detected in 32/5,167 live and clinically healthy birds sampled between 23 November 2016 and 28 January 2017 in the Netherlands ***[Chapter 2.2]***. The virus-positive species detected in active surveillance activities were largely in agreement with those found in passive surveillance [52, 53], although the non-lethal infections in mallards from January 2017 would have been missed without active surveillance efforts. Genetic analyses of the Dutch HPAI H5N8 viruses showed that these viruses were reassortants between group B viruses as detected in Eastern China in 2014 and Eurasian LPAI viruses ***[Chapter 2.2]***. This genetic constellation was similar to that of viruses detected in Russia in May 2016 and in Germany in late 2016, although additional local reassortment events of the NP and PA genes were observed, similar to those described for German viruses [49]. In contrast to the 2014/15 outbreak, viruses of group B showed frequent reassortment with local LPAI viruses, resembling the 2014/15 situation in the United States [22, 23]. During the 2016/17 outbreak, these local reassortment events led to the emergence and detection of multiple group B H5N5 and one H5N6 virus [53]. Recently, group B H5N8 viruses continued to be reported, but less frequently from Europe and more frequently from the Middle East and Africa [53, 54]. Serological assays performed on sera obtained during the winter of 2015/16 and 2016/17 revealed an antibody incidence of 3.5% (12/347) and 4.2% (18/431), respectively ***[Chapter 2.1 and 2.2]***. In contrast to the 2014/15 outbreak, the antibody incidence at the start of the 2016/17 outbreak was rather low (2.0%) but increased over time. This might be indicative for a different timing of virus

amplification, before arrival at the European wintering grounds in 2014/15, versus at the European wintering grounds in 2016/17 ***[Chapter 2.1 and 2.2]***. The avian species identified to have been infected by serological methods were similar to those detected in virological assays; Eurasian wigeons, mute swans, mallards and black-headed gulls *(Chroicocephalus ridibundus)* ***[Chapter 2.2]***. To date, group B clade 2.3.4.4 viruses have caused the largest global HPAI H5 epidemic in number of poultry outbreaks, and rate of mortality in wild birds [7, 49, 51, 55].

2017/18: HPAI clade 2.3.4.4 H5N6 group B viruses

HPAI group B H5N6 viruses were first detected in Japan, South Korea [56-58] and the Netherlands [59] ***[Chapter 3]*** in late 2017. Starting in mid-December 2017, HPAI H5N6 group B viruses were also detected in wild birds in Iran and other European countries including Switzerland, the United Kingdom, Germany, Sweden, Ireland, Denmark, Slovakia and Finland [53, 54]. Outbreaks in poultry were only reported in the Netherlands and Germany. Although most H5N6 virus detections in wild birds were based on passive surveillance activities, these viruses did not cause mass mortality events in wild birds, in contrast to the group B H5N8 viruses circulating the previous year [51] ***[Chapter 2.2]***. Interestingly, birds of prey were frequently found dead and infected, mainly white-taled eagles *(Haliaeetus albicilla)*. Group B H5N6 viruses were also detected in living and clinically healthy Eurasian wigeons in the Netherlands and a mallard and an Armenian gull *(Larus armenicus)* in the Republic of Georgia ***[Chapter 3]***. Genetic analyses of these group B H5N6 viruses have shown that they most likely did not originate in Southeast Asia, but were new reassortant viruses between European group B H5N8 viruses and Eurasian LPAI viruses, most likely originating on the Palearctic breeding grounds during 2017. This would explain the simultaneous first detections of these viruses in wild birds in late 2017 in both Europe and Southeast Asia ***[Chapter 3;*** *[57, 59]*.

The applicability of NGS in outbreak situations

In recent years, sequencing has been applied routinely in virus diagnostics and virus characterization, enabling comparison analyses of avian influenza viruses at the genome level for epidemiological studies. Especially in outbreak situations, like those of clade 2.3.4.4 viruses, sequence analysis was an indispensable tool to determine the source of the virus and the individual gene segments ***[Chapter 2.2 and 3]***. Next-generation sequencing (NGS) methods have facilitated sequencing of hundreds to thousands of avian influenza virus particles in real-time from a single sample, enabling the detection of single nucleotide variants (SNVs) that are used for genetic tracking of viruses, and minority SNVs (mSNV) that could potentially be used for

more detailed epidemiological tracking of viruses, to identify host adaptation markers, or explain differences in clinical presentation of infection ***[Chapter 1]***. Despite the lack of a gold standard technique, the number of studies cross-validating NGS data generated with different sequence platforms (SP) and bio-informatics data-processing pipelines (DPP) is very limited. In the light of the EU Horizon 2020 COMPARE programme, the reproducibility of NGS data analyses between three laboratories was tested using HPAI H5N8 group A outbreak viruses. To determine the comparability and reproducibility of generating consensus sequences and identifying mSNVs, the raw sequence data from three SPs and three DPPs were shared between the laboratories ***[Chapter 4]***. Consensus sequences were reliably determined regardless of the SP/DPP combination used. However, the reproducibility of mSNVs was suboptimal, with only 40% of high frequent minority variants (occurring in >10% of the sequences) confirmed in any of the other tested SP/DPP combinations. This is not surprising considering the many factors influencing all stages of NGS data generation. In addition to SP-dependent error-profiles [60-63], PCR-amplification steps [64-67], differences in nucleotide coverage (number of reads) [68, 69], and individually set quality parameters for data inclusion and mSNV identification have large influences on the resulting data ***[Chapter 4]***. In conclusion, NGS technologies can be reliably used to generate consensus sequence data in an outbreak situation. In the future, mSNV detection might prove to be a valuable tool for more detailed tracing of viruses, host-adaptation or clinical manifestation. However, before mSNV data can be reliably used, NGS techniques need further (cross-) validation to improve the reproducibility between workflows and laboratories ***[chapter 4]***.

Recommendations to optimise (inter)national surveillance efforts

The joint worldwide efforts made in avian influenza surveillance programmes have provided many insights that formed the basis of evidence-based hypotheses about the epidemiology of HPAI viruses. However, we currently lack a true early warning system for incursions of HPAI viruses. In order to further improve surveillance and scientific output, some challenges with regard to location of surveillance, timing of surveillance, species responsible for long-distance dispersal of virus, surveillance strategies, host migratory behaviour, avian influenza diagnostics, and data sharing need addressing.

Reviewing the most recent three incursions of HPAI H5 viruses in Europe and comparing the dynamics to the earlier ones revealed insights into the earliest virus detection patterns, in agreement with those previously described [37], that may be considered as 'early warning signals'. Generally, the HPAI H5 outbreaks in Europe were preceded by detections of the virus in Southeast Asia, followed by virus spread

to other Asian countries in the first months of the year. This was followed by spreading west to north-central China (Qinghai Lake), Mongolia including the Russia-Mongolia border area (Uvs-Nuur and Khovsgol Lake), and the Kazakhstan-Russia border area in the spring, with subsequent further geographical spread westwards. Early detection sites in Europe were the Black Sea area, the Baltic Sea area and Lake Constance. Increased surveillance activities at the Asian and Russian early detection sites may provide an early warning system for future virus spread to Europe. Moreover, other, possibly currently unsurveyed, early detection sites may exist. For instance, the widespread Palearctic (post-) breeding sites are suspected to serve as virus hotspots, because of the aggregation of many birds from different geographical locations and the birth of many immunologically naïve juveniles. Although monitoring for virus prevalence during the breeding period is close to impossible at these sites, post-breeding moulting sites could provide insight into the virus events that occurred during breeding, and might be used to catch birds that are impossible to catch otherwise.

Preceding detections in previous HPAI H5 incursions also provided leads about the optimal timing for surveillance. After Southeast Asia, Qinghai Lake is the first early detection site visited by spring migrants in May/June. Thereafter, birds continue their migration to the Palearctic breeding sites, with detections in Russia/Mongolia both before (May/June) and after (July – September) breeding. The timing of the first virus detections in Europe coincided with the early arrival of long-distance migrants in September/October.

Long-distance migrants identified in previous HPAI H5 incursions, including Eurasian wigeons, tufted ducks, common coots and Eurasian teals, might act as key species for global virus spread. Although many avian species have been found infected with HPAI H5 viruses, most are local species or short-distant migrants, probably playing only a limited role in global virus dispersal. This information should be better represented in the EU law. This currently only contains a high-risk species list (EU Commission Decision 2005-726) for passive surveillance, that has not been updated since 2005, although a more up-to-date lists for passive surveillance has already been published [70], and mentions species that deserve extra attention if found diseased or dead. For instance, large gulls, mute swans and birds of prey seem to act as sentinels, even in the absence of mortality in wild duck species. However, neither of these lists mention species that should be tested live because of their possible involvement in long-distance dispersal of the virus, or species to test in case of absence of observed mortality in wild birds. An alternative list prioritising species likely involved in long-distance virus movement versus species likely acting as local sentinels should be made to guide active surveillance programmes for true early warning purposes.

Passive surveillance efforts on Asian early detection sites, including the Asian part of Russia, should in spring and summer be complemented with active surveillance activities, especially in the absence of wild bird mortality [70]. To maximise the chance of early detection of viruses entering Europe, European national surveillance programmes should focus on sampling long-distance migratory species upon early arrival from early autumn through early winter, guided by detections of HPAI viruses at early detection sites if available. Environmental sampling (fresh faeces) of these species provides a cost-effective and statistically high-powered sampling system [71]. In case live birds can be caught, taking blood samples in addition to cloacal and oropharyngeal swabs would enable to determine both the current and recent HPAI H5 infection status.

With the revision of the identification of high-risk species for HPAI dispersal, additional knowledge on their migratory behaviour, including inter- and intra-species differences is needed. Recent developments in GPS-tagging techniques now enable accurate, long-term and cost-effective tracking of birds (e.g. www.movebank.org). The low costs of GPS-tagging may also allow the tracking of populations tested positive for HPAI virus at Asian early detection sites (including the Asian part of Russia), to track virus vector movements in real time. This information might also reveal a more optimal timing or more accessible locations for surveillance.

With regard to diagnostics, there is a need for accurate, quicker and more cost-effective methods. The development of rapid (subtype specific) diagnostic tests (equivalent to human point of care [POC] tests) to detect the presence of avian influenza viruses in diagnostic material is needed to allow for cheap, real-time on-site screening under field conditions. Secondly, there is a need for sequencing/NGS methods that are practical under field conditions and/or to enable cheap and real-time genotype determination in a laboratory setting (e.g. Oxford Nanopores MinION). Also, to determine target species for surveillance, there is a need for robust, quick and cheap validated serological assays.

Avian influenza epidemiology studies are highly dependent on the public availability of genome sequence data and associated metadata. Although platforms like GISAID provide safe environments to share genomic data, some researchers and countries remain hesitant to do so, which negatively influences accurate and real-time epidemiological tracing of viruses and risk-evaluation. Additionally, sharing clinical samples and viruses across borders is a cumbersome process that limits and sometimes even prohibits sharing of materials and thereby international collaboration.

Conclusion

Various surveillance programmes have provided substantial new knowledge on avian influenza in wild birds since the first European outbreak with Asian HPAI H5 viruses in 2005. This knowledge can guide the optimization of future surveillance programmes and maximize the accompanying scientific output. Despite currently remaining gaps in knowledge, future evidence-based hypothesis-driven studies can reveal additional crucial insights into the dynamics of HPAI viruses. Such future studies would benefit from interdisciplinary international collaborations between virologists, ornithologists, ecologists, epidemiologists, pathologists, wildlife health experts, veterinarians and immunologists to work towards a global understanding of HPAI virus epidemiology and the design of appropriate preventive measures. International (animal) health organisations, national and international policy makers and politicians, and legal experts should facilitate such "One World, One Health" approaches on influenza.

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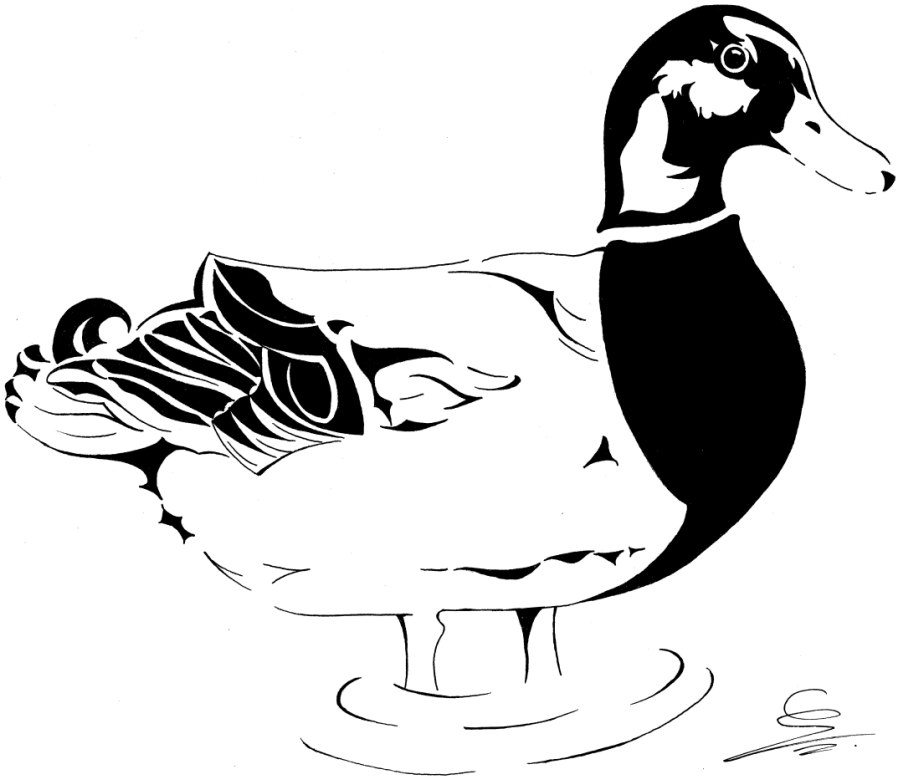
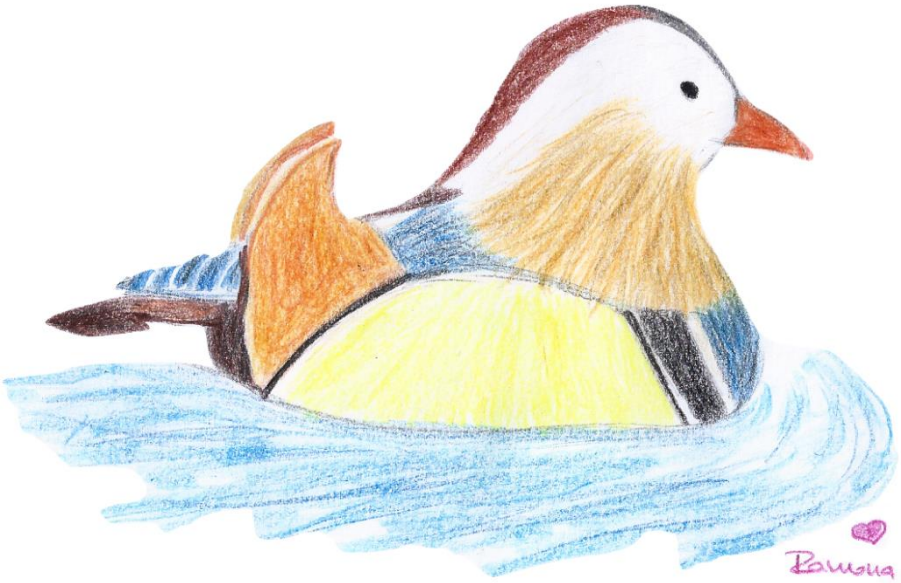


Carolien

Chapter 6

Dutch summary



Ramona

Chapter 6

Nederlandstalige samenvatting

Vogelgriepvirussen (aviaire influenzavirussen) staan wereldwijd in de belangstelling doordat deze virussen een constante bedreiging vormen voor de gezondheid van mens en dier. Vogelgriepvirussen kennen twee verschijningsvormen, als laagpathogene aviaire influenza (LPAI) en als hoogpathogene aviaire influenza (HPAI) virussen. Wilde vogels, met name soorten behorende tot de ordes *Anseriformes* (vooral eenden, ganzen en zwanen) en *Charadriiformes* (onder andere meeuwachtigen), zijn de natuurlijke gastheersoorten voor LPAI virussen. LPAI virussen van de subtypen H5 en H7 kunnen in pluimvee veranderen (muteren) naar de gevaarlijker HPAI vorm. In tegenstelling tot LPAI virussen zorgen HPAI virussen voor snelle en massale sterfte in pluimvee en wisselende sterfte in wilde vogels. Ook zijn sommige HPAI virussen in staat om mensen te infecteren. In Zuidoost-Azië circuleren sinds 1996 HPAI H5 virussen die afstammen van het virus A/Goose/Guangdong/1/1996 (GSGD). Virussen die tot de GSGD groep behoren hebben zich in de tijd verder ontwikkeld waardoor ze in verschillende subgroepen in te delen zijn, 'clades' geheten *(hoofdstuk 1)*. Sinds 2014 hebben HPAI H5 virussen van subclade 2.3.4.4 gezorgd voor drie opeenvolgende virusuitbraken in Europa. Vóór 2014 is Europa twee keer getroffen door HPAI virussen uit de GSGD groep: in 2005/06 hebben clade 2.2. virussen veel schade aangericht in heel Europa en in 2010 zijn clade 2.3.2.1 virussen in Oost-Europa gevonden. Gebaseerd op de epidemiologie en de verspreidingspatronen van deze virussen werden wilde vogels verdacht als mogelijke vectoren. Migrerende wilde vogels zijn in staat om in korte tijd grote afstanden af te leggen, met name tijdens de migratie tussen de broedgebieden in Siberië en de overwinteringsgebieden in Europa, Azië, het Midden-Oosten of Afrika. Het samenkomen van grote aantallen vogels afkomstig uit verschillende gebieden biedt de mogelijkheid voor virussen om zich verder te verspreiden. Dit proefschrift beschrijft het onderzoek dat verricht is naar de rol die wilde vogels spelen in de verspreiding van vogelgriep in drie opeenvolgende Europese virusuitbraken vanaf 2014. Daarnaast is er gekeken naar de bruikbaarheid van serologie en 'next-generation sequencing' (NGS) als diagnostische methoden in uitbraaksituaties.

De eerste uitbraak van clade 2.3.4.4 virussen: HPAI H5N8 groep A

Vanaf begin 2014 veroorzaakten clade 2.3.4.4 H5N8 virussen uitbraken in pluimvee en wilde vogels in Azië. Deze virussen werden ingedeeld in twee groepen op basis van hun genetische samenstelling, groep A en groep B. Na de eerste detectie hebben virussen van groep A zich verder verspreid naar Europa en Noord-Amerika,

respectievelijk in november en december 2014. In tegenstelling tot de grote problemen die dit virus in Noord-Amerika heeft veroorzaakt in met name pluimvee, bleef het aantal detecties van dit virus in pluimvee en wilde vogels in Europa relatief gering, zonder waarneembare sterfte van wilde vogels. De laatste waarneming van dit virus in Europa was in februari 2015. Naar aanleiding van de eerste virusdetectie in Europa werd het in Nederland lopende actieve wildevogelsurveillanceprogramma, het testen van levende en gezonde dieren, geïntensiveerd. Wilde vogels van vele soorten werden getest op aanwezigheid van het H5N8 virus ten tijde van de uitbraak (n=5.389) en gedurende het opvolgende jaar (n=5.968). Het virus werd gevonden in drie smienten in december 2014 en februari 2015 *(hoofdstuk 2.1)*. Deze resultaten kwamen overeen met de bevindingen in andere Europese landen waar ook weinig viruspositieve wilde vogels werden gevonden. Wanneer een vogel een infectie doormaakt, kunnen antistoffen tegen het virus gevormd worden die in het bloed komen. Eerder onderzoek had aangetoond dat met serologische testen (het testen van antistoffen in het bloed) onderscheid gemaakt kon worden tussen antistoffen die gevormd waren tegen verschillende specifieke HPAI H5 clades. Om de toepasbaarheid van deze serologische testen in een uitbraaksituatie te testen, werden ten tijde van de uitbraak bloedmonsters van wilde vogels genomen (n=945) om te bepalen welke vogelsoorten een infectie hadden doorgemaakt en mogelijk gastheersoorten zijn die een rol kunnen hebben in de verspreiding van het virus. Om de serologische test te valideren werden ook bloedmonsters van vóór de uitbraak en een jaar erna meegenomen. Antistoffen specifiek gericht tegen deze clade 2.3.4.4 virussen waren zoals verwacht afwezig in bloedmonsters die genomen waren vóór 2014, maar aanwezig in bloedmonsters genomen tijdens (4,6%) en in mindere mate een jaar na de uitbraak (3,5%) *(hoofdstuk 2.1)*. De resultaten van de serologie en virologie wezen erop dat HPAI H5N8 virussen na de winter van 2014/15 niet in hoge mate in wilde vogels hadden gecirculeerd en dat het niet aannemelijk was dat deze virussen zich zouden handhaven in de wildevogelpopulatie.

De tweede uitbraak van clade 2.3.4.4 virussen: HPAI H5N8 groep B

Eind 2016 doken er opnieuw HPAI H5N8 virussen van clade 2.3.4.4 op in Europa na eerdere detecties van dit virus in Azië (inclusief het Aziatische deel van Rusland). Genetische analyses van dit virus toonden aan dat het tot groep B behoorde. Dit werd daarmee de tweede onafhankelijke introductie van een Aziatische clade 2.3.4.4 virus in Europa. In tegenstelling tot de 2014/15 uitbraak vertoonden deze H5N8 virussen uitwisseling van gensegmenten ('reassortment') met lokaal circulerende LPAI virussen. Deze tweede uitbraak van H5N8 virussen ging gepaard met hoge sterfte van wilde vogels in meerdere Europese landen. De vogelgroepen die vaak dood gevonden werden waren eendachtigen, meeuwen en roofvogels. De laatste groep raakte waarschijnlijk geïnfecteerd door het eten van zieke of dode watervogels. Ook in reactie op deze tweede uitbraak van H5N8 virussen werd de

surveillance in wilde vogels in Nederland geïntensiveerd. HPAI H5N8 virussen konden worden aangetoond in 57 wilde vogels van 12 soorten tijdens actieve (32/5.167) en passieve (25/36) surveillance-activiteiten, respectievelijk het testen van levende en dode vogels, tussen november 2016 en april 2017 *(hoofdstuk 2.2)*. De vogelsoorten die viruspositief werden gevonden in actieve en passieve surveillance kwamen grotendeels overeen. Ook de soorten waarin antistoffen tegen deze clade 2.3.4.4 virussen werden gevonden kwamen grotendeels overeen met de soorten die positief werden gevonden tijdens de eerste uitbraak; smienten, knobbelzwanen, kokmeeuwen en wilde eenden. Opvallend was dat het percentage dieren met antilichamen aan het begin van deze tweede uitbraak erg laag lag (2,0%) en gedurende de uitbraak steeg naar een gemiddelde incidentie van 4,2% *(hoofdstuk 2.2)*. Tijdens de 2014/15 uitbraak werden in een relatief hoog percentage vogels antistoffen gevonden terwijl het virus maar in een zeer laag aantal vogels werd gevonden. In tegenstelling daarmee werden er in de 2016/17 uitbraak in minder vogels antistoffen gevonden terwijl het virus juist in veel vogels werd aangetoond. Dit kan duiden op een andere timing van de piek in virusinfecties; in 2014/15 vóór aankomst en in 2016/17 na aankomst op de Europese overwinteringsgebieden.

De derde uitbraak van clade 2.3.4.4 virussen: HPAI H5N6 groep B

Vanaf december 2017 werden meerdere Europese landen getroffen door HPAI H5N6 clade 2.3.4.4 virussen die uitbraken in (hobby)pluimvee en wilde vogels veroorzaakten. Tegelijkertijd kwamen er meldingen uit Zuid Korea en Japan waar deze virussen ook voor het eerst werden aangetroffen. In tegenstelling tot de eerste twee uitbraken met H5N8 virussen, was dit H5N6 virus niet eerder ontdekt in Zuidoost Azië of Rusland. Genetische analyses van dit virus toonden aan dat dit virus waarschijnlijk tijdens 2017 ontstaan is uit een combinatie van genen afkomstig van het 2016/17 Europese HPAI H5N8 virus en Europees/Aziatische LPAI virussen *(hoofdstuk 3)*. HPAI H5N6 virussen werden in Nederland gevonden in levende gezonde smienten en in de Republiek Georgië in een levende Armeense meeuw en een gejaagde wilde eend *(hoofdstuk 3)*. Hoewel er geen massale sterfte van onder wilde vogels is opgetreden, viel het op dat er relatief vaak melding gemaakt werd solitair gevonden zieke of dode roofvogels.

Het gebruik van next-generation sequencing methoden in uitbraaksituaties

De genetische code van vogelgriepvirussen kan worden verkregen door de sequentie te bepalen van het erfelijk materiaal (RNA). De genetische code kan gebruikt worden voor fylogenetische analyses waarmee bepaald kan worden waar en wanneer de meest verwante virussen/virusgenen vóórkwamen. Traditionele sequencing methoden bepalen de consensussequentie, d.w.z. de genetische code die in de meerderheid van de virusdeeltjes in een (klinisch) monster aanwezig is. Nieuwere technieken, zogeheten 'next-generation sequencing' (NGS) technieken,

zijn in staat om de genetische code van individuele virusdeeltjes in een monster te bepalen. Hierdoor kunnen ook variaties die in een minderheid van de virusdeeltjes voorkomen achterhaald worden, de zogeheten minderheid single nucleotide varianten (mSNV). NGS technieken worden steeds populairder om virusgenomen te analyseren in uitbraaksituaties. Met name het bepalen van mSNVs kan helpen bij epidemiologisch traceren van virussen, d.w.z. het opsporen van de bron. Echter, er is weinig onderzoek gedaan naar de invloed van verschillende technieken en data-analyse methoden op de betrouwbaarheid en vergelijkbaarheid van de resultaten. Om hier meer inzicht in te krijgen zijn drie HPAI H5N8 virussen, de NGS data en de analyse resultaten gedeeld en vergeleken tussen drie laboratoria *(hoofdstuk 4)*. Deze studie toonde aan dat consensussequenties betrouwbaar waren, ongeacht de gebruikte sequencingtechniek of analysemethode, maar dat de vergelijkbaarheid en reproduceerbaarheid van mSNVs suboptimaal was *(hoofdstuk 4)*. Voor het gebruik van mSNV data in onder andere epidemiologische studies moeten NGS technieken dus verder worden gestandaardiseerd en gevalideerd.

Conclusie

Wereldwijd wordt er veel onderzoek gedaan naar vogelgriep, dat heeft geleid tot kennis die de wetenschappelijke basis vormt van nieuwe hypotheses over de epidemiologie van HPAI virussen. Om surveillanceactiviteiten te optimaliseren en onze wetenschappelijke kennis verder te vergroten behoeven een paar gebieden verbetering, zoals de kennis omtrent de locatie van surveillance, de timing van surveillance, de vogelsoorten betrokken bij de spreiding van vogelgriep, de methode van surveillance, gastheersoortgedrag, diagnostische methoden en het delen van data.

Europa is meerdere keren getroffen door uitbraken van Aziatisch hoog pathogene vogelgriepvirussen. Het is opvallend dat er overeenkomstige patronen zijn waarin deze virussen hun weg richting Europa vonden. Na circulatie van het virus in Zuidoost Azië spreidde het ten tijde van de voorjaarsmigratie naar meer noordelijke gebieden zoals het Qinghaimeer in China (mei/juni) en de Russische grensgebieden met Mongolië (de meren Uvs Nuur en Hövsgöl Nuur) en Kazachstan. Op de Russische grensgebieden werd het virus zowel gevonden vóór het broedseizoen (mei/juni) als na het broedseizoen (juli tot september). Verdere spreiding van het virus vanaf oktober/november naar Europa werd vaak in een vroeg stadium gezien op locaties zoals de Zwarte Zee, de Oostzee en het Bodenmeer. Deze Aziatische, Russische en Europese locaties kunnen daarom gezien worden als zogeheten "early detection sites" voor vogelgriepvirussen.

Vogelsoorten die een rol spelen in de verspreiding van HPAI virussen zijn trekvogels die over lange afstanden migreren, zoals smienten, kuifeenden, meerkoeten en wintertalingen. Andere vogelsoorten die vaak besmet zijn gevonden, zoals

knobbelzwanen en meeuwen, zijn in Nederland standvogels of vogels die alleen over kortere afstanden migreren waardoor hun rol in lange-afstandsspreiding van vogelgriepvirussen minder groot zal zijn. Het is sterk aanbevolen om recente inzichten, zoals de hiervoor genoemde vogelsoortprioritering, te verwerken in de EU wetgeving. De huidige "Lijst van in het wild levende vogelsoorten die een hoger risico op aviaire influenza opleveren" (EU Beschikking van de Commissie 2005-726), is verouderd en alleen gericht op passieve surveillance. Ook vogelsoorten, zoals grote meeuwen, knobbelzwanen en roofvogels, die vaak ziek of dood gevonden worden in uitbraaksituaties, ook wanneer er geen duidelijke sterfte van eendachtigen wordt gezien, zijn hier niet allemaal in opgenomen. Het zou goed zijn een tweede lijst toe te voegen met daarin soorten die een mogelijke rol spelen in de verspreiding van HPAI virussen, zodat deze gebruikt kan worden in actieve surveillance programma's als een "early warning system", met name in afwezigheid van wilde vogel sterfte.

De surveillance-activiteiten op de Aziatische en Russische "early detection sites" richten zich voornamelijk op passieve surveillance. Idealiter zou dit in de lente en zomer worden aangevuld met actieve surveillance-activiteiten, met name wanneer er geen wildevogelsterfte wordt gezien, om zo de kans op vroege detectie van HPAI virussen te vergroten. Om de detectiekans van binnenkomend virus in Europa in een vroeg stadium te vergroten, moeten nationale surveillanceprogramma's zich richten op het testen van lange afstand vliegende trekvogels vanaf het eerste moment dat zij Europa binnenkomen in de herfst/winter. Vogelsoorten die hetzelfde jaar op andere locaties geïnfecteerd zijn gevonden kunnen hierbij als leidraad dienen. Om dit systeem kosteneffectief te houden kunnen verse fecesmonsters verzameld en getest worden. In het geval levendevogelvangsten mogelijk zijn, zal het nemen van een bloedmonster ten behoeve van serologie naast keel- en cloacaswabs inzicht geven in de huidige en recente HPAI H5 infectiestatus.

Met de toenemende kennis over de gastheersoorten voor HPAI virussen, komen er ook meer vragen over hun migratiepatronen. Met het steeds makkelijker en goedkoper worden van technieken om vogels te voorzien van GPS zenders, zou dit kunnen worden ingezet om meer kennis te vergaren over migratiegedrag in het algemeen, of om vogelsoorten die positief worden getest voor HPAI virussen in Azië of Rusland te voorzien van een GPS zender om zo de gastheer te kunnen vervolgen op zijn weg naar Europa.
Qua diagnostiek is er behoefte aan de ontwikkeling van goedkope en snelle methoden die in veldomstandigheden uit te voeren zijn en direct resultaat geven, zoals influenzasneltesten (point of care tests [POC]) en snelle sequening/NGS technieken zoals bijvoorbeeld MinION sequencing (Oxford Nanapope Technologies) om een direct inzicht te krijgen in het genotype van de circulerende virussen. In toevoeging hierop moeten er voor het toespitsen van surveillance robuuste, snelle,

goedkope en gevalideerde serologische assays komen waarin mogelijke gastheersoorten snel en betrouwbaar geïdentificeerd kunnen worden. Vervolganalyses zoals epidemiologische en fylogenetische studies zijn sterk afhankelijk van de hoeveelheid publiekelijk toegankelijke data. Ondanks dat er platforms zijn, zoals GISAID, die een veilige omgeving bieden om data te delen, lijken sommige onderzoekers terughoudend om virus- en gastheerdata onvertraagd te delen, hetgeen snelle en accurate epidemiologische analyses en risicoanalyses sterk negatief beïnvloed. Daarnaast is ook het delen van de virussen en klinische monsters zelf tussen landen een lastig proces dat vaak traag verloopt of soms zelfs onmogelijk blijkt waardoor internationale samenwerkingen worden bemoeilijkt.

Internationale (dier)gezondheidsorganisaties, nationale en internationale politici, beleidsmakers en juristen dienden zich hard te maken voor het faciliteren van een "One World, One Health" aanpak met betrekking tot (vogel)griep.

Marjolein

Chapter 7

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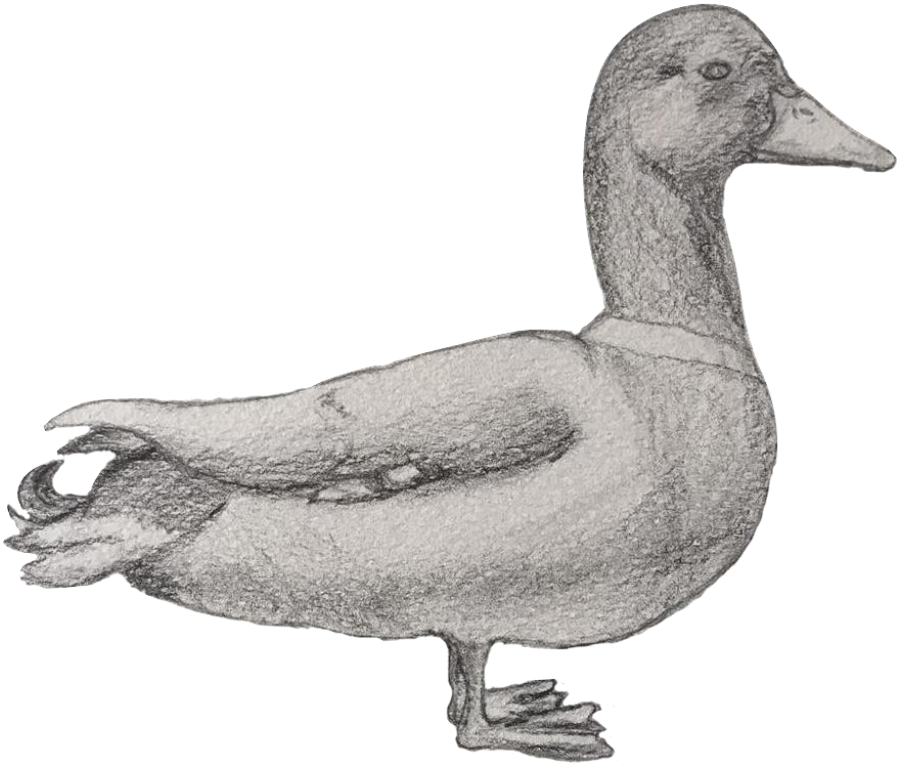

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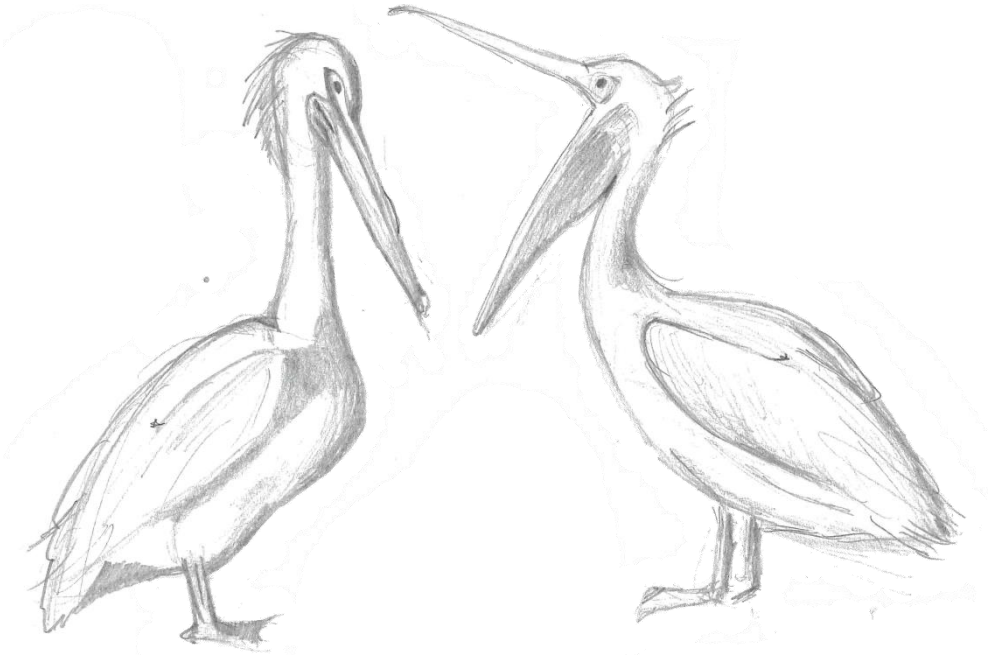
Curriculum Vitae

Marjolein Poen was born on the 31st of January 1987 in Haarlemmermeer, the Netherlands. After she finished pre-university secondary education at Atheneum College Hageveld in Heemstede, she started to study Biomedical Sciences at the University of Amsterdam in 2005. To broaden her view, she started with a compressed bachelor program in General Economics aside, also at the University of Amsterdam. During her studies she obtained an interest in infectious diseases with an emphasis on zoonotic diseases. To cover both the human and animal health areas of these diseases, she decided to start studying Veterinary Medicine at Utrecht University in 2009. For her thesis research she got the opportunity to work with Prof.Dr. Ab Osterhaus and Dr. Rogier Bodewes at the Department of Viroscience at the Erasmus MC, Rotterdam, where she worked on detecting influenza virus specific antibodies in seal sera. After she graduated from Veterinary Medicine, she joined the group of Prof.Dr. Ron Fouchier at the Department of Viroscience as a PhD candidate to coordinate the wild bird influenza surveillance project. Here, she worked on the subsequent highly pathogenic avian influenza H5 outbreaks in Europe, resulting in this thesis.



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Research school	Post-graduate Molecular Medicine (MolMed)
PhD period	2015 – 2019
Promotors	Prof.dr. Ron A.M. Fouchier Prof.dr. Thijs Kuiken

Education

2015 – 2019	PhD program, Erasmus MC, Rotterdam, the Netherlands. PhD thesis: Highly Pathogenic Avian Influenza In Wild Birds: Towards evidence-based surveillance
2012 – 2015	Master of Science in Veterinary Medicine. Major in Farm Animal Health and Veterinary Public Health. Utrecht University, the Netherlands
2009 – 2012	Bachelor of Science in Veterinary Medicine. Utrecht University, the Netherlands
2005 – 2009	Bachelor of Science in Biomedical Sciences. University of Amsterdam, the Netherlands

PhD training

Courses

2018	Career Development, MolMed research school, Rotterdam, the Netherlands
2017	IRD/ViPR hands-on workshop, MolMed research school, Rotterdam, the Netherlands
2016	22nd International Bioinformatics Workshop on Virus Evolution and Molecular Epidemiology, KU Leuven, Seoul, South-Korea
2016	Bleeding and intubation techniques ferrets and rabbits, Utrecht University, the Netherlands
2016	Survival analysis course, MolMed research School, Rotterdam, the Netherlands
2016	Advanced course Excel, MolMed research school, Rotterdam, the Netherlands

2016 Basic course Excel, MolMed Research school, Rotterdam, the Netherlands
2016 Management of Infections and Diseases in Animal Populations, Wageningen Research University, Wageningen, the Netherlands
2016 MolMed Course, MolMed Research School, Rotterdam, the Netherlands
2016 Course on R, MolMed Research School, Rotterdam, the Netherlands
2016 KNAW Course on Laboratory Animal Science – species specific module: Birds
2015 Research Integrity Course, MolMed research School, Rotterdam, the Netherlands

Presentations at (inter)national meeting

2019 CEIRS Surveillance Meeting, Columbus, Ohio, USA: Oral presentation
2019 Young COMPARE Meeting, Copenhagen, Denmark: Oral presentation
2018 8th Orthomyxovirus Research Conference, Hanoi, Vietnam. Oral presentation
2018 and 11th Annual CEIRS Network Meeting, New York, USA. Poster presentation and invited session moderator
2018 24th Joined Annual Meetings of the Nation Reference Laboratories for Avian Influenza and Newcastle Disease, Windsor, United Kingdom. Invited speaker.
2018 10th International Symposium on Avian Influenza, Brighton, United Kingdom. Oral presentation
2018 Young COMPARE Meeting, Copenhagen, Denmark. Oral presentation
2017 CEIRS Surveillance Meeting, Bergen, the Netherlands. Oral presentation
2017 23rd Joined Annual Meetings of the Nation Reference Laboratories for Avian Influenza and Newcastle Disease, Zagreb, Croatia. Invited speaker
2017 Ecology Meets Epidemiology Conference, Kalmar, Sweden. Oral and poster presentation
2017 General COMPARE Meeting, Rotterdam, the Netherlands. Poster presentation
2017 Young COMPARE Meeting, Rotterdam, the Netherlands. Oral presentation.
2016 CEIRS Surveillance Meeting, Columbus, USA. Oral presentation
2016 DSWH-BWDS Symposium – Wildlife crossing borders. Invited speaker
2016 22nd International Bioinformatics Workshop on Virus Evolution and Molecular Epidemiology, Seoul, South-Korea. Poster presentation
2016 9th Annual CEIRS Network Meeting, Memphis, USA. Poster presentation
2016 22nd Joined Annual Meetings of the Nation Reference Laboratories for Avian Influenza and Newcastle Disease, Copenhagen, Denmark. Oral presentation
2016 Dutch Ornithology Conference, Haren, the Netherlands. Invited speaker

2015 Predigone (Predemics/Antigone) meeting, Rotterdam, the Netherlands. Poster presentation

Conferences attended

2018 NCOH Science Café, Utrecht, the Netherlands
2018 VEEC/DSWH Joined Symposium, Utrecht, the Netherlands: Joined conference moderator
2017 DSWH Symposium, Utrecht, the Netherlands
2017 10th Annual CEIRS Network Meeting, Atlanta, Georgia, USA: Invited session moderator
2016 9th Annual CEIRS Network Meeting, Memphis, Tennessee, USA
2016 Dutch Annual Virology Symposium, Amsterdam, the Netherlands
2015 Antigone Meeting, Rotterdam, the Netherlands
2015 CEIRS Surveillance Meeting, Cambridge, Massachusetts, USA
2015 7th Orthomyxovirus Research Conference, Toulouse, France
2015 DSWH Symposium, Utrecht, the Netherlands

Teaching

Lecturing, tutoring, supervising

2018/19 Supervision of 4th year student HU University of Applied Sciences Utrecht: Competition assay seasonal influenza
2018 Lab rotations Infection & Immunity Master students; Avian influenza wild birds, Rotterdam, the Netherlands
2017 Viruskenner, Amsterdam/Rotterdam, the Netherlands
2017 Hygieia; Update on Avian Influenza, Veterinary Science, Utrecht, the Netherlands
2017 Lab rotations Infection & Immunity Master students; Avian influenza wild birds, Rotterdam, the Netherlands
2017 Supervision 3rd year student Rotterdam University of Applied Sciences; Serology in gulls
2016 Instruction birdsampling, NIOO, Wageningen, the Netherlands
2016 Viruskenner, Amsterdam/Rotterdam, the Netherlands
2016 Lab rotations Infection & Immunity Master students; Avian influenza wild birds, Rotterdam, the Netherlands
2015 Lab rotations Infection & Immunity Master students; Avian influenza wild birds, Rotterdam, the Netherlands

Heidi

Publications

2019 **Marjolein J. Poen**, Ron A.M. Fouchier, Mohamed El Zowalaty. Evidence of low incidence of avian influenza viruses in wild waterfowl in South Africa, 2018. In preparation

Vigeveno RM, **Poen MJ**, Parker E, Holwerda M, De Haan K, Lewis NS, Bestebroer TM, Fouchier RAM, De Jong MD. and Eggink D. Highly Pathogenic Avian influenza (A)H5N8 virus polymerase complexes and their role in virulence'. In preparation

Josanne H. Verhagen, **Marjolein J. Poen**, David E. Stallknecht, Stefan van Vliet, Pascal Lexmond, Srinand Sreevatsan, Scott Krauss, Janice C. Pedersen, Rebecca L.Poulson, Shinichiro Enomoto, Jonas Waldenström, Justin D. Brown, Ron A.M. Fouchier, Camille Lebarbenchon. Phylogeography and antigenic diversity of low pathogenic avian influenza H13 and H16 viruses. In preparation

Marjolein J. Poen, Anne Pohlmann, Clara Amid, Theo M. Bestebroer, Sharon M. Brooks, Ian H. Brown, Helen Everett, Claudia M.E. Schapendonk, Rachel D. Scheuer, Saskia L. Smits, Martin Beer, Ron A.M. Fouchier, Richard J. Ellis. Comparison of sequencing methods and data processing pipelines for whole genome sequencing and minority single nucleotide variant (mSNV) analysis during an influenza A/H5N8 outbreak. Revised version submitted to PLoSONE

Saskia A. Bergervoet, Sylvia Pritz-Verschuren, Jose L. Gonzales, Alex Bossers, **Marjolein J. Poen**, H. van Bakel, Ruth Bouwstra, Ron A.M. Fouchier, Nancy Beerens. Circulation of low pathogenic avian influenza (LPAI) viruses in wild birds and poultry in the Netherlands, 2006-2016. Submitted to Scientific Reports

Marjolein J. Poen, Divya Venkatesh, Theo M. Bestebroer, Oanh Vuong, Rachel D. Scheuer, Bas B. Oude Munnink, Dennis de Meulder, Mathilde Richard, Thijs Kuiken, Marion P.G. Koopmans, Leon Kelder, Yong-Joo Kim,

Youn-Jeong Lee, Mieke Steensel, Benedicte Lambrecht, Adam Dan, Anne Pohlmann, Martin Beer, Vladimir Savic, Ian H. Brown, Ron A.M. Fouchier, Nicola S. Lewis. Co-circulation of genetically distinct highly pathogenic avian influenza A clade 2.3.4.4 (H5N6) viruses in wild waterfowl and poultry in Europe and East Asia, 2017-18. Virus Evolution 2019, Vol.5, Iss.1 Jan 2019

2018 Divya Venkatesh, **Marjolein J. Poen**, Theo M. Bestebroer, Rachel D. Scheuer, Oanh Vuong, Mzia Chkhaidze, Anna Machablishvili, Jimsher Mamuchadze, Levan Ninua, Nadia B. Fedorova, Rebecca A. Halpin, Xudong Lin, Amy Ransier, Timothy B Stockwell, David E. Wentworth, Divya Kriti, Jayeeta Dutta, Harm van Bakel, Anita Puranik, Marek J Slomka, Steve Essen, Ian H. Brown, Ron A.M. Fouchier, and Nicola S. Lewis. Avian influenza viruses in wild birds: virus evolution in a multi-host ecosystem. Journal of Virology 2018, JVI.00433-18

Marjolein Poen, Oanh Vuong, Rachel Scheuer, Ron Fouchier. Vogelgrieponderzoek door het Erasmus MC, 2012-2016. WREN nieuwbrief 4, April 2018

Marjolein J Poen, Theo M Bestebroer, Oanh Vuong, Rachel D Scheuer, Henk P van der Jeugd, Erik Kleyheeg, Dirk Eggink, Pascal Lexmond, Judith MA van den Brand, Lineke Begeman, Stefan van der Vliet, Gerhard JDM Müskens, Frank A Majoor, Marion PG Koopmans, Thijs Kuiken, Ron AM Fouchier. Local amplification of highly pathogenic avian influenza H5N8 viruses in wild birds in the Netherlands, 2016 to 2017. Eurosurveillance 2018, Vol. 23, Iss. 4

2017 SA Bergervoet, R Heutink, SBE Pritz-Verschuren, **MJ Poen**, RJ Bouwstra, RAM Fouchier, N Beerens. A41: Diversity and evolution of aviain influenza (AI) viruses in poultry and wild birds. Virus Evolution, 2017, Vol1, Suppl. 1, p.15

MJ Poen, JH Verhagen, FA Majoor, NS Lewis, T Kuiken, MCM De Jong, RAM Fouchier. A43: Modelling the ecology and evolution of H13 and H16 avian influenza A subtypes in black-headed gulls to understand influenza disease dynamics. Virus Evolution, 2017, Vol. 3, Suppl. 1. P.16

Daisy Duncan, Kate Harris, **Marjolein Poen**, Stefan Kowalezyk, Ron Fouchier, Christoph Staubach, Thijs Kuiken. LPAI detection in wild birds and LPAI spread between European holdings in the period 2005-2015. EFSA Supporting publication 2017. Vol.14, Iss.10:EN- 1286

Daisy Duncan, Kate Harris, **Marjolein Poen**, Stefan Kowalezyk, Ron Fouchier, Christoph Staubach, Thijs Kuiken. Report about HPAI introduction into Europe, HPAI detection in wild birds and HPAI spread between European holdings in the period 2005-2015. EFSA Supporting publication 2017. Vol.14, Iss.10:EN-1284

Erik Kleyheeg, Roy Slaterus, Rogier Bodewes, Jolianne M Rijks, Marcel AH Spierenburg, Nancy Beerens, Leon Kelder, **Marjolein J Poen**, Jan A Stegeman, Ron AM Fouchier, Thijs Kuiken, Henk P van der Jeugd. Deaths among Wild Birds during Highly Pathogenic Avian Influenza A(H5N8) Virus Outbreak, the Netherlands. Emerging Infectious diseases 2017, Vol. 23, No, 12

2016 **Poen MJ**, Verhagen JH, Manvell RJ, Brown I, Bestebroer TM, van der Vliet S, Vuong O, Scheuer RD, van der Jeugd HP, Nolet BA, Kleyheeg E, Müskens GJDM, Majoor FA, Grund C, Fouchier RAM. Lack of virological and serological evidence for continued circulation of highly pathogenic avian influenza H5N8 virus in wild birds in the Netherlands, 14 November 2014 to31 January 2016. Euro Surveill. 2016; Vol.21, Iss.38

2015 Thijs Kuiken, **Marjolein Poen**, Ron Fouchier. 4.2.2 Monitoring zoönosen bij levende wilde vogels. Staat van Zoönosen 2014, RIVM Report 2015-0151

2014 Koenders, K., Steenaert, M., **Poen, M.J.** Administering Meloxicam to postpartum sows reduces within litter variation of piglet IgG titres. Proceedings 23rd IPVS Congress, Jun 2014 O.114, pp.199

2013 Bodewes, R., Rubio García, A., Wiersma, L.C.M., Getu, S., Beukers, M., Schapendonk, C.M.E., Van Run, P.R.W.A., Van De Bildt, M.W.G., **Poen, M.J.**, Osinga, N., Sánchez Contreras, G.J., Kuiken, T., Smits, S.L., Osterhaus, A.D.M.E. Novel B19-Like Parvovirus in the Brain of a Harbor Seal. Plos One Nov. 2013, Vol.8, Iss.11

2012 **Poen, M.**J. De GGD , die is toch voor mensen? Tijdschrift voor Diergeneeskunde, No.137, Iss:7, 1 juli 2012, pp: 474-475

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