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Genetic Characterisation of Highly Pathogenic Avian Influenza Viruses Based on Novel Real-Time Nanopore Sequencing

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It never hurts to keep looking for sunshine

– *Eeyore*

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CONTENTS

CHAPTER I: INTRODUCTION

I. INTRODUCTION

Alike many viral pathogens, influenza A viruses (IAV) are characterised by their constant evolution and subsequent emergence of novel strains (1). Over the past decade, IAV have been the elicitor of four pandemics and numerous epidemics, as well as having drastic impacts on the poultry and swine production sectors (2, 3). In addition to the pronounced bearing on the livestock industry, the zoonotic propensity of certain IAV strains poses a threat to public health (1, 4). Concomitant circulation of IAV in waterfowl, the natural reservoir of IAV, rapid adaptation to environmental changes and frequent spill over events into animal hosts and the human population upholds the advancement of novel IAV strains (5, 6). IAV can be classified depending on the host of origin, accordingly termed avian influenza virus (AIV), swine influenza A virus (swIAV), or elsewise conferring to the affected species (7, 8). Further, AIV of subtypes H5 and H7 can be divided into two groups according to the level of pathogenicity: Low pathogenic AIV (LPAIV) and highly pathogenic AIV (HPAIV) (9).

Due to the broad diversity of IAV, surveillance by full genomic evaluation has become indispensable. In an outbreak scenario whole-genome sequencing (WGS) is utilised to identify the emerging, potentially zoonotic IAV (10). Thereafter, genetic characterisation of the novel IAV assists in the establishment of preventive measures. The nucleotide-level resolution of in-depth WGS permits phylogenetic analysis and molecular epidemiology studies to achieve a detailed understanding of the outbreak (11). Although WGS with expensive, stationary, slow and laborious second-generation devices produces high quality data, this thesis describes the establishment of a rapid, portable, inexpensive, real-time third-generation sequencing approach to improve outbreak response and WGS availability. In addition, application of the proposed protocol in a recent HPAIV H5 outbreak in Germany, 2020, is included in this thesis.

The most widespread HPAIV H5 lineage emerged in 1996, affecting geese in the Chinese Guangdong province (goose/Guangdong lineage, gs/GD) (12). Aided by the global spread via migratory birds and unprecedented tendency for reassortment, a plethora of clades, sub- and genotypes causing high mortality as well as zoonotic strains have evolved from the respective H5 lineage (3, 13, 14). Since the initial detection of the first gs/GD HPAI H5N1 virus in Germany, 2006 (15), a recurring influx of reassortant HPAIV H5 subtypes including H5N1, H5N8, H5N5 and H5N6 in assorted genotype constellations have been identified (16-20). Recurring clade 2.3.4.4b HPAIV H5 outbreaks have been repeatedly detected in Germany since 2016. The most severe HPAIV clade 2.3.4.4b epidemic was recorded in Germany during winter 2016/2017, affecting an unprecedented number of (commercial) poultry holdings and triggering high mortality in the wild bird population (18, 19, 21, 22). As curtailing of novel pandemic threats is believed to be best accomplished by effectively controlling AIV in poultry,

precise understanding of the role of poultry in outbreak scenarios by phylogenetic analysis can aid in the establishment of prevention measures (23). The conducted in-depth phylogenetic analyses of clade 2.2.3.4b HPAIV H5 outbreaks by WGS included in this thesis cast a light on the genetic relations, outbreak dynamics, and zoonotic propensity of the German clade 2.3.4.4b HPAIV H5 2016 – 2020 outbreaks.

Likewise, the introduction of clade 2.3.4.4b HPAI H5 viruses to Egypt in 2016 led to the emergence of multiple novel reassorted HPAIV (24, 25). Again, the utilisation of WGS for genetic characterisation of the novel strains allowed exact dissection of the genetic backbone and reassortment pattern (26), as described in this thesis. Due to the endemic HPAIV situation in Egypt, precise identification and classification of novel HPAIV H5 strains is of utmost importance (26, 27).

Collectively, this thesis focusses on (i) the establishment and application of a rapid, portable, third-generation nanopore sequencing workflow for IAV, (ii) molecular epidemiology, zoonotic potential and genetic evaluation of the 2016 – 2020 HPAIV H5 outbreaks in Germany, and (iii) the genetic characterisation of novel HPAI H5 viruses in Egypt.

CHAPTER II: LITERATURE REVIEW

II. LITERATURE REVIEW

1. Whole-Genome Sequencing

Since the discovery of the structure of deoxyribose nucleic acid (DNA) in 1953 (28), a multitude of sequencing techniques have emerged. All pursuing the goal of obtaining the nucleic acid sequences of entire (viral) genomes, this process is now referred to as whole-genome sequencing (WGS). Knowledge of the full genome allows genetic characterisation, molecular epidemiology studies and classification of novel and known pathogens.

1.1. Employment and Value of Sequencing in Viral Outbreak Scenarios

A glance at the size and growth of the international nucleotide sequence database shows the integral role of sequencing in most scientific research applications, especially in the field of virology (29-31). Human history is abounding with viral disease outbreaks causing devastating repercussions for entire populations, for instance the 1918 Spanish flu pandemic (32) and 2009 H1N1 influenza pandemic (33), the ongoing human immunodeficiency virus (HIV) pandemic (34), and the recent 2014-2015 Ebola virus (EBOV) epidemic (35). Highlighting the significance of viral pathogens, the 2019 annually reviewed list of diseases recommended for prioritised research and development by the World Health Organization (WHO) included seven viral diseases and an unknown, emerging disease yet to be discovered, “Disease X”, also most likely to be of viral origin (36).

Although epidemiological methods (contact tracing, mathematical modelling) and molecular biology tools (genotypic and phenotypic methods) have successfully assisted in disease control, rapid identification of the causative agent of emerging and re-emerging infectious diseases by WGS has become indispensable in outbreak scenarios (37, 38). Full genomic evaluation facilitates nucleotide-level resolution, permitting phylogenetic and variant analyses to aid in establishing preventive and therapeutic measures (39, 40).

In December 2019, reports of a novel respiratory disease in Wuhan, China, led to the detection of the first “Disease X” (41, 42). Rapid WGS allowed the identification and classification of the causative agent: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (42, 43). In addition to initial metagenomic identification of SARS-CoV-2, this pandemic has demonstrated the enormous capacity and importance of WGS to study emerging infectious diseases by tracing outbreak origin and drivers, establishing transmission chains and monitoring virus evolution (43, 44). In light of the SARS-CoV-2 pandemic, the employment of rapid, accessible and inexpensive WGS techniques in outbreak scenarios is crucial.

1.2. The Era of High-Throughput Sequencing

Before the era of high-throughput sequencing (also called next-generation sequencing, NGS), the first-generation chain termination method by Sanger was primarily used for sequencing ventures (45). Here, incorporation of radiolabelled dideoxynucleosidtriphosphates (ddNTP) led to random chain termination during elongation. For the determination of the base order, the resulting fragments of varying lengths were visualised by radiography after electrophoresis on a polyacrylamide gel. Optimisation of the respective first-generation sequencing method involved different fluorescently labelled ddNTP to allow chain termination in one tube (instead of four for each radiolabelled ddNTP), and subsequent automated separation by capillary electrophoresis and optical detection of the bases (46).

Although Sanger sequencing led to the discovery of many ground breaking sequences, including the determination of the first human genome (47, 48), the time consuming, expensive and laborious approach called for a second generation of high-throughput, accurate and low cost sequencers. Characterised by the simultaneous parallel generation of short sequences (reads), the first high-throughput second-generation sequencer, the 454 Genome Sequencer 20 developed by 454 Life Sciences (now Roche), was introduced in 2005. The highly competitive field of second-generation sequencers led to the discontinuation of the 454 platform in 2013 due to dated technology, and today the Illumina (Illumina Inc.) and IonTorrent (Thermo Fisher Scientific) platforms dominate the second-generation market (49).

Independent of the executing platform, second-generation sequencing (SGS) comprises four steps: Sample preparation, clonal amplification, sequencing and bioinformatic analysis (50, 51). Initially, the originating genetic material (ribonucleic acid, RNA/DNA) is prepared for sequencing. While DNA can be directly employed, RNA must be transcribed into complementary DNA (cDNA) by reverse transcription. After random fragmentation of the DNA/cDNA (fragment size dependent on the sequencing platform, typically 75 – 500 base pairs, bp), known oligonucleotide adapters encompassing sequences essential for amplification and polymerase binding are ligated to the 3’ and 5’ termini. Clonal amplification is performed to boost the generated sequencing signal. Based on sequencing-by-synthesis, integrated nucleotides are registered during elongation of the synthesised chain conferring to the template sequence. Contingent on the sequencing platform, the integrated nucleotides are detected by fluorescence signals (Illumina) or electric potential changes (IonTorrent) (52).

Data processing (basecalling) of the amassed sequencing signals can result in billions of output reads per sequencing run. Due to the challenges of working with massive datasets, further bioinformatic analysis calls for large processing capacities. In most cases, either a de-novo (overlapping reads are

combined to produce larger sequences) or map-to-reference (reads are mapped to a known reference genome) approach is utilised to achieve whole genome sequences (53, 54).

1.3. Nanopore Sequencing Methods – Long-Read, Real-Time Sequencing

Over the past 15 years, tremendous progress has been made in the development, employment and commercialisation of NGS, including the achievement of the US$1000 human genome and 1000 Genomes Project (55-57). While short-read, second-generation sequencers revolutionised scientific research applications in terms of cost efficiency, throughput volume and speed, a demand for technologies producing longer reads in real time resulted in the advent of third-generation sequencing (TGS).

Major differences between SGS and TGS lie in the longer read lengths (75-500 bp vs. dependent on the sample molecule length), lack of need for clonal amplification and single molecule sequencing (58). Pacific Biosciences (PacBio) introduced the first successful single-molecule real time (SMRT) platform in 2011. To date, the stationary, costly and large PacBio Sequel sequencer can achieve mean read lengths of up to 15,000 bp with 5 – 10 gigabytes (GB) of throughput per four hour run, but carries significantly higher error rates (10 – 15 %) than second-generation sequencers (<1 %) (58-60).

A novel third-generation nanopore sequencer was introduced to the market by Oxford Nanopore Technologies (ONT) in 2014. Dubbed MinION, the pocket-sized, portable device is capable of producing long reads limited only by the length of the sample molecule and can produce up to 30 GB of data in 48 hours. Alike the PacBio platform, the MinION struggles with high error rates (10 – 15 %) especially in homopolymer regions, although the rapid evolution of new chemistries and bioinformatic workflows has considerably reduced this value (5 – 8 %) and improved homopolymer basecalling (58, 61, 62).

The foundation for nanopore sequencing was laid in the 1980s when the idea of sequencing a single strand of nucleic acid by employing electrophoresis to draw it through a nanoscopic pore in a membrane was born (63). It took until 2012 for the first successful sequencing reports, followed by the commercialisation two years later in a large-scale collaborative MinION Access Program (MAP) (62, 63). For sequencing, a MinION Mk1B device is coupled with a flow cell and powered by USB connection to a computer/laptop. The flow cell contains multiple protein nanopores set in an electrically resistant polymer membrane. By setting a voltage across this membrane, an ionic current flows through the nanopores and nucleic acid strands are driven through the pores. The transition of nucleic acid through the pores disrupts the current and changes in pattern or magnitude can be measured and characterised. A sensor measures the ionic current several thousand times per second and relays the accumulated data to an application-specific integrated circuit (ASIC) microchip (Figure 1). Final data processing and basecalling is subsequently conducted by the ONT MinKNOW software (61, 64).

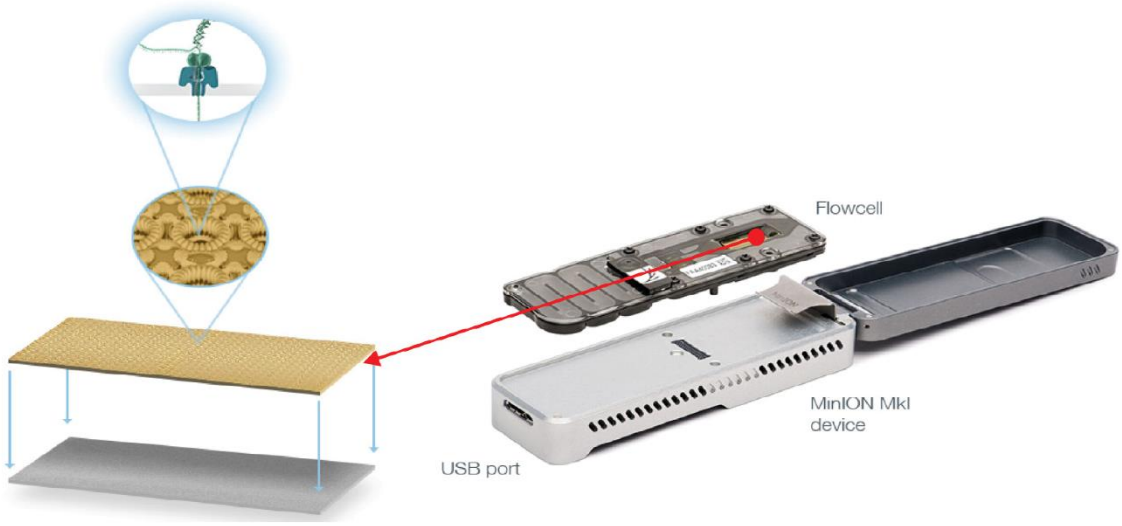

Figure 1. The MinION (Mk1B) sequencing device (15 cm in length, right) and flow cell (13 cm in length, right). Sequencing is performed by adding the sample to the flow cell. The DNA molecule passes through the nanopore (1 nm, top left) set in a channel (middle left), resulting in a change of current in the nanopore. The change is measured by a sensor and passed to the ASIC (1 cm in length, bottom left) (61). For permission rights see Chapter X, Supplementary Material, Permission for Reproduction.

ONT have introduced a wide array of novel and improved products over the past years. To help with the large processing capacities needed for data acquisition and basecalling, the MinIT was introduced as a companion to the MinION Mk1B in 2018, carrying multiple threading graphics processing units (GPU), 8 GB random-access memory (RAM) and 512 GB solid-state drive (SSD), thus eliminating the need for a dedicated laptop. In 2019, ONT announced the MinION Mk1C. This sequencer syndicates the original MinION Mk1B and MinIT setup by combining the real-time, portable sequencing of the MinION with a powerful integrated compute (preinstalled basecalling and analysis software) and a high-resolution touchscreen. Additionally, two larger, stationary high-throughput sequencers are available: The benchtop GridION can run five flow cells at once (up to 150 GB of data) while the ultra-high throughput PromethION device can run 48 flow cells at once to create up to eight terabytes (TB) of data, especially suitable for population-scale sequencing (62, 64).

The MinION and GridION flow cells contain 512 nanopore channels (ASIC measurement notions), allowing the simultaneous sequencing of 512 independent DNA/RNA molecules. Each channel is connected to four wells, from which one well at a time can provide data. Every well can contain zero to several pores, ideally carrying one pore per well. Therefore, the MinION and GridION flow cells carry 2048 wells – four wells for each of the 512 channels, and ideally 2048 pores with one pore per well. Conversely, some wells can carry no pores, several pores, blocked or damaged pores (Figure 1). Quality criteria by ONT call for >800 pores per new flow cell. The MinION and GridION flow cells are available in two different pore chemistries, the standard R9.4.1 and novel R10.3. While the R9.4.1 pores have

one short reader head, the R10.3 pores carry a dual reader head and longer reading barrel. This drastically improves the read accuracy (achieving on average 95% accuracy), especially in homopolymer regions.

As with SGS, the samples must undergo library preparation prior to sequencing. Dependent on the research aim, ONT offers 14 assorted preparation kits for whole genome or targeted DNA and RNA sequencing. The samples can be fragmented or directly employed to sequence direct RNA, cDNA, genomic DNA or polymerase chain reaction (PCR) amplicons. All kits share the essential addition of a specific Y-shaped sequencing adapter with motor protein by end-ligation or transposase activity to allow docking and transit of the sample through the nanopore. To reduce costs and increase throughput, multiplexing is possible: The addition of known oligonucleotides ("barcodes") to samples allows simultaneous sequencing of multiple libraries. Produced reads can later be allocated to their respective barcode based on the oligonucleotide sequence (Figure 2). Library preparation is much less time consuming than SGS protocols, with transposase-based kits allowing sample preparation in only 10 minutes.

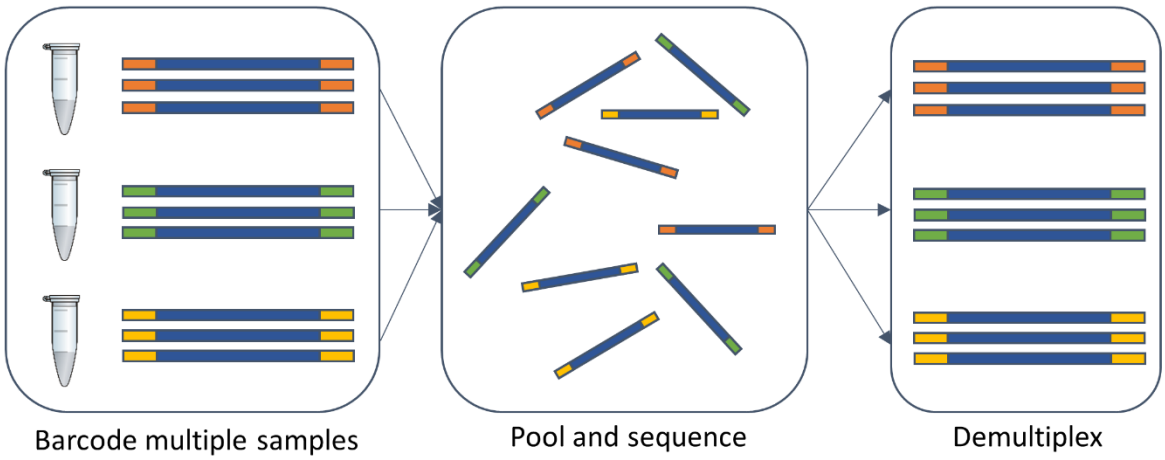

Figure 2. Barcoding/multiplexing of samples. The addition of a known oligonucleotide sequence (barcode) allows pooling of samples for sequencing. Subsequently, assignment of reads depending on the barcode (demultiplexing) allows further analysis.

At an initial cost of US$1000 for the MinION Starter Pack (including the MinION Mk1B, two R9.4.1 flow cells, one kit of choice and a flow cell wash kit), nanopore sequencing has become accessible and affordable for many research groups. Long-read technologies are enabling the exploration of genomes at an unprecedented resolution, allowing de novo genome assemblies, the determination of structural variants and insights into long-stretch repetitive sequences (65, 66). Additionally, unamplified direct sequencing permits the observation of base modifications such as methylations (67, 68).

Although the main asset lies in the production of long reads, the ability of real-time, portable and inexpensive sequencing are irreplaceable in outbreak scenarios. Deployment of the MinION to the International Space Station, the Canadian High Arctic and a working swine exhibition emphasises the versatility and portability of the device (69-71). Successful outbreak or surveillance sequencing has been conducted for *inter alia*, swIAV (71), Zika virus (72), EBOV (73, 74) und most recently, the SARS-CoV-2 virus pandemic (75).

2. Influenza A Virus

IAV are highly contagious, potentially zoonotic viral pathogens that cause subclinical, mild or severe respiratory disease (76, 77). The ability to adapt quickly to environmental (host) changes and the continuous circulation of IAV in waterfowl, the natural reservoir of IAV, with spill over events into several avian, animal and human hosts sustains the evolution of novel IAV strains. Alongside carrying the potential for (human) epidemics or pandemics, IAV are of utmost importance in the livestock industry, in particular the poultry and swine production sectors (1, 6, 77, 78).

2.1. Taxonomy, Host Range and Classification

IAV belong to the family *Orthomyxoviridae* (78). This family comprises six further genera including influenza B, C, and D viruses, Thogotovirus, infectious salmon anemia virus, and Quaranjavirus (79, 80).

Depending on the host of origin, IAV can be classified as AIV, swIAV or other types of animal influenza viruses (7). Human, swine, equine, feline, canine and marine mammals have been reported to carry IAV, yet wild aquatic birds of the orders *Anseriformes* (ducks, geese and swans) and *Charadriiformes* (gulls, terns and waders) form the virus reservoir in nature (1, 81).

IAV are highly variable. The antigenic variation based on the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) allows the theoretical classification into 154 subtypes, as eighteen subtypes of HA (H1 – H18) and eleven subtypes of NA (N1 – N11) have been identified to date (82). Subtypes H1 – H16 and N1 – N9 have been described in waterfowl (78, 81, 82), while H17N10 and H18N11 have only been found in bats and fail to reassort with conventional IAV (8, 83).

In addition to classification based on the antigenic properties of HA, AIV can be classified according to their pathogenic phenotype in chickens: LPAIV and HPAIV. The majority of AIV are LPAIV and cause mild or subclinical disease in birds. In contrast, two influenza subtypes (H5 and H7) have the potential to occur as HPAIV with up to 100% morbidity and mortality within a few days (9).

In 1980, a nomenclature system for influenza viruses was established by the WHO (84). Included is the influenza type (A, B, C or D), host of origin (omitted for human-derived viruses), geographical location, strain or laboratory number, year of isolation and the HA/NA subtype (e.g. A/turkey/Germany-NI/AI00334/2020 (H5N8)).

2.2. Virus Structure and Genome Organisation

Pleomorphic IAV particles are spherical (approximately 100 – 120 nanometres – nm, in diameter) or filamentous (over 300 nm in length) in shape (80). The IAV virion comprises a host-cell derived lipid bilayer membrane studded with HA and NA glycoprotein spikes in a ratio of four to one (Figure 3). A further surface protein, the matrix-2 (M2) protein, forms a transmembrane ion channel to traverse the

lipid envelope. Six internal proteins complete the virion, including polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), nucleocapsid protein (NP), matrix protein (MP) and non-structural protein (NS2) (1, 81).

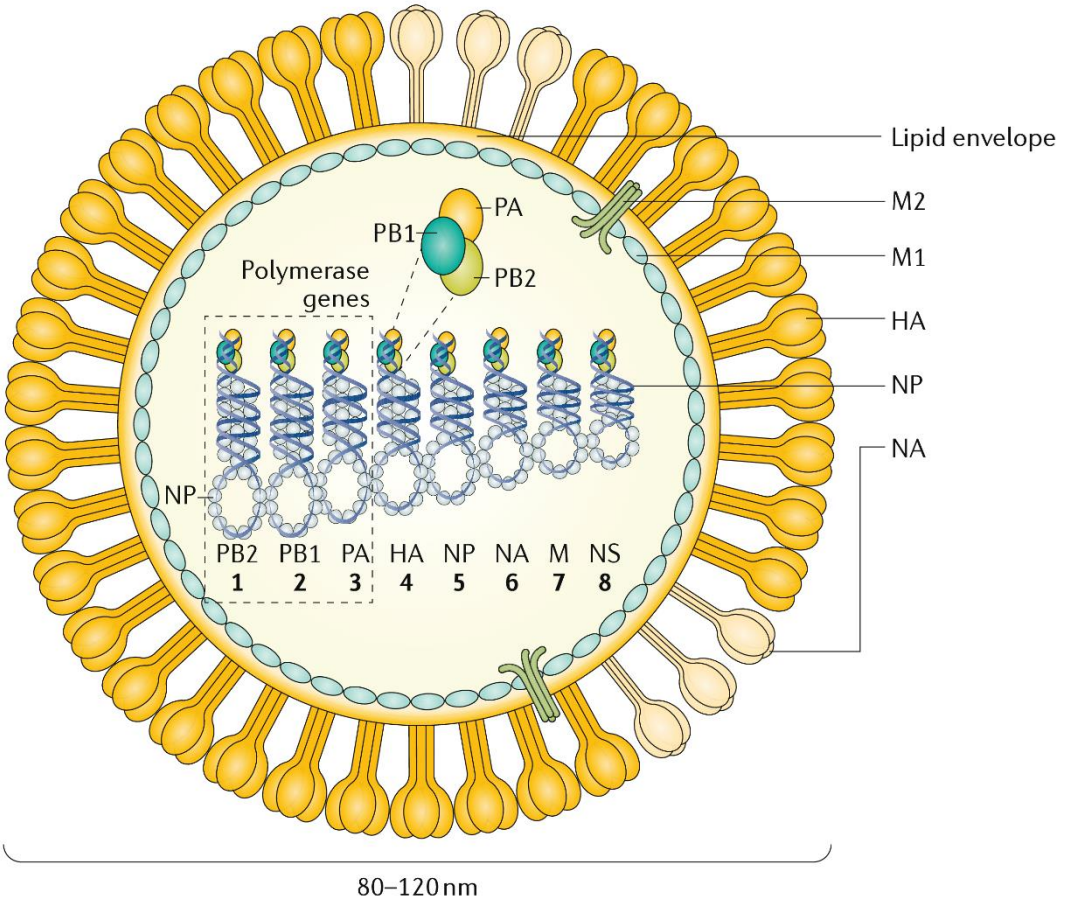

Figure 3. Structure of an influenza A virus particle (78). For permission rights see Chapter X, Supplementary Material, Permission for Reproduction.

Orthomyxoviridae harbour a negative sense, single-stranded, encapsidated, eight-fold segmented ribonucleic acid (RNA) genome of approximately 13,500 base pairs (bp). All genetic information necessary for replication and assembly of progeny virions is encoded in the IAV genome (80). The eight gene segments of IAV are numbered in order of decreasing length, holding coding capacities for nine structural proteins (PB2, PB1, PA, HA, NP, NA, M1, M2, and NS2) and up to five non-structural proteins identified *in vitro* (PB2-S1, PB1-F2, PB1-N40, PA-X, and NS1) (1, 6, 85, 86). Highly conserved regions can be found at the 3' terminus (12 nucleotides) and 5' terminus (13 nucleotides) of all gene segments. Partially complementary, the conserved regions form a short double-stranded RNA structure known as the "panhandle" and function as the promotor for viral RNA replication and transcription (87-89).

Segments 1 – 3 encode the polymerase proteins: PB2, PB1 and PA. The PB2 protein is responsible for the recognition and binding of 5' cap structures of host cell viral messenger RNA (mRNA). The PB1

protein binds the 3' and 5' termini of viral RNA (vRNA) and complementary RNA (cRNA), initiating the process of genome transcription and replication. The role of the PA protein has yet to be fully dissected. The three described polymerase subunits form a heterotrimeric viral RNA-dependent RNA polymerase (RdRP) complex required for replication (Figure 3) (1, 80, 82, 90, 91).

The viral surface glycoproteins HA and NA are encoded by segments 4 and 6, respectively (80). The HA protein is responsible for binding of sialic acids (viral host cell receptors) and fusion of viral and host cell membranes ensuing endocytosis (92). For activation of the HA protein, the initially synthesised single polypeptide precursor protein (HA_0) undergoes posttranslational endoproteolytic cleavage into subunits HA_1 and HA_2 by cellular proteases (93). The NA protein, a sialidase, cleaves the sialic acids residue attached to newly produced virions, thus facilitating virus release (94).

Segment 5 encodes the NP protein, responsible for binding vRNA with the amino terminus RNA-binding domain. Together with the heterotrimeric RdRP complex, vRNA segments coated in NP protein form the ribonucleoprotein (RNP) complex (Figure 3). The RNP complex plays a fundamental role in the transcription, replication and intracellular transport of vRNA as well as viral genome packaging into progeny particles (95).

The MP protein, encoded by segment 7, includes the viral matrix structural protein M1 and the ion channel protein M2. A matrix of M1 protein encloses the virion core as a major structural component of the viral envelope. M2 protein forms a transmembrane ion channel to traverse the lipid envelope (96).

Segment 8 encodes for two major NS proteins, NS1 and NS2. As an antagonist of interferon expression of infected cells, NS1 suppresses the host innate immune response and interferes with host gene expression. NS2, also known as nucleic export protein (NEP), plays a role in the nuclear export of RNP into the cytoplasm prior to virion assembly (97, 98).

2.3. Genetic and Antigenic Viral Evolution

IAV exhibit rapid evolutionary dynamics, irrespective of host range and viral subtype (99). Driven by large host populations, short generation intervals and high mutation rates, IAV pose the risk of zoonotic infection, host switch and the generation of pandemic viruses (3). Four human flu pandemics have been recorded over the past 100 years: 1918 H5N1 Spanish flu, 1957 H2N2 Asian flu, 1968 H3N2 Hong Kong flu and 2009 H1N1 swine flu (3, 100-103). Although not classified as a pandemic, the HPAI H5N1 virus has caused more than 1000 deaths with mortality rates of about 55% since its emergence in 1996 (2).

Ongoing viral evolution is expedited by three mechanisms: Point mutations (genetic drift), reassortment (genetic shift), and RNA recombination (104).

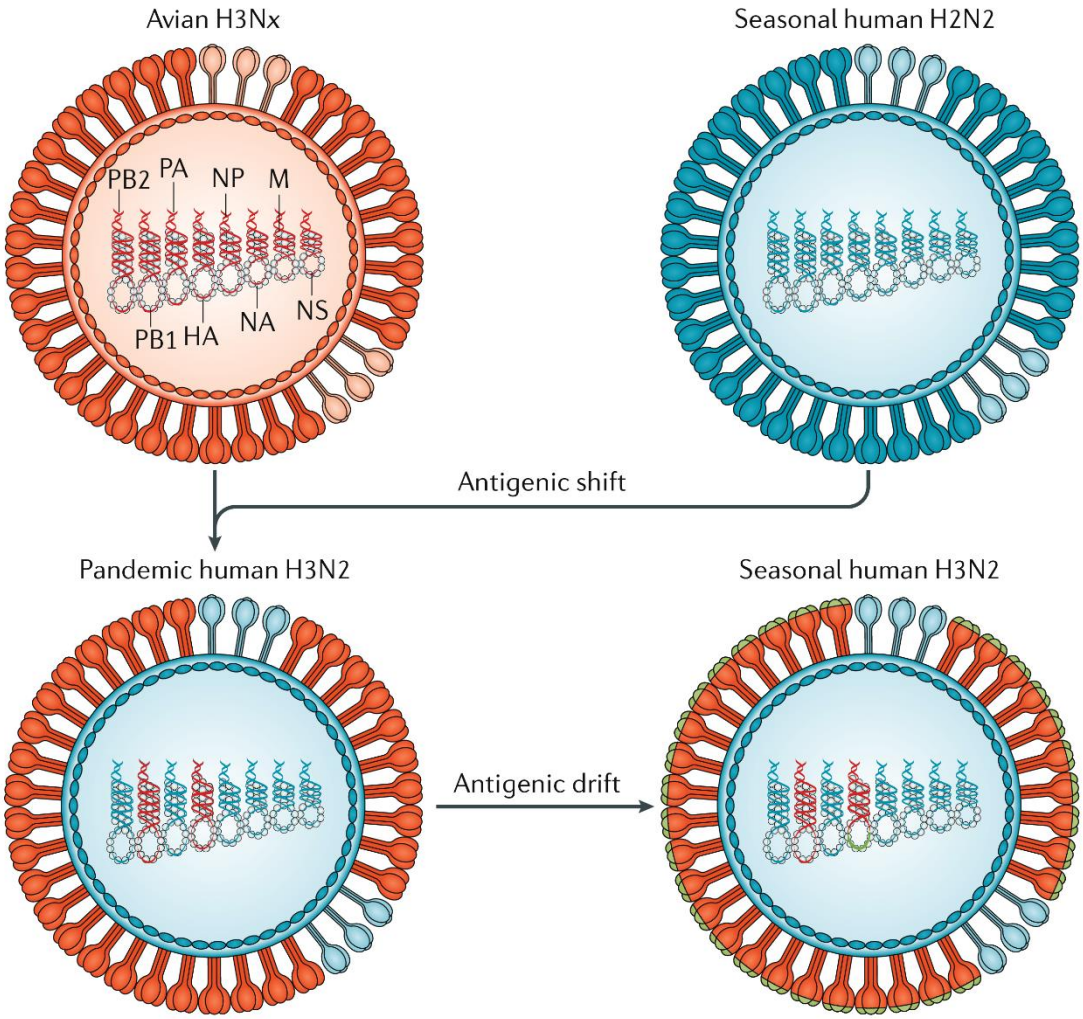

Figure 4. Antigenic drift (point mutations) and antigenic shift (reassortment), demonstrated by the example of the pandemic human H3N2 virus from 1968 (78). For permission rights see Chapter X, Supplementary Material, Permission for Reproduction.

Gradual evolution of the IAV genome results from high rates of synonymous and nonsynonymous point mutations (>1 x 10^{-3} substitutions per site, per year (99)), attributed to the error-prone RdRP activity and lack of a proofreading mechanism in IAV RNA replication (99, 105). Significant mutations affecting the major surface glycoproteins (HA and NA) can elicit antigen migration and result in new antigenic variants. This process is often referred to as *antigenic drift* (Figure 4). The host immune protection (neutralising antibodies acquired by previous infection or immunisation) might subsequently be no longer effective against the novel antigenic virus. Every mutation can potentially lead to evasion of the host immune system, positive selection and thus, wider and more effective distribution and infection (106).

Due to the segmented genome of IAV, co-infection of one host cell with two varying parental influenza viruses (sub- and/or genotype) can result in the exchange of complete gene segments during virion assembly (Figure 4). Termed *reassortment*, the genesis of progeny viruses with gene segments from both parental viruses alters the genotype (107-109). Involvement of the HA and NA gene segments results in subtype change and differing antigenic properties, and is consequently referred to as *antigenic shift*. All four flu pandemics were linked to reassortant IAV of avian (1918, 1957, 1968) or swine origin (2009) (3).

RNA *recombination* occurs in many RNA viruses, yet is rarely detected in negative-sensed, single stranded RNA viruses like IAV (110). Two main mechanisms of recombination can be distinguished: Non-homologous recombination between two different RNA fragments (111), and homologous recombination with template switching during virus replication (112). In China, the circulating zoonotic H7N9 AIV may carry a PB1 segment recombined with a highly pathogenic fragment from a HPAI H5N1 virus, potentially the reason for the high fatality rate among patients infected with the respective H7N9 strain (113).

2.4. Highly Pathogenic and Low Pathogenic Avian Influenza Viruses

AIV, also termed “bird flu/bird plague” was first described in poultry in Italy, 1878 (114). Aside from causing huge (economic) losses in both the poultry industry and wild bird population, some strains can carry zoonotic potential with the possibility of causing major worldwide pandemics as mentioned above. The classification of AIV into two pathotypes, LPAIV and HPAIV, can be achieved depending on the pathogenicity in chickens and the HA endoproteolytic cleavage site (HACS) (115).

LPAIV are most frequently detected in the natural reservoir, wild aquatic birds, in a wild array of HA/NA subtype combinations and count for the vast majority of AIV. The widespread occurrence of LPAIV in (migratory) wild birds cause an asymptomatic infection course (116, 117). Sporadic transmission to gallinaceous poultry commonly induces either no clinical symptoms or mild disease. LPAIV infections with H7 and H9 are most frequently detected in poultry (118, 119). Although generally no clinical signs are visible, LPAIV play an important role in the reassortment and emergence of novel AIV, potentially carrying different antigenic and pathogenic properties (117).

Of all HA subtypes, only subtypes H5 and H7 have the potential to evolve from LPAIV to HPAIV (1, 120, 121). The classification of HPAIV builds on two conclusive characteristics: An intravenous pathogenicity index of >1.2 in chickens and a multibasic HACS (122). After infection of domestic poultry (especially chickens and turkeys) with LPAIV from the wild bird reservoir, host adaptation is believed to be the driver for the emergence of HPAIV variants (123). Initially, the HA protein stands as a single polypeptide precursor (HA_0). The monobasic HA_0 cleavage site of LPAIV allows cleavage into the subunits HA_1 and

HA_2 by trypsin-like proteases found in the respiratory and intestinal tract. In contrast, HPAIV carry a polybasic HA_0 cleavage site cleaved by ubiquitously expressed subtilisin-like cellular proteases, resulting in systemic viral replication and lethal infection with mortality rates of up to 100% in birds (93).

2.5. **Review:** Genetics of Highly Pathogenic Avian Influenza H5 Viruses in Germany

A conclusive summary of the genetics of HPAI subtype H5 viruses in Germany from 2006 to 2020 is presented here as a review publication. The reference section is presented in the style of the respective journal and is not included at the end of this document. The numeration of figures and tables corresponds with the published form.

Review

The Genetics of Highly Pathogenic Avian Influenza Viruses Subtype H5 in Germany, 2006 – 2020

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REVIEW

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The genetics of highly pathogenic avian influenza viruses of subtype H5 in Germany, 2006–2020

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Abstract

The H5 A/Goose/Guangdong/1/1996 (gs/GD) lineage emerged in China in 1996. Rooted in the respective gs/GD lineage, the hemagglutinin (HA) gene of highly pathogenic avian influenza viruses (HPAIV) has genetically diversified into a plethora of clades and subclades and evolved into an assortment of sub- and genotypes. Some caused substantial losses in the poultry industry and had a major impact on wild bird populations alongside public health implications due to a zoonotic potential of certain clades. After the primary introduction of the HPAI H5N1 gs/GD lineage into Europe in autumn 2005 and winter 2005/2006, Germany has seen recurring incursions of four varying H5Nx subtypes (H5N1, H5N8, H5N5, H5N6) carrying multiple distinct reassortants, all descendants of the gs/GD virus. The first HPAIV H5 epidemic in Germany during 2006/2007 was caused by a clade 2.2 subtype H5N1 virus. Phylogenetic analysis confirmed three distinct clusters belonging to clades 2.2.1, 2.2.2 and 2.2, concurring with geographic and temporal structures. From 2014 onwards, HPAIV clade 2.3.4.4 has dominated the epidemiological situation in Germany. The initial clade 2.3.4.4a HPAIV H5N8, reaching Germany in November 2014, caused a limited epidemic affecting five poultry holdings, one zoo in Northern Germany and few wild birds. After November 2016, HPAIV of clade 2.3.4.4b have dominated the situation to date. The most extensive HPAIV H5 epidemic on record reached Germany in winter 2016/2017, encompassing multiple incursion events with two subtypes (H5N8, H5N5) and entailing five reassortants. A novel H5N6 clade 2.3.4.4b strain affected Germany from December 2017 onwards, instigating low-level infection in smallholdings and wild birds. Recently, in spring 2020, a novel incursion of a genetically distinct HPAI clade 2.3.4.4b H5N8 virus caused another epidemic in Europe, which affected a small number of poultry holdings, one zoo and two wild birds throughout Germany.

KEYWORDS

Clade 2.2, Clade 2.3.4.4, highly pathogenic avian influenza viruses, HPAIV, reassortment, subtype H5

[Correction added on 01 October, after first online publication: Projekt DEAL funding statement has been added.]

1 | INTRODUCTION

Since the first detection of the A/Goose/Guangdong/1/1996 (gs/GD) lineage in domestic geese located in the Guangdong province of China, 1996 (Xu et al., 1999), highly pathogenic avian influenza viruses (HPAIV) of the respective H5N1 gs/GD lineage have caused devastating losses worldwide within both the wild bird and the poultry population over the past 24 years (Alarcon et al., 2018; Guan & Smith, 2013; Harfoot & Webby, 2017; Nunez & Ross, 2019; Sutton, 2018; Yamaji et al., 2020).

Initially, the identified HPAIV of subtype H5N1 was limited to poultry species and geographically confined to Southeast China. One year after its emergence, in 1997, a gs/GD-related HPAIV H5N1 reassortant caused outbreaks in poultry and zoonotic transmissions to humans in Hong Kong (Claas et al., 1998; Shortridge et al., 1998). Of the 18 human cases recorded in the particular epidemic, six proved to be fatal, shining a light on the zoonotic potential and subsequent serious public health implications of this lineage (Webby & Webster, 2001; Webster, 2002). After several years of low level, yet likely endemic circulation in aquatic poultry species in Southern China, re-emergence of the HPAIV subtype H5N1 in 2003 caused not only large-scale poultry outbreaks in several Asian countries (Cauthen et al., 2000; Guan et al., 2002; Li et al., 2010; Webster et al., 2002), but was also transmitted to the migratory aquatic wild bird population (Chen et al., 2005; Ellis et al., 2004). From December 2003 to February 2004, HPAIV H5N1 outbreaks were detected almost simultaneously in the Republic of Korea, Japan, Cambodia, Indonesia and Thailand, affecting not only poultry, but also a small amount of wild birds, felines and humans (Alexander, 2007; WHO, 2012).

Over the course of eight years (1996–2004), the gs/GD lineage was perceived as a problem confined to East Asia. However, the situation dramatically transformed after the initial identification of the respective virus in migratory wild birds in 2003 (Chen et al., 2005; Ellis et al., 2004). Intercontinental dissemination of the subsequently designated clade 2.2 HPAIV H5N1 gs/GD lineage from Asia began in April 2005 after the respective virus was detected as the cause of high wild bird mortality rates at Lake Qinghai, Northwest China, affecting numerous species within the orders Anseriformes (waterfowl, e.g. ducks, geese and swans) and Charadriiformes (gulls and shorebirds; Chen et al., 2005, 2006; Lvov et al., 2010; Zhou et al., 2006). From late 2005 onwards, the gs/GD lineage rapidly spread westwards through Russia, Mongolia, Kazakhstan and Turkey to reach Europe, the Middle East, and Africa in 2005 and 2006 (Olsen et al., 2006; Takekawa et al., 2010). Almost simultaneously within a one-week time frame in February 2006, HPAIV H5N1 surfaced in several Central European countries, including Greece, Italy, Bulgaria, Slovenia, Austria and Germany (Brown, 2010; WHO, 2012). Multiple incursion routes into Europe, including wild bird migration and illegal domestic poultry and exotic bird trade, were discussed as potential drivers of the H5N1 epidemic (Serratosa et al., 2007; van den Berg, 2009; Van Borm et al., 2005; Yee et al., 2009).

The continuous and extensive circulation of descendants of the HPAIV H5N1 gs/GD lineage in poultry and wild birds led to the appearance of multiple individual genotypes achieved by reassortment events; genetic drift by point mutations in the hemagglutinin (HA) gene which is under considerable selection pressure led to its diversification into 10 distinct genetic clades (0–9) and multiple subclades (Guan & Smith, 2013; WHO/OIE/FAO, 2008). To date, over 80 countries in Asia, Africa, Europe and North America have reported gs/GD lineage HPAIV H5Nx outbreaks, meriting the term 'pandemic' in birds due to the severity, size and geographic distribution (Guan & Smith, 2013; Lee et al., 2017). Since the initial introduction of the HPAIV H5N1 gs/GD lineage to Europe in autumn 2005 (Weber et al., 2007), the source of all HPAIV H5 epidemics has been traced back to the East of Europe, mainly in Central and Southeast Asia, while Central and West Europe have become a 'sink' for gs/GD HPAIV infections, experiencing multiple novel incursions from sources to the East: a model that is similar to the one proposed for the ecology of seasonal human influenza virus strains (Rambaut et al., 2008). The only subtype H5 HPAIV reported from Europe that was not rooted in the gs/GD lineage recently had caused multiple regional outbreaks in the duck-fattening industry of France (Briand et al., 2017).

Germany has experienced a recurring influx of four HPAIV H5Nx subtypes carrying multiple distinct reassortants, all clustering as descendants of the primary gs/GD lineage (Harder et al., 2015; King et al., 2020; Pohlmann et al., 2018, 2019; Starick et al., 2008). This includes the 2006/2007 clade 2.2 H5N1 epidemic, the 2014/2015 clade 2.3.4.4a H5N8 epidemic, the major 2016/2017 clade 2.3.4.4b H5N8 and H5N5 epidemic, the 2017/2018 clade 2.3.4.4b H5N6 events, and the recent 2020 clade 2.3.4.4b H5N8 scenario. For updates on the current HPAIV situation in Germany, please check the risk assessment available from the Friedrich-Loeffler-Institut website (https://www.fli.de/en/publications/risk-assessments/).

To date, a comprehensive and conclusive genetic review of all German HPAIV H5Nx occurrences has yet to be compiled. This paper aims to assess and present the genetic evolution and molecular epidemiology of all assorted HPAIV H5 outbreaks in Germany since 2006 in a temporal manner.

2 | 2006–2007: CLADE 2.2—H5N1

As of December 2009, the respective clade 2.2 HPAI H5N1 virus had caused poultry or wild bird outbreaks in 62 countries reaching over three continents, including 24 European states (Cattoli, Fusaro, et al., 2009). After the first European cases were identified in Romania (Oprisan et al., 2006), Croatia (Savic et al., 2010) and the Ukraine (Muzyka et al., 2019) in summer–winter of 2005, Germany alongside 18 other European countries between Southern Sweden and Italy recorded their first cases almost simultaneously in January/February 2006 (Alexander, 2007; Cattoli, Fusaro, et al., 2009; WHO, 2012).

In Germany, the first cases confirmed on 14 February 2006, comprised of multiple wild birds, predominantly swans (*Cygnus* sp.) and geese (*Anser* sp.), geographically concentrated on the Baltic Coast and with the Island of Ruegen as a small epicentre covering 46% of all German 2006 HPAIV H5N1 cases (Globig et al., 2009). Two weeks after the initial outbreak in Northern Germany, a second epicentre was established in Southern Germany at Lake Constance (Baden-Wuerttemberg), followed by further wild bird infections along the Danube River and in wetlands in Bavaria. The HPAIV H5N1 spread rapidly in a radial manner within the following weeks, affecting seven federal states in Germany and over 30 wild bird species. Overall, in 2006, 343 HPAIV H5N1-infected wild birds were diagnosed in Germany, alongside virus recovery from three stray cats and one stone marten (*Martes foina*; Globig et al., 2009; Harder et al., 2009). In addition, a single mixed commercial turkey/chicken/geese holding located in Saxony was affected in April 2006. The epidemic came to a preliminary halt on 12 May 2006, when the final wild bird case involving two eagle owls (*Bubo bubo*) was detected in Bavaria. From this date on, only one more isolated case, involving a black swan (*Cygnus atratus*) held in captivity, was recorded as HPAIV H5N1 positive in August 2006. Otherwise, no further poultry outbreaks or wild bird cases were detected in 2006 (Figure 1a; Alexander, 2007; Globig et al., 2009; Hesterberg et al., 2009).

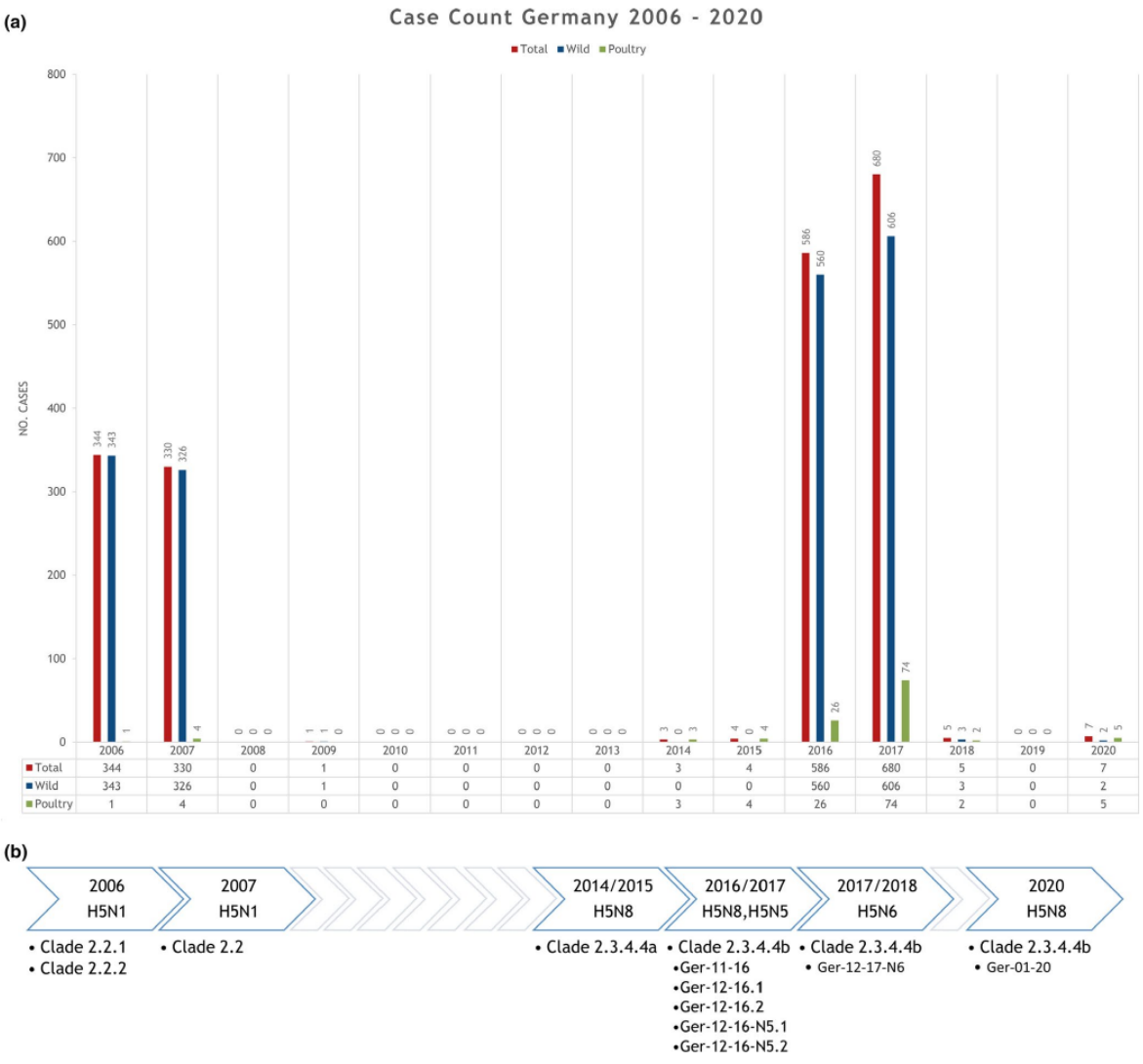

FIGURE 1 (a) Case counts of all HPAIV H5 reports in Germany retrieved from the German animal disease notification system (Tierseuchen-Nachrichtensystem, TSN, accessed 26.05.2020) with added wild bird cases from the German National Reference Laboratory for Avian Influenza, 2006–2020. Numbers of cases are divided into total, wild bird and poultry reports. (b)Timeline of occurrence and genetic background of HPAIV H5 viruses in Germany, 2006–2020. Details are given in the text

In the following year, several European countries (Hungary, UK, Czech Republic and the European region of Russia) reported novel HPAIV H5N1 outbreaks in poultry (Brown, 2010; Irvine et al., 2007; Nagy et al., 2009; Szeleczky et al., 2009). The HPAIV H5N1 virus re-emerged in Southeast Germany in June 2007, identified in a mute swan (*Cygnus olor*) on June 19, 2007, in Bavaria (Globig et al., 2009). During this month, France and the Czech Republic also reported further HPAIV H5N1 poultry and wild bird infections (Gall-Recule et al., 2008; Nagy et al., 2009; WHO, 2012). To conclude, 2007 brought a further wave of wild bird cases similar in size to the previous year, counting 326 infected wild birds distributed throughout the southern federal states of Bavaria, Thuringia, Saxony and Saxony-Anhalt in June–August, 2007 (Figure 1a). In addition, two large duck-fattening holdings, in Bavaria, reported outbreaks with the respective subtype (Globig et al., 2009). The final HPAIV H5N1 outbreaks took place in December 2007, affecting three individual backyard holdings in Brandenburg which were epidemiologically linked to the one in fattening ducks in Bavaria in 2007 (Harder et al., 2009).

The initial genetic evaluation revealed a high identity rate between all German outbreak sequences and Qinghai-like viruses, of both bird and mammalian origin, but clearly distinct from other HPAI H5N1 viruses of the gs/GD lineage (Cattoli, Fusaro, et al., 2009; Chen et al., 2006; Shestopalov et al., 2006). All investigated German and other European HPAIV H5N1 isolates shared characteristics concurring with analogous HPAIV H5N1 strains of Asian origin, for example the identical polybasic hemagglutinin cleavage site (PQGERRRKKR*GLF) and a typical 20 amino acid deletion in the neuraminidase stalk region (Fink et al., 2010; Lee, Bertran, et al., 2017; Smietanka et al., 2010; Starick et al., 2008), described as a strong marker for increased virulence in galliform poultry (Li et al., 2014; Munier et al., 2010).

According to the official nomenclature provided for the gs/GD lineage by the WHO/OIE/FAO (2008) Evolution Working Group, the allocated clade 2.2 HPAIV H5N1 epidemic was further divided into two distinct subclades, 2.2.1 and 2.2.2 (WHO/OIE/FAO, 2008, 2009, 2012). Nonetheless, Starick et al. reported a third subclade 2.2.3 (2008), which was, however, only proposed and so far not officially designated as a separate third degree clade by the WHO/OIE/FAO (2009) Evolution Working Group. The proposed clade 2.2.3 is officially regarded as part of clade 2.2 (Figure S3). Also often termed clade 2.2 A, B and C or in accordance with the geographic origin European–Middle East–African (EMA) 1–3 (Salzberg et al., 2007), the German 2006/2007 epidemic encompassed all three clades, often coinciding with geographic and temporal metadata. Within the German 2006/2007 HPAIV H5N1 epidemic, the phylogenetic clades were temporally divided, including clades 2.2.1 and 2.2.2 in 2006 and the third clade 2.2 in 2007 (Figure 1b). Additionally, all cases from the primary 2006 German epidemic were not only geographically clustered into a northern and southern type, but also revealed a genetic classification, with few exceptions, allocating clade 2.2.2 to the northern cluster and clade 2.2.1 to the southern cluster. Analyses of neuraminidase (NA) sequences equally mirrored the described classification based on the HA sequences (Starick et al., 2008).

On closer inspection, the HA gene of clade 2.2.1 in Southern Germany shared great sequence identity levels of >99% with other European, yet also Asian, Middle East and African countries. Prior to the initial German outbreak scenario, equally high identity levels were established with sequences from Croatia, Russia, Mongolia and China (Table S1). These findings highlight the potential initial restriction of the clade 2.2.1 situation in Southern Germany, pointing towards an introduction from Asia via Mongolia and Central Russia, followed by the Southern European country Croatia in 2005 (Coulombier et al., 2005; Gilbert et al., 2012; Lipatov et al., 2007; Savic et al., 2010). During the epidemic in 2006, values of over 99% identity were established with sequences from Southern Europe, pinpointing the continuous circulation of clade 2.2.1 in the Southern European countries concurring with the geographic dynamics of the respective clade in Southern Germany (Brown, 2010; Fink et al., 2010; Nagy et al., 2009; Szeleczky et al., 2009; WHO, 2012). The additional identification of highly similar sequences in Turkey and Nigeria indicates the further spread of clade 2.2.1 onto the African continent (Table S1; Cattoli, Monne, et al., 2009; Fusaro et al., 2009; Oner et al., 2006; Salzberg et al., 2007; Zhou et al., 2016). In the federal state of Bavaria, two further genotypically distinct groups (termed Bavaria 1 and Bavaria 2) of isolates were identified, both clustering within clade 2.2.1 (Rinder et al., 2007). This also underlines the continuous circulation of clade 2.2.1 in the surrounding European countries, potentially introducing Bavaria 1 and 2 into Germany by separate entries (Fink et al., 2010; Haase et al., 2010; Nagy et al., 2007; Rinder et al., 2007; Savic et al., 2010; Starick et al., 2008).

In comparison with clade 2.2.1, outbreaks clustering within clade 2.2.2, demonstrated here by the index strain A/*Cygnus cygnus*/Germany/R65/2006 (R65/06), showed extreme homogeneity between singular cases. The shorter circulation time frame of clade 2.2.2 in comparison with clade 2.2.1 could be the reason for the high similarity levels, and points towards a solitary introduction of the clade 2.2.2 into the area and subsequent spread from the Island of Ruegen into the surrounding regions (Harder et al., 2009; Starick et al., 2008). All segments of R65/06 ranged between sequence identity levels of 99% (polymerase basic 2 protein, PB2; polymerase basic 1 protein, PB1; polymerase acidic protein, PA; HA; nucleoprotein, NP; NA; non-structural protein, NS) and 100% (matrix protein, MP) when aligned with a HPAIV H5N1 strain from the Astrakhan region of Central Russia, indicating a novel introduction into Germany, likewise from Asia (Brown, 2010; Coulombier et al., 2005; Lipatov et al., 2007; Shestopalov et al., 2006; Starick et al., 2008; Takekawa et al., 2010; Zhou et al., 2016).

Further analyses of the respective HA segment allowed the establishment of over 99% sequence identity between R65/06 and sequences found in Eurasia (Table S1). In accordance with the identified strains, the geographic distribution shows a differing circulation in comparison with clade 2.2.1. In agreement with the northern occurrence of clade 2.2.2 in Germany

predominantly along the Baltic Coast, the identified related strains sampled in Northern Europe, affecting *inter alia*, Poland (Smietanka et al., 2010), Denmark (Bragstad et al., 2007) and the Czech Republic (Nagy et al., 2007, 2009). Additionally, Sweden reported a range of cases or outbreaks, partly clustering within clade 2.2.2 (Kiss et al., 2008; Zohari et al., 2008). The analysed sequences of three stray cats and one stone marten identified on the Island of Ruegen all clustered within clade 2.2.2 (Harder et al., 2009). The infection was highly likely a result of direct contact to HPAIV H5N1-infected wild birds in the area, presumably by scavenging; susceptibility of cats through the oral route was subsequently proven experimentally (Harder & Vahlenkamp, 2010; Vahlenkamp & Harder, 2006; Vahlenkamp et al., 2010). Sequence identity levels between the R65/06 strain and a full-genome sequence of an HPAIV H5N1-infected cat (A/cat/Germany/R606/2006) were exceedingly high, with only two varying amino acid substitutions: a phenylalanine-to-leucine substitution in the PA protein (F4L) and N110D substitution in the HA1 protein. None of the identified substitutions had previously been described as an adaptive mutation aiding infection in mammals (Harder et al., 2009).

The final clade 2.2 initially identified during June 2007 was slightly more distinct in its composition compared to the previous clades (Starick et al., 2008). By utilizing the index strain A/*Cygnus olor*/Germany/R1359/2007 (R1359/07), the highest HA similarity values between R1359/07 and an HPAIV H5N1 from the Krasnodar region in Southern Russia were attained. In addition, identity levels of over 99% were shared with sequences detected in Eurasia (Table S1; Chen et al., 2006; Gall-Recule et al., 2008; Lipatov et al., 2007; Nagy et al., 2009; Shestopalov et al., 2006). Interestingly, a single HPAIV H5N1 sequence from Italy identified in 2006 also clustered within clade 2.2, yet formed a separate branch within the respective clade and shares the closest relations to samples collected in Russia, Iran and Dagestan in 2006 (Nagy et al., 2009). In this light, clade 2.2 was not entirely novel to Europe; however, the epidemic in Europe in 2007 represented the first stable occurrence of the respective clade. According to Haase et al. (2010), the ancestor of clade 2.2 viruses based on a set of 28 HA segments most likely resided in Germany, independently spreading to France, Russia and the Czech Republic. The final cases of the clade 2.2 outbreak in Germany took place in Brandenburg during December 2007 (Harder et al., 2009). Interestingly, no cases in the poultry or wild bird population were detected after August 2007 until three backyard chicken holdings experienced analogous clinical symptoms and mortality rates within a 2-week period. Epidemiological and phylogenetic analyses established a potential transmission pathway between the backyard holdings and industrial duck-fattening farms in Bavaria, previously identified with subclinical/silent clade 2.2 HPAIV H5N1 infections in summer 2007 by analysis of retained slaughter batches from the affected duck holding. All backyard holdings had access to uncooked offal from commercial deep-frozen duck carcasses prior to the infection. The high identity levels of the HA sequences of the backyard holdings and frozen duck carcasses brand an infection via the food chain highly probable, although no direct connection was established in the concurring epidemiological investigations (Harder et al., 2009).

After the final backyard cases in December 2007, no further HPAIV H5N1 cases with the exception of a singular wild bird case (common pochard duck—*Aythya ferina*) in 2009 were identified in Germany to date (WHO, 2012).

3 | 2014/2015: CLADE 2.3.4.4a—H5N8

Germany was the first European country to report the incursion of a novel Eurasian clade 2.3.4.4 HPAIV of subtype H5N8 in November 2014 (Figure 1b; WHO, 2014). Within the succeeding months until January 2015, a minor epidemic confined mostly to poultry holdings occurred in Northern Germany, affecting the federal states Mecklenburg-Western Pomerania and Lower Saxony. Overall, five poultry holdings and one zoo were reported as HPAIV H5N8 positive, showing signs of increased mortality and severe illness (Figure 1a; Conraths et al., 2016). In the same period, outbreaks with the respective clade 2.3.4.4 HPAIV H5N8 were reported in the Netherlands (Bouwstra et al., 2015), UK (Hanna et al., 2015), Hungary and Italy (Adlhoch et al., 2014). Interestingly, intensified surveillance of the wild bird population led to the identification of infected, yet clinically healthy aquatic wild bird species (ducks, swans and a gull) in Germany, the Netherlands and Sweden, proving the presence of these viruses in the wild bird population (Globig, Starick, et al., 2017; Harder et al., 2015; Verhagen et al., 2015).

Initial phylogenetic analyses of the German index case A/turkey/Germany-MV/AR2472/2014 (AR2472/14) and all other European outbreaks clustered the novel reassortant HPAIV H5N8 virus within clade 2.3.4.4 (Lee, Bertran, et al., 2017; Smith, Donis, & WHO, 2015). The precursor subtype H5N8 HPAIV most likely originated from China, initially isolated in 2009–2010 (Wu et al., 2014; Zhao et al., 2013). South Korea reported the first H5N8 epidemic outside China in January 2014, when two HPAIV H5N8 sister lineages clustering within clade 2.3.4.4 were detected and subsequently split into group A, termed Donglim-like, aka 2.3.4.4a and group B, termed Gochang-like, aka 2.3.4.4b (Figure S4; Kang et al., 2015; Lee et al., 2014). The genetic composition confirmed close relations of all segments between the AR2472/14 and the described South Korean HPAIV H5N8 clade 2.3.4.4a viruses (Harder et al., 2015). In turn, the Donglim-like isolate is most likely a reassortant of the precursor A/duck/Jiangsu/k1203/2010 from China and other AI viruses co-circulating among birds in East Asia during 2009–2012 (Figure S1; Gu et al., 2013; Kim et al., 2014).

Prior to the outbreak situation in Europe in late 2014, a further highly similar clade 2.3.4.4a HPAIV H5N8 isolate sharing over 99% identity with AR2472/17 for all genome segments was identified in the Russian Far East, September 2014 (Marchenko et al., 2015), in an area situated at the intersection of several wild bird migration flyways (Global Consortium for H5N8 & Related Influenza Viruses, 2016). According to Hanna et al. (2015), the most recent common ancestor for the European and Japanese HA segment cluster was calculated

for June 2014 (95% highest posterior density level: April 2014–September 2014), while the ancestor of viruses from Europe, Japan and Korea was approximately estimated for October 2013 (95% highest posterior density level: June 2013–January 2014).

Of the six outbreaks in captive birds recorded in Germany in 2014–2015, genetic and geographic connections could be established within Germany (Conraths et al., 2016; Globig, Starick, et al., 2017). The first poultry outbreak in Mecklenburg-Western Pomerania clustered with a wild gull found approximately one month later in Lower Saxony, and further cases reported from the Netherlands and England (Bouwstra et al., 2015; Hanna et al., 2015). Two poultry outbreaks recorded during December 2014 (located in close geographic proximity in Lower Saxony), and a backyard holding affected in January 2015 (Mecklenburg-Western Pomerania) equally shared high identity levels for all segments. All samples analysed from the Rostock Zoo (Mecklenburg-Western Pomerania) presented high identity levels, although several bird species were affected, for example a white stork (*Ciconia ciconia*), scarlet ibis (*Eudocimus ruber*) and hooded merganser (*Lophodytes cucullatus*). The respective outbreak in zoo birds shared close relations with mute swans identified in Sweden, April 2015 (Globig, Starick, et al., 2017). Overall, for the HA segment identity values of 99.5%–99.9% were established between all German and other European clade 2.3.4.4a HPAIV H5N8 outbreaks or cases from November 2014 to April 2015. Interestingly, equal identity levels were recorded for the European datasets in comparison with further Asian sequences and likewise for the Russian Sakha/14 strain (Table S2). These findings, alongside the most recent common ancestor dates previously mentioned (Hanna et al., 2015), indicate the co-circulation of extremely similar clade 2.3.4.4a HPAIV H5N8 contemporaneously in East Asia and Europe (Ozawa et al., 2015; Saito et al., 2015; Si et al., 2016), underlining the great likelihood of virus dissemination via wild migratory birds after congregation at bird nesting and moulting grounds in Russia and subsequent dissemination to known wintering grounds in Europe, North America and Asia (Global Consortium for H5N8 & Related Influenza Viruses, 2016). In addition, North America reported further clade 2.3.4.4a HPAIV H5N8 cases equally from November 2014 onwards, additionally emphasizing these findings (Ip et al., 2016; Lee et al., 2015, 2018). In North America, the respective HPAIV H5N8 underwent reassortment with North American low pathogenic avian influenza viruses (LPAIV), leading to the establishment of HPAIV subtype H5N2 (dominating the 2014/2015 outbreak), H5N1 and additional H5N8 (Lee et al., 2016; Lee, Bertran, et al., 2017).

To date, no further clade 2.3.4.4a HPAIV H5N8 cases have been reported in Germany after the concluding outbreak in January 2015 (Figure 1b).

4 | 2016–2017: CLADE 2.3.4.4b—H5N8, H5N5

During 2016–2017, a novel HPAIV H5Nx clade 2.3.4.4, in this case belonging to the cluster B Gochang-like lineage, caused the largest European HPAIV epidemic on record to date. Unprecedented in geographic distribution, spectrum of species affected and magnitude of outbreaks, 30 countries in Europe reported 1,590 wild bird mortality events and 24 European Union countries experienced 1,207 HPAIV outbreaks in poultry holdings between October 2016 and August 2017 (Alarcon et al., 2018; Brown et al., 2017; Napp et al., 2018). All genome segments of the novel clade 2.3.4.4b HPAIV H5Nx viruses differed significantly from clade 2.3.4.4a detected in 2014–2015 (Harder et al., 2015; Pohlmann et al., 2017).

This event transpired to become the largest HPAIV epidemic ever reported in Germany, peaking during the winter of 2016/2017 (November 2016–March 2017). Two H5Nx subtypes (H5N8, H5N5) and five reassortants (reassortant designation termed Ger-11-16, Ger-12-16.1 and Ger-12-16.2, Ger-12-16-N5.1 and Ger-12-16-N5.2 as suggested by Pohlmann et al. (2018)) were identified within the epidemic time frame, affecting over 1,100 wild birds, 92 poultry holdings and 15 zoos/wildlife parks in Germany alone (Figure 1a,b). All linked by a shared HA segment rooted in the HPAIV H5N8 clade 2.3.4.4b Gochang-like lineage, the individual reassortants distinguish themselves by distinct PB2, PB1, PA, NP, NA and NS segments (Figure 2, Figure S4), shining a light on the 'promiscuous' propensity of this clade to form new sub- and genotypes, often capable of rapid and global spread (Claes et al., 2016; Global Consortium for H5N8 & Related Influenza Viruses, 2016; Lee, Bertran, et al., 2017). By the end of the epidemic, every federal state in Germany had reported both wild bird and poultry cases (Globig, Staubach, et al., 2017).

Prior to the European 2016–2017 epidemic and alike the incursion patterns of the previously described outbreaks, analogous HPAIV H5N8 clade 2.3.4.4b reassortants were detected as the causative agent for mass mortality in waterfowl at the Uvs-Nuur Lake located between Mongolia and the Republic of Tyva (Russia) in late May–early June 2016, followed by further reports in the Kurgan region three months later and almost simultaneous reports from Tatarstan and Lake Chany in October 2016 (Lee, Sharshov, et al., 2017; Li et al., 2017; Marchenko et al., 2017; Voronina et al., 2018). Due to the genetic characteristics of the novel clade 2.3.4.4b, the close relations to Central Asian isolates described and the lack of identification of other HPAIV H5Nx strains in wild birds from November 2014 to January 2016 in the Netherlands (Poen et al., 2016, 2018), the 2016–2017 epidemic most likely represents at least one new incursion into Europe.

5 | 2016/2017: Reassortant Ger-11–16—H5N8

The first HPAIV H5N8 reassortant of the unprecedented epidemic was reported in diving duck species on November 7, 2016, almost simultaneously in the North (Lake Plön, Schleswig-Holstein) and South (Lake Constance, Baden-Wuerttemberg) of Germany (Globig, Staubach, et al., 2017). Comparable to the 2006/2007 HPAIV H5N1 outbreak, the initial cases were reported in wild birds (Globig et al., 2009), followed by poultry outbreaks shortly thereafter

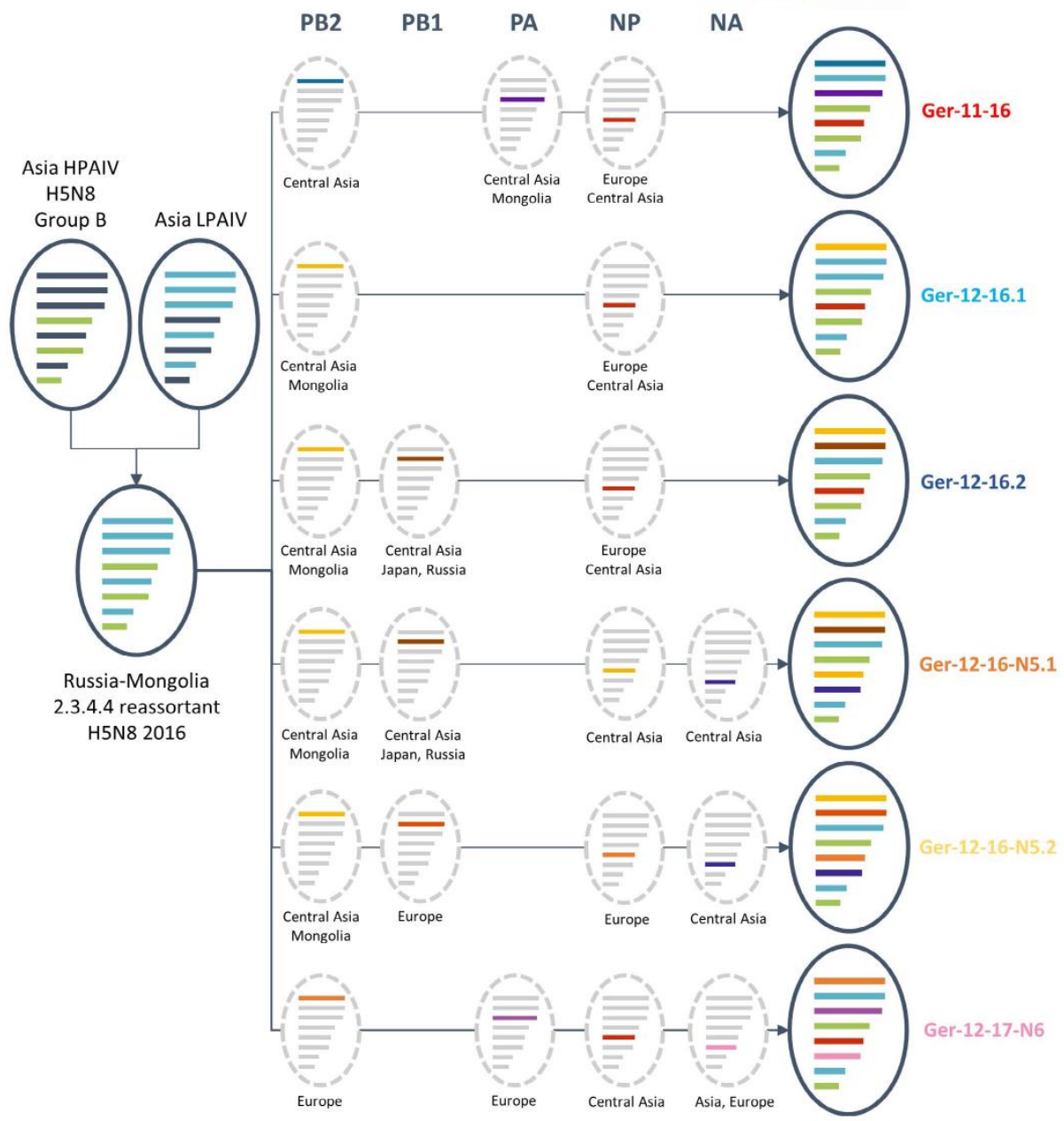

FIGURE 2 Visual representation of the reassorted HPAIV clade 2.3.4.4b viruses identified from 2016 to 2018, including Ger-11-16, Ger-12-16.1 and Ger-12-16.2 (H5N8), Ger-12-16-N5.1 and Ger-12-16-N5.2 (H5N5), and Ger-12-17-N6 (H5N6). Colours are designated in accordance with Pohlmann et al. (2018, 2019). Dashed lines indicate putative precursors

(Starick et al., 2008). Here, the majority of primary cases were reported in wild birds along the Baltic Coast, before spreading further west- and southwards affecting a wide variety of commercial and non-commercial poultry holdings, and subsequently continuing to spread throughout Europe (Globig, Staubach, et al., 2017).

Phylogenetic analysis of the novel German HPAIV H5N8 clade 2.3.4.4b isolate revealed the first described reassortant virus within the respective epidemic (Pohlmann et al., 2017). Termed Ger-11-16 in accordance with the initial detection date in November 2016, the genetic backbone comprises of HPAIV H5N8 from the 2016 Russia–Mongolia 2.3.4.4b reassortant, however carrying differing PA and NP segments (Figure 2; Lee, Sharshov, et al., 2017). In addition, all H5N8 reassortants show a C-terminal truncation of 13 amino acids in the NS1 protein in comparison with the formerly

described Gochang-like lineage (Pohlmann et al., 2017), previously observed in various AI viruses mainly identified in gallinaceous poultry (Abdelwhab et al., 2016). Unlike the previous 2006/2007 clade 2.2 HPAIV H5N1 lineage, where the genetic distinction coincided with geographic distribution and the circulation in Europe was formerly detected in 2005 (Starick et al., 2008), only very few genetic variances could be established between the northern and southern outbreaks of Ger-11-16 and Europe saw an unprecedented, simultaneous influx of the novel clade 2.3.4.4b HPAIV H5Nx reassortants during winter 2016 (Beerens et al., 2017; Napp et al., 2018; Pohlmann et al., 2017).

On closer examination, the Ger-11-16 PA and NP segments are of diverse Eurasian origin. The PA segment shares 97% identity with LPAIV found in Asia. In comparison, the NP segment can be traced back to varying AI viruses found in Central Europe and in Central/Western Asia (Figure 2, Table S2). HPAIV H5N8 detected in wild birds during August 2016 in Russia and an almost identical isolate reported in Poland shortly before the primary German outbreak at the beginning of November share over 99% identity throughout all segments with the concurring Ger-11-16 reassortant (Beerens et al., 2017; El-Shesheny et al., 2017; Lee, Sharshov, et al., 2017; Marchenko et al., 2018, 2017; Poen et al., 2018; Pohlmann et al., 2017; Souvestre et al., 2019; Swieton & Smietanka, 2018). Further outbreaks with the equivalent Ger-11-16 reassortant were reported in, *inter alia*, Italy (Fusaro et al., 2017), the Netherlands (Beerens et al., 2017), Czech Republic (Nagy et al., 2018), Switzerland (Meier et al., 2017), France (Guinat et al., 2018) and Poland (Swieton & Smietanka, 2018).

Within the Ger-11-16 reassortant cluster, 99.0%–99.9% identity was recorded for concatenated full-genome sequences. The final cases of Ger-11-16 were reported in August 2017, affecting two swans in Saxony-Anhalt. Thereafter, no further Ger-11-16 outbreaks were recorded in Germany (Globig, Staubach, et al., 2017).

6 | 2016/2017: Reassortant Ger-12–16—H5N8

From December 2016 onwards, two additional corresponding HPAIV H5N8 reassortants were discovered during the epidemic, termed Ger-12-16.1 and Ger-12-16.2. Both groups could genetically be distinguished by their varying PB1 segment, presenting drastically differing outbreak progressions (Pohlmann et al., 2018).

After the primary detection of Ger-12-16.1 in a mute swan in Bavaria, January 2017, this reassortant overall led to merely eight cases or outbreaks in total during January–February 2017 (Figure 1a,b), of which two were reported from poultry holdings (one commercial and one smallholding). Geographically, this reassortant concentrated on Southern Germany, affecting the federal states Bavaria, Baden-Wuerttemberg, Hesse, Thuringia and Saxony (Globig, Staubach, et al., 2017).

In comparison, Ger-12-16.2 encompassed the largest case count of all identified reassortants of the epidemic, also responsible for the largest poultry HPAIV H5 hotspot located in Lower Saxony. Circulating from December 2016 to May 2017, Central Germany was primarily affected with reports from the federal states Lower Saxony, Brandenburg, North Rhine-Westphalia, Rhineland-Palatinate, Saxony, Hesse, Thuringia, Saxony-Anhalt and Northern Bavaria (Globig, Staubach, et al., 2017).

Both Ger-12-16 reassortants share the same genetic backbone as the previously described Ger-11-16 reassortant: The 2016 HPAIV H5N8 Russia–Mongolia 2.3.4.4b isolate, A/great crested grebe/Uvs-Nuur Lake/341/2016 (Lee, Sharshov, et al., 2017). Varying PB2 and PA segments allow the differentiation of Ger-12-16 and Ger-11-16, traced back to LPAIV detected in Asia (Pohlmann et al., 2018). The PB2 segment clusters with LPAIV reported in Central Asia (El-Shesheny et al., 2017; Marchenko et al., 2018). Correspondingly, the PA segment shares 98% identity with additional LPAIV detected in Central and Southeast Asia (Figure 2, Table S2; Pohlmann et al., 2018).

The differentiation of both Ger-12-16 reassortants is based on a unique PB1 segment carried by Ger-12-16.2 (Pohlmann et al., 2018). Very high identity levels of over 99% were obtained for LPAIV rooted in Asia (Figure 2; Lee, Bertran, et al., 2017; Pohlmann et al., 2018).

The Ger-12-16 reassortants cluster separately with sequence data from various countries. Ger-12-16.1 viruses were primarily identified in Southern European countries (Nagy et al., 2018; Napp et al., 2018). This displays the concurrent circulation of Ger-12-16.1 in neighbouring countries (Globig, Staubach, et al., 2017; Pohlmann et al., 2018). In contrast, the Ger-12-16.2 reassortant clusters with HPAIV H5N8 detected in Belgium, Poland, Hungary and Southern Russia (Table S2). These established connections suggest a divergent incursion route for Ger-12-16.2, introduced into Central Germany after detections in Central/Northern Europe (Marchenko et al., 2018; Swieton & Smietanka, 2018; Venkatesh et al., 2018). This was supported by a spatial–temporal analysis of the 2016–2017 reassortants that suggested distinct entry routes for the different reassortant (Lycett et al., 2020).

7 | 2016/2017: Reassortant Ger-12–16-N5—H5N5

The 2016–2017 epidemic encompassed numerous subtypes and distinctive reassortants. Alongside the respective HPAIV H5N8, HPAIV H5N5 isolates were identified, further clustering into two novel H5N5 reassortant groups in Germany, Ger-12-16-N5.1 and Ger-12-16-N5.2 (Figure 1b; Pohlmann et al., 2018).

The first Ger-12-16-N5.1 outbreak caused by this reassortant was detected in a wild swan in Saxony, December 2016. In conjunction with a small group of infected wild birds, a commercial turkey holding in Northern Germany reported an outbreak with the respective reassortant at the end of January 2017. Unlike the majority of previously described outbreaks (Alarcon et al., 2018), the first worldwide detection of a second H5N5 reassortant Ger-12-16-N5.2 was within a turkey holding, surprisingly the identical holding simultaneously infected with Ger-12-16-N5.1. After the

concurrent poultry outbreak with both HPAIV H5N5 reassortants in January 2017, additional wild birds in the surrounding area in Northern Germany tested positive for Ger-12-16-N5.2 (Pohlmann et al., 2018).

In line with the previously analysed outbreaks, both H5N5 reassortants share the same 2016 Russia–Mongolia 2.3.4.4 reassortant backbone, differing by a novel N5 segment and a non-truncated NS1 protein with several amino acid substitutions (Pohlmann et al., 2018). The novel NA segment was previously identified in various Central Asian isolates. Both H5N5 reassortants cluster with PB2 and PA segments of H5N8 Ger-12-6 viruses (Figure 2). Interestingly, an environmental sample taken in October 2016 in the Kamchatka Peninsula of the Russian Federation shares over 99% identity for all segments of Ger-12-16-N5.1 (Table S2), suggesting Ger-12-16-N5.1 shares the same descendent with the reassorted H5N5 virus previously detected in Kamchatka, the Russian Federation (Bergervoet et al., 2019; Fusaro et al., 2017; Marchenko et al., 2018; Pohlmann et al., 2019; Swieton & Smietanka, 2018).

When inspecting the Ger-12-16-N5.2 reassortant, all but the PB1 and NP segments are congruent with the findings of Ger-12–16-N5.1. In the case of the PB1 segment, the highest identity levels of 98%–99% were documented in LPAIV found in the Netherlands in the years prior. Likewise, the closest relations of the NP segment were established in viruses identified in the Netherlands (Figure 2). These findings substantiate that LPAIV circulating in the European wild bird population subsequently served as the source of the reassorted segments (Bergervoet et al., 2019; Pohlmann et al., 2019).

While the Ger-12-16-N5.1 reassortant was identified in six European countries (the Netherlands, Poland, Italy, Croatia, Hungary and Germany; Beerens et al., 2017; Fusaro et al., 2017; Nagy et al., 2018; Napp et al., 2018; Pohlmann et al., 2019; Swieton & Smietanka, 2018), only one further case with a virus genetically closely related to the Ger-12-16-N5.2 reassortant has been detected worldwide, in a wild goose in the Netherlands, May 2017 (A/Go/NL-Utrecht/17006881-001/2017, Table S2) (Bergervoet et al., 2019; Brown et al., 2017; Pohlmann et al., 2019). According to the genetic analysis, temporal course and geographic distribution, the poultry holding in Northern Germany infected with both HPAIV H5N5 reassortants is suggested as the most likely location of reassortment yielding Ger-12-16-N5.2. From there, a consecutive spill over event from the poultry premises into the wild bird population and subsequent limited spread in Northern Europe, that is to the Netherlands, ensued (Bergervoet et al., 2019; Pohlmann et al., 2018; Swieton & Smietanka, 2018).

8 | 2017/2018: CLADE 2.3.4.4b, H5N6—REASSORTANT GER-12–17-N6

Initially emerging in December 2017 in Germany, a solitary H5N6 reassortant, Ger-12-17-N6, accounted for three wild bird cases and two poultry outbreaks over a 9-month period, concluding with the final case in late August 2018 (Figure 1b). Listed conferring to the infection date, the federal states Bavaria (common pochard—December 2017), Schleswig-Holstein (chicken, turkey, goose and domestic duck smallholding, March 2018), Lower Saxony (white stork—*Ciconia ciconia*, April 2018), North Rhine-Westphalia (buzzard—*Buteo buteo*, May 2018) and Mecklenburg-Western Pomerania (domestic duck smallholding, August 2018) were affected. Although two poultry outbreaks were recorded within the Ger-12-17-N6 timeframe, both classified as smallholdings and no larger commercial poultry farms were recorded (Figure 1a; Pohlmann et al., 2019).

The conducted phylogenetic analyses of Ger-12-17-N6 coincided with previous studies (Beerens et al., 2018, 2019; Poen et al., 2019; Pohlmann et al., 2019; Susloparov et al., 2019), highlighting the similarity of the segments PB1, HA, NP, MP and NS with the aforementioned Ger-11-16 H5N8 virus. The NA segment of the respective H5N6 reassortant derives from Eurasian LPAIV. In addition, the PA segment showed a high identity of 99% to HPAI H5N8 viruses detected in the Netherlands in 2016, while the PB2 segment proved to be alike LPAIV identified in Europe (Figure 2, Table S2; Pohlmann et al., 2019).

These findings finally allow a definitive distinction between the Ger-12-17-N6 reassortant and a varying H5N6 reassortant concurrently circulating in Europe (Greece) and East Asia (Taiwan, South Korea, Japan), distinguishable mainly by their PA and PB2 genes (Beerens et al., 2019; Poen et al., 2019).

9 | 2020: CLADE 2.3.4.4b, H5N8—REASSORTANT GER-01–20

The most recent HPAIV H5 series of outbreaks and cases reported in Germany was likewise caused by a novel clade 2.3.4.4b HPAIV H5N8 reassortant, termed Ger-01–20 (in accordance with suggested reassortant designation; King et al., 2020). First identified in mid-January 2020, seven cases or outbreaks across Brandenburg (white-fronted goose—*Anser albifrons*), Baden-Wuerttemberg (chicken smallholding), Saxony (steamer duck—*Tachyeres* (zoo), common buzzard, and chicken smallholding), Lower Saxony (commercial turkey holding) and Saxony-Anhalt (commercial turkey holding) have been recorded (Figure 1a,b). Prior to the initial German case in Brandenburg, several outbreaks of HPAIV H5N8 had been reported in Central and Eastern Europe (OIE, 2020).

In comparison, Ger-01-20 comprises of eight segments, all with the exception of PB1 and NP reverting to a HPAIV H5N8 backbone, newly introduced to Germany and differing from previous German H5 isolates and reassortants worldwide (Figure S4). Phylogenetic analyses show 98% sequence identity of the segments PB2, PA, HA, NA, MP and NS to a preceding H5N8 reassortant previously detected on multiple continents during 2016/2017 (Figure S2, Table S2). This includes Asia/Eurasia, Europe and Africa (Abolnik et al., 2019; Fusaro et al., 2017; Kwon et al., 2018; Nagarajan et al., 2017; Voronina et al., 2018; Yehia et al., 2018). Slightly higher identity levels of 98.9%-99.6% were detected for the PB2, PA, HA, MP and NS segments in combination with a Nigerian HPAIV H5N8 isolate detected in July 2019 (Adlhoch et al., 2020).

LPAIV H3N8 detected in waterfowl carried PB1 and NP segments identified as closest related to the novel reassortant Ger-01-20. A green sandpiper isolate sampled in the Kurgan region of Central Russia, August 2018, shared, for example identity levels of more than 99% with segment PB2. Equal identity values were established for the NP segment from a LPAIV H3N8 detected in a gadwall sampled at Lake Chany, Russia, October 2018 (Figure S2; Adlhoch et al., 2020; King et al., 2020).

Phylogenetic analysis of the available European full-genome HPAIV H5N8 2019/2020 sequencing data shows clustering of three German cases in Saxony, March 2020, all located in close geographic proximity (A/chicken/German-SN/AI00276/2020, smallholding; A/buzzard/German-SN/AI00285/2020, wild bird; A/steamer duck/Germany-SN/AI00346/2020, captive bird). Close relations could likewise be established between Hungarian (A/turkey/Hungary/1020/20VIR749-1/2020, commercial holding), Polish (A/hawk/Poland/003/2020, wild; A/turkey/Poland/23/2019, commercial holding), Czech (A/turkey/Czech Republic/3071/2020, commercial holding) and German (A/white-fronted goose/Germany-BB/AI00018/2020; A/turkey/Germany-ST/AI00352/2020, commercial holding) sequences. A third cluster consisting of HPAIV H5N8 from the Czech Republic (A/chicken/Czech Republic/1175-1/2020, smallholding) and Germany (A/chicken/Germany-BW/AI00049/2020, smallholding; A/turkey/Germany-NI/AI00334/2020, commercial holding) was also detected (Figure S4; Adlhoch et al., 2020; King et al., 2020). The incursion of the novel clade 2.3.4.4b HPAIV H5N8 into Europe in late 2019/early 2020 most likely follows a similar pattern to the previously described clade 2.3.4.4 situation (Lycett et al., 2020). After the probable initial migratory bird dissemination of the virus from Eurasia and/or Africa into East and Central Europe, local dissemination and thus detached evolution of the reassortant led to partitioning of the described clusters (Adlhoch et al., 2020).

10 | CONCLUDING REMARKS

Over the past 24 years, Germany has seen an influx of varying H5 reassortants, subtypes and clades rooted in the Asian 1996 H5 gs/GD lineage. After the first introduction of the HPAIV clade 2.2 H5N1 subtype in 2006, evolving clade 2.3.4.4 viruses dominated outbreaks from 2014 onwards. Ranging from outbreaks of minor scope to unprecedented magnitude, immense wild bird mortality to the immersion of poultry holdings, each novel outbreak situation conveyed a new assortment of genetic and epidemiological characteristics. The vast emergence of, in particular, clade 2.3.4.4 H5 reassortants underlines the promiscuity of this clade and its tendency for reassortment. The necessity of continued active and passive surveillance on all afflicted continents in combination with real-time whole-genome sequencing of identified positive samples and virus isolates to allow precise and detailed phylogenetic analyses is apparent when regarding the dimensions of the previous epidemics. Close monitoring and surveillance of migratory birds along their migrations routes and networks is essential for the early detection of novel viruses. This would allow specific preparation and containment strategies in the 'sink' countries, possibly avoiding further epidemics, as imminent introductions of novel HPAI H5 viruses in Germany are highly probable in the future.

ETHICS STATEMENT

No ethical approval was required for this review article. The authors confirm that the ethical policies of the journal have been adhered to as stated on the journal's author guidelines page.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study. Data acknowledgements can be found in Table S3.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supplemental Material

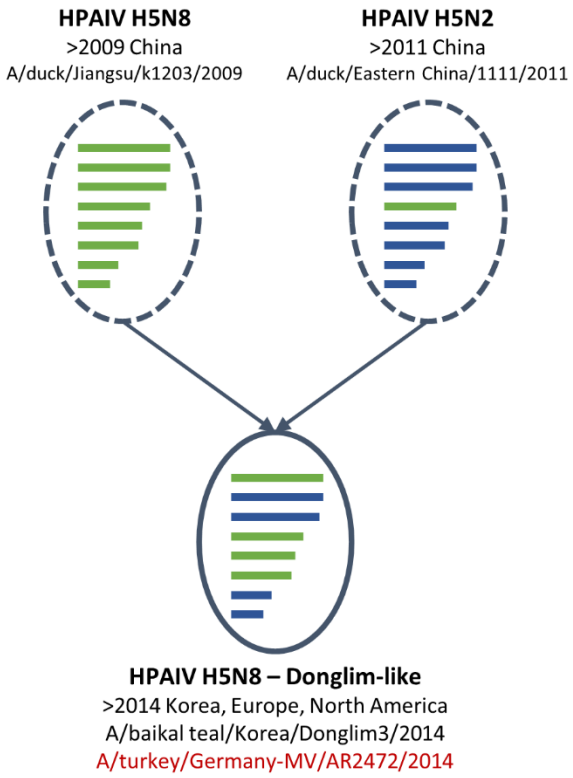

Figure S1. Visual representation of proposed reassortment events for clade 2.3.4.4a HPAIV H5N8. Dashed lines indicate putative precursors.

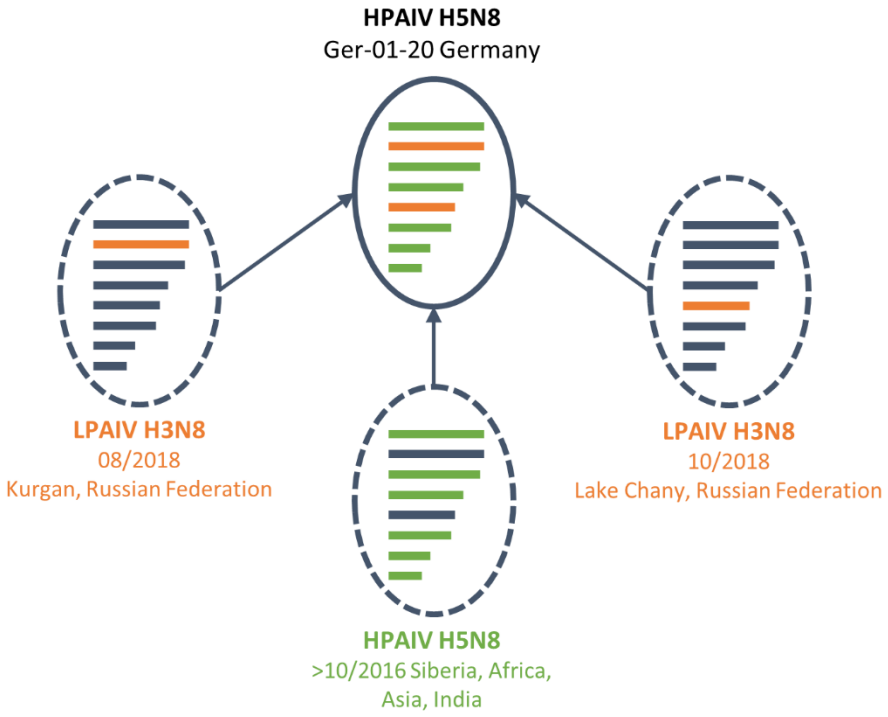

Figure S2. Visual representation of proposed reassortment event for clade 2.3.4.4b HPAIV H5N8 reassortant Ger-01-20. Dashed lines indicate putative precursors.

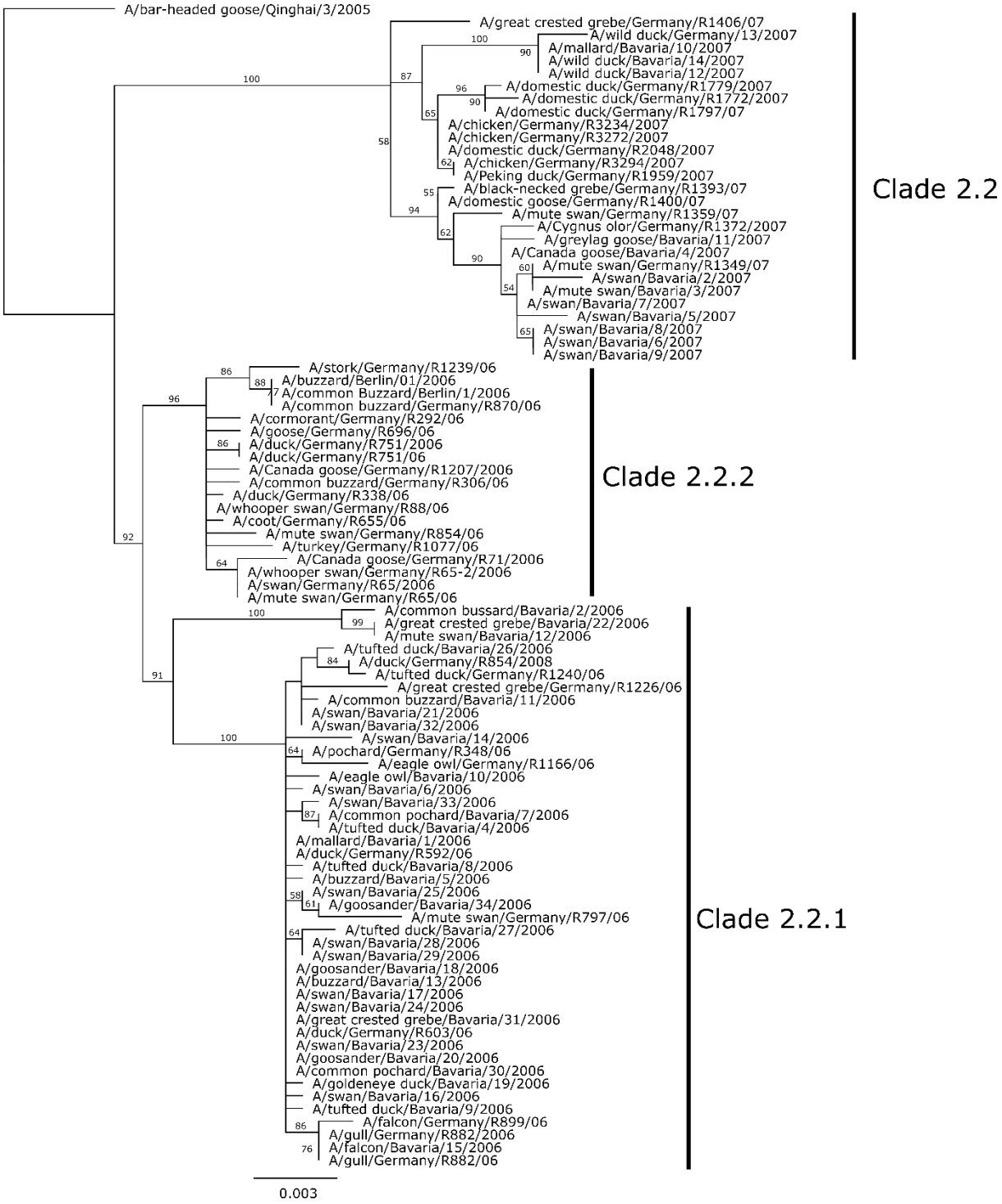

Figure S3. Phylogenetic analyses of German clade 2.2 HA sequences done by Maximum Likelihood trees using RAxML with a bootstrap value of 1000 cycles. Trees include all available German clade 2.2 HA segments from the 2006/2007 epidemic and reference strain A/bar-headed goose/Qinghai/3/2005 (H5N1) for clade 2.2 (root) as designated by the World Health Organization.

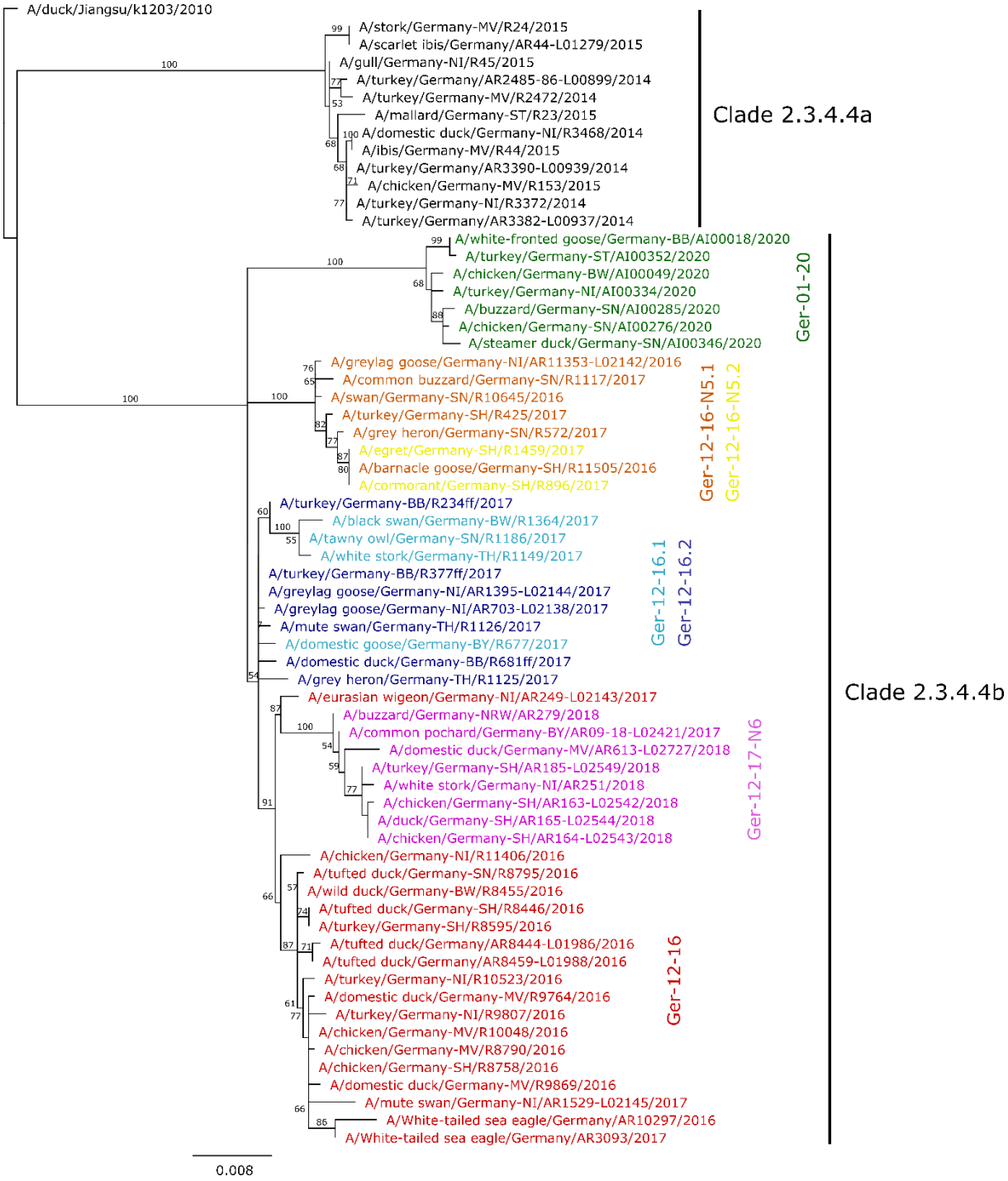

Figure S4. Phylogenetic analyses of German clade 2.3.4.4 HA sequences epidemic done by Maximum Likelihood trees using RAxML with a bootstrap value of 1000 cycles. Trees include all available German clade 2.3.4.4 HA segments from 2014 – 2020 and a reference strain for clade 2.3.4.4 (root) as designated by the World Health Organization.

Table S1. Sequence identity (%) of respective index strains from the 2006 – 2007 HPAIV H5N1 epidemic in comparison to the top ten BLAST results, displaying one exemplary case per country and excluding other German cases (status as of 23.07.2020 on EpiFlu™).

H5N1	German Index Strain	Isolate ID	Corresponding Strains	Identity level (%)
Clade 2.2.1	A/pochard/Germany/R348/2006	EPI171706	A/*Anas platyrhynchos*/Slovenia/359/2006	99.88
		EPI120044	A/common pochard/Switzerland/V592/2006	99.88
		EPI14330	A/turkey/France/06222-1.1/2006	99.88
		EPI231242	A/swan/Schaerding/1499/2006	99.88
		EPI184079	A/goose/Hungary/14756/2006	99.71
		EPI97191	A/bar-headed goose/Qinghai/62/05	99.35
		EPI232420	A/whooper swan/Mongolia/244/2005	99.29
		EPI45943	A/chicken/Nigeria/641/2006	99.29
		EPI47200	A/cygnus olor/Croatia/1/2005	99.23
Clade 2.2.2	A/*Cygnus cygnus*/Germany/R65/2006	EPI241681	A/peregrine/Denmark/6632/2006	99.94
		EPI156821	A/swan/Poland/467-136V08/2006	99.88
		EPI114800	A/goose/Krasnoozerskoe/627/05	99.71
		EPI136605	A/*Cygnus olor*/Czech Republic/10814/06	99.65
		EPI171691	A/chicken/Romania/3293/05	99.60
		EPI101483	A/*Cygnus olor*/Astrakhan/Ast05-2-2/2005	99.48
		EPI227150	A/duck/Novosibirsk/02/05	99.43
		EPI171701	A/*Ardea cinerea*/Slovenia/185/06	99.26
		EPI114798	A/goose/Suzdalka/10/05	99.15
		EPI171687	A/peregrine falcon/Slovakia/Vh242/2006	99.04
Clade 2.2	A/*Cygnus olor*/Germany/R1359/2007	EPI184642	A/mute swan/France/070203/2007	99.53
		EPI132663	A/chicken/Kuwait/KISR8/2007	99.53
		EPI126535	A/*Cygnus cygnus*/Krasnodar/329/07	99.48
		EPI136631	A/*Cygnus olor*/Czech Republic/10732/07	99.31
		EPI1780	A/whooper swan/Mongolia/2/06	99.13
		EPI106728	A/grebe/Tyva/Tyv06-1/2006	99.08
		EPI126212	A/chicken/Korea/CA7/2006	98.97
		EPI225053	A/common gull/Chany/P/2006	98.91
		EPI175729	A/chicken/Manshera/NARC10337/2007	98.86
		EPI993179	A/chicken/Okayama/T6/2007	98.80

Table S2. Sequence identity (%) of respective index strains from clade 2.3.4.4 and corresponding strains described in the manuscript, displaying one exemplary case per country and excluding other German cases (status as of 24.07.2020 on EpiFlu™).

Clade	Isolate ID	German Index Strain	ID	Corresponding Strains	Identity level (%)
Clade 2.3.4.4a	EPI_ISL_167140	A/turkey/Germany-MV/AR2472/2014	EPI_ISL_169427	A/wigeon/Sakha/1/2014	99.83
			EPI_ISL_167904	A/duck/England/36254/14	99.82
			EPI_ISL_167905	A/chicken/Netherlands/14016437/2014	99.82
			EPI_ISL_174398	A/mallard/Korea/H1924-6/2014	99.78
			EPI_ISL_168025	A/duck/Chiba/26-372-61/2014	99.77
			EPI_ISL_175534	A/mute swan/Sweden/SVA150311KU0277/SZ502/2015	99.73
			EPI_ISL_177584	A/domestic duck/Hungary/7341/2015	99.72
			EPI_ISL_169429	A/environment/Kagoshima/KU-ngr-H/2014	99.72
Clade 2.3.4.4b					
Ger-11-16	EPI_ISL_237944	A/tufted duck/Germany/AR8444-L01987/2016	EPI_ISL_257699	A/tufted Duck/Switzerland/V237/2016	99.92
			EPI_ISL_247713	A/tufted duck/Denmark/17740-1/2016	99.92
			EPI_ISL_255209	A/common tern/Hungary/8187/2017	99.89
			EPI_ISL_237921	A/wild duck/Poland/82A/2016	99.84
			EPI_ISL_268678	A/T_Dk/NL-Zeewolde/16013976-005/2016	99.83
			EPI_ISL_238895	A/chicken/Sweden/SVA161122KU0453/SZ0209318/2016	99.76
			EPI_ISL_256298	A/gadwall/Kurgan/2442/2016	99.60
			EPI_ISL_239801	A/turkey/England/052131/2016	99.55
			EPI_ISL_247721	A/turkey/Rostov-on-Don/11/2017	99.54
Ger-12-16.1	EPI_ISL_288746	A/mute swan/Germany-BY/AR857/2017	EPI_ISL_240012	A/duck/France/161108h/2016	99.82
			EPI_ISL_255933	A/cormorant/Hungary/6102/2017	99.79
			EPI_ISL_240104	A/chicken/Poland/79A/2016	99.74
			EPI_ISL_309196	A/*Buteo buteo*/Belgium/3022/2017	99.69
			EPI_ISL_250885	A/mute swan/Czech Republic/54-17_2/2017	99.66
			EPI_ISL_238196	A/mute swan/Croatia/70/2016	99.64
			EPI_ISL_387143	A/goose/Spain/IA17CR02699/2017	99.57
			EPI_ISL_419350	A/duck/Bulgaria/Dobrich/407/2017	99.54
Ger-12-16.2	EPI_ISL_240892	A/turkey/Germany-NI/R10523/2016	EPI_ISL_240102	A/domestic goose/Poland/33/2016	99.72
			EPI_ISL_309195	A/*Cygnus olor*/Belgium/2967/2017	99.55
			EPI_ISL_297234	A/chicken/Rostov-on-Don/1321/2017	99.52
			EPI_ISL_257004	A/chicken/Czech Republic/206-17_2/2017	99.40
			EPI_ISL_255174	A/goose/Hungary/65817/2016	99.35

Clade	Isolate ID	German Index Strain	ID	Corresponding Strains	Identity level (%)
Ger-12-16-N5.1	EPI_ISL_262056	A/greylag goose/Germany-NI/AR11353-L02142/2016	EPI_ISL_287565	A/M_Swan/NL-Groningen/16015826-001/2016	99.92
			EPI_ISL_256462	A/mute swan/Hungary/5879/2017	99.85
			EPI_ISL_255189	A/gadwall/Italy/17VIR133-2/2017	99.84
			EPI_ISL_240101	A/mute swan/Croatia/102/2016	99.83
			EPI_ISL_300746	A/swan/Poland/81/2017	99.75
			EPI_ISL_256301	A/environment/Kamchatka/18/2016	99.54
Ger-12-16-N5.2	EPI_ISL_260059	A/cormorant/Germany-SH/R896/2017	EPI_ISL_288411	A/Go/NL-Utrecht/17006881-001/2017	99.76
Ger-12-17-N6	EPI_ISL_305453	A/chicken/Germany-SH/AR163-L02542/2018	EPI_ISL_332435	A/chicken/Netherlands/18000887-005/2018	99.67
			EPI_ISL_291110	A/mute swan/Switzerland/V361-L02422/2017	99.58
			EPI_ISL_292225	A/canada goose/England/AV58_18OPpoolEP1/2018	99.53
Ger-01-20	EPI_ISL_404993	A/white-fronted goose/Germany-BB/AI00018/2020	EPI_ISL_405813	A/hawk/Poland/003/2020	99.83
			EPI_ISL_402134	A/turkey/Poland/23/2019	99.83
			EPI_ISL_419220	A/turkey/Hungary/1020_20VIR749-1/2020	99.79
			EPI_ISL_418266	A/chicken/Czech Republic/1175-1/2020	99.61
			EPI_ISL_405391	A/turkey/Czech Republic/3071/2020	99.61
			EPI_ISL_405278	A/guinea fowl/Nigeria/OG-GF11T_19VIR8424-7/2019	97.59

Table S3. Data acknowledgement – we acknowledge the following laboratories for providing sequences in the EpiFlu™ Database.

Isolate ID	Isolate Name	Submitting Lab
EPI_ISL_169427	A/wigeon/Sakha/1/2014	State Research Center of Virology and Biotechnology (VECTOR)
EPI_ISL_167904	A/duck/England/36254/14	Animal and Plant Health Agency (APHA)
EPI_ISL_167905	A/chicken/Netherlands/14016437/2014	Wageningen Bioveterinary Research
EPI_ISL_174398	A/mallard/Korea/H1924-6/2014	Animal and Plant Quarantine Agency (S-2026)
EPI_ISL_168025	A/duck/Chiba/26-372-61/2014	National Institute of Animal Health Japan
EPI_ISL_175534	A/mute swan/Sweden/SVA150311KU0277/SZ502/2015	National Veterinary Institute Sweden
EPI_ISL_177584	A/domestic duck/Hungary/7341/2015	Danam Vet Molbiol Hungary
EPI_ISL_169429	A/environment/Kagoshima/KU-ngr-H/2014	Kagoshima University Japan
EPI_ISL_257699	A/tufted Duck/Switzerland/V237/2016	Faculty of Veterinary Medicine at the University of Bern
EPI_ISL_247713	A/tufted duck/Denmark/17740-1/2016	Technical University of Denmark
EPI_ISL_255209	A/common tern/Hungary/8187/2017	Danam Vet Molbiol Hungary
EPI_ISL_237921	A/wild duck/Poland/82A/2016	National Veterinary Research Institut Poland (PIWet-PIB)
EPI_ISL_268678	A/T_Dk/NL-Zeewolde/16013976-005/2016	Wageningen Bioveterinary Research
EPI_ISL_238895	A/chicken/Sweden/SVA161122KU0453/SZ0209318/2016	National Veterinary Institute Sweden
EPI_ISL_256298	A/gadwall/Kurgan/2442/2016	State Research Center of Virology and Biotechnology (VECTOR)
EPI_ISL_239801	A/turkey/England/052131/2016	Animal and Plant Health Agency (APHA)
EPI_ISL_247721	A/turkey/Rostov-on-Don/11/2017	State Research Center of Virology and Biotechnology (VECTOR)
EPI_ISL_240012	A/duck/France/161108h/2016	Agence Nationale De Securite Sanitaire De L'alimentation (ANSES)
EPI_ISL_255933	A/cormorant/Hungary/6102/2017	Danam Vet Molbiol Hungary
EPI_ISL_240104	A/chicken/Poland/79A/2016	National Veterinary Research Institut Poland (PIWet-PIB)
EPI_ISL_250885	A/mute swan/Czech Republic/54-17_2/2017	State Veterinary Institute Prague
EPI_ISL_238196	A/mute swan/Croatia/70/2016	Croatian Veterinary Institute
EPI_ISL_419350	A/duck/Bulgaria/Dobrich/407/2017	National Diagnostic and Research Veterinary Medical Institute Bulgaria (NDRVMI)
EPI_ISL_240102	A/domestic goose/Poland/33/2016	National Veterinary Research Institut Poland (PIWet-PIB)
EPI_ISL_297234	A/chicken/Rostov-on-Don/1321/2017	State Research Center of Virology and Biotechnology (VECTOR)
EPI_ISL_255174	A/goose/Hungary/65817/2016	Danam Vet Molbiol Hungary
EPI_ISL_287565	A/M_Swan/NL-Groningen/16015826-001/2016	Wageningen Bioveterinary Research
EPI_ISL_256462	A/mute swan/Hungary/5879/2017	Danam Vet Molbiol Hungary
EPI_ISL_255189	A/gadwall/Italy/17VIR133-2/2017	Istituto Zooprofilattico Sperimentale delle Venezie
EPI_ISL_240101	A/mute swan/Croatia/102/2016	Croatian Veterinary Institute
EPI_ISL_300746	A/swan/Poland/81/2017	National Veterinary Research Institut Poland (PIWet-PIB)
EPI_ISL_256301	A/environment/Kamchatka/18/2016	State Research Center of Virology and Biotechnology (VECTOR)
EPI_ISL_288411	A/Go/NL-Utrecht/17006881-001/2017	Wageningen Bioveterinary Research
EPI_ISL_332435	A/chicken/Netherlands/18000887-005/2018	Wageningen Bioveterinary Research
EPI_ISL_291110	A/mute swan/Switzerland/V361-L02422/2017	Institut für Virologie und Immunologie - Bundesamt für Lebensmittelsicherheit und Veterinärwesen
EPI_ISL_292225	A/canada goose/England/AV58_18OPpoolEP1/2018	Animal and Plant Health Agency (APHA)

Isolate ID	Isolate Name	Submitting Lab
EPI_ISL_405813	A/hawk/Poland/003/2020	National Veterinary Research Institut Poland (PIWet-PIB)
EPI_ISL_402134	A/turkey/Poland/23/2019	National Veterinary Research Institut Poland (PIWet-PIB)
EPI_ISL_419220	A/turkey/Hungary/1020_20VIR749-1/2020	Istituto Zooprofilattico Sperimentale delle Venezie
EPI_ISL_418266	A/chicken/Czech Republic/1175-1/2020	State Veterinary Institute Prague
EPI_ISL_405391	A/turkey/Czech Republic/3071/2020	State Veterinary Institute Prague
EPI_ISL_405278	A/guinea fowl/Nigeria/OG-GF11T_19VIR8424-7/2019	Istituto Zooprofilattico Sperimentale delle Venezie

2.6. Circulation of Avian Influenza Viruses in Egypt

The circulation of a wide variety of LPAIV and HPAIV in Egypt has led to the emergence of many reassorted AIV, of which many pose a serious poultry production and public health risk. Catalysed by lack of biosecurity measures in poultry production systems and high prevalence of backyard farming, the country constantly carries the threat of a major outbreak (27). To date, three major AIV lineages circulate in Egypt, including the HPAIV subtype H5N1 of clade 2.2.1 gs/GD lineage (2006), LPAIV subtype H9N2 of the G1 lineage (2011), and HPAIV subtype H5N8 of the clade 2.3.4.4b gs/GD lineage (2016) (24, 26, 124, 125).

The first HPAIV H5N1 case in Egypt was reported in 2006 and the country was declared enzootic in 2008 (126). The zoonotic potential of the circulating H5N1 strain has caused around 200 human cases with a mortality rate of 36% and remains a challenge for the public health of Egypt (127). The utilisation of vaccinations in both commercial poultry holdings and backyard flocks as a control measure was unable to stifle the spread of H5N1 and even enabled further mutation and genetic drift. As a result, two subclades (2.2.1 and 2.2.1.1) co-circulated from late 2009 through 2011. From 2012 onwards, clade 2.2.1.1 viruses were rarely detected, yet clade 2.2.1 viruses continue to circulate and evolve (124, 128).

LPAIV H9N2 of the Eurasian G1 lineage was first identified in 1966 in North America. Prior to the detection of LPAIV H9N2 in Egypt in 2011, the respective subtype had been circulating in Middle Eastern and Central-Asian countries from 1998-2010, of which many countries are now enzootic with H9N2 in poultry (129, 130). Although wild birds and poultry show no clinical illness, H9N2 viruses are capable of infecting humans and have traceably played a role in the genetic evolution of many zoonotic AIV (131). The co-circulation of H5N1 and H9N2 in susceptible host populations can increase the likelihood of the emergence of novel reassortant viruses carrying public health implications (132).

Alike the extensive 2016-2017 HPAIV H5N8 outbreak in Europe, the same clade 2.3.4.4b reassortants reached Egypt in 2016 (133). Following the unprecedented tendency for reassortment of the respective clade, the probability of the generation of novel reassortant viruses, especially in a country with multiple co-circulating (zoonotic) strains, poses a great threat to the public health and poultry production sector. Since the introduction of HPAIV H5N8 in 2016, multiple novel reassortants have been identified (134).

CHAPTER III: STUDY OBJECTIVES

III. STUDY OBJECTIVES

Objective I: Establishment and application of a novel and rapid MinION nanopore sequencing workflow for IAV

Publication I, III

Due to the expedited evolution of IAV and the concomitant risk of novel antigenic and genetic, potentially zoonotic strains, rapid, accessible and real-time WGS is of utmost importance to aid in outbreak detection and surveillance studies. While the characterisation of emerging and re-emerging IAV has mainly been driven by SGS over the past decade, restrictive factors such as high costs, laborious protocols, expenditure of time and large, stationary equipment limit their application, especially in outbreak scenarios. Thus, attaining a real-time, rapid, portable sequencing system with the MinION third-generation nanopore sequencer to allow direct identification of potentially novel IAV strains builds and advances the foundation for IAV surveillance and detection.

Objective II: Genetic characterisation, outbreak dynamics and molecular epidemiology of clade 2.3.4.4b HPAIV subtype H5 in Germany, 2016 – 2020

Publication II, III

Since the emergence of the H5 gs/GD lineage in China, 1996, the HPAIV H5 gene has evolved into a plethora of clades and genotypes, causing significant losses in the poultry industry, majorly affecting the wild bird population and influencing public health due to the zoonotic propensity of certain strains. Germany has seen a recurring influx of various reassortant clade 2.3.4.4b HPAI H5 viruses since 2016, a clade notorious for its unprecedented tendency for reassortment. With the help of WGS involving tools described under Objective I, the genetic characterisation, reassortant designation and temporal analysis of the occurring HPAI H5 viruses in Germany from 2016 – 2020 has permitted molecular epidemiology studies resulting in profound knowledge of the dynamics and relations of individual outbreaks. In addition, zoonotic risk assessment of IAV strains by the identification of genetic mutations linked to host adaptation can be achieved with WGS.

Objective III: Surveillance for the identification and genetic characterisation of novel reasssortant HPAIV subtype H5 circulating in Egypt, 2019

Publication IV

Co-circulation of multiple, partially zoonotic (HP)AIV lineages and the enzootic presence of H5N1, H9N2 and H5N8 in the Egyptian poultry sector has led to a wide variety of novel AIV by point mutations and, in more recent times, reassortment. Surveillance by WGS is of paramount importance to rapidly identify new AIV strains and estimate their zoonotic potential. Only WGS can give insights into the

exact arrangement of reassorted segments and allow full genetic dissection of harboured segments. Publication IV targets the identification of novel reassorted HPAI H5 viruses collected in farms reporting clinical respiratory symptoms in chickens.

CHAPTER IV: RESULTS

IV. RESULTS

The publications included in this thesis are grouped according to their topic and presented as a part of results section. The reference section of each manuscript is presented in the style of the respective journal and is not included at the end of this thesis. The numeration of figures and tables corresponds with the published form of each respective manuscript.

1. **Publication I:** Rapid Multiplex MinION Nanopore Sequencing Workflow for IAV

Publication I

Rapid Multiplex MinION Nanopore Sequencing Workflow for Influenza A Viruses

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TECHNICAL ADVANCE Open Access

Rapid multiplex MinION nanopore sequencing workflow for Influenza A viruses

Jacqueline King, Timm Harder, Martin Beer and Anne Pohlmann*

Abstract

Background: Due to the frequent reassortment and zoonotic potential of influenza A viruses, rapid gain of sequence information is crucial. Alongside established next-generation sequencing protocols, the MinION sequencing device (Oxford Nanopore Technologies) has become a serious competitor for routine whole-genome sequencing. Here, we established a novel, rapid and high-throughput MinION multiplexing workflow based on a universal RT-PCR.

Methods: Twelve representative influenza A virus samples of multiple subtypes were universally amplified in a one-step RT-PCR and subsequently sequenced on the MinION instrument in conjunction with a barcoding library preparation kit from the rapid family and the MinIT performing live base-calling. The identical PCR products were sequenced on an IonTorrent platform and, after final consensus assembly, all data was compared for validation. To prove the practicability of the MinION-MinIT method in human and veterinary diagnostics, we sequenced recent and historical influenza strains for further benchmarking.

Results: The MinION-MinIT combination generated over two million reads for twelve samples in a six-hour sequencing run, from which a total of 72% classified as quality screened, trimmed and mapped influenza reads to produce full genome sequences. Identities between the datasets of > 99.9% were achieved, with 100% coverage of all segments alongside a sufficient confidence and 4492fold mean depth. From RNA extraction to finished sequences, only 14 h were required.

Conclusions: Overall, we developed and validated a novel and rapid multiplex workflow for influenza A virus sequencing. This protocol suits both clinical and academic settings, aiding in real time diagnostics and passive surveillance.

Keywords: Nanopore sequencing, MinION, Influenza A viruses, Full genome sequencing, Next-generation sequencing, Avian influenza viruses, Multiplexing

Background

Next-generation sequencing (NGS) methods, especially second-generation sequencers, have shown their capability of whole-genome sequencing (WGS) over the past decade for a wide spectrum of pathogens including influenza A viruses (IAV) [1, 2]. Due to widespread avian influenza virus (AIV) outbreaks with high mortality among poultry and wild birds in combination with the unceasing risk of zoonosis, avian origin IAV has devastating economic and anthropological impacts [3, 4]. Frequent reassortment events and vast genetic diversity of these viruses show the necessity for fast and accurate WGS [5].

While the characterisation of IAV has greatly benefitted from WGS utilising first- and second-generation sequencers, limiting factors such as high costs, process

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duration, extensive protocols and large, stationary equipment leave room for improvement [6]. In recent times, the new era of third-generation sequencers has started to fill this gap. Among these, the portable MinION third-generation nanopore sequencing device (Oxford Nanopore Technologies, Oxford, UK; ONT) has developed to become a serious competitor [7], especially in regard to real-time sequencing and multiplex barcoding possibilities [8].

Employing the MinION with a broad range of IAV subtypes of both avian and human origin, we developed and validated a high-throughput sequencing workflow and speedy screening method for unknown IAV samples. During an outbreak situation, this method could dramatically reduce the cost and time for WGS, thus accelerating the response and aiding in disease control.

Methods

Nucleic acid extraction

Twelve egg-grown avian virus isolates (Table 1), four human IAV isolates and two avian swab samples (Additional File 1, Table S1) of different subtypes were collected in the German National Reference Laboratory for Avian Influenza, located at the Friedrich-Loeffler-Institut, Insel Riems, Germany. RNA was extracted using TRIZOL LS (Thermo Fisher Scientific, Waltham, USA) and the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

IAV-End-RT-PCR and purification

RNA was amplified with one pair of influenza-specific primers (forward and reverse) at a 10 pmol/μl concentration [9] using Invitrogen Superscript III One-Step RT-PCR with Platinum Taq (Thermo Fisher Scientific). The IAV-End-RT-PCR included 5 μl RNA template, 1 μl forward and 1 μl reverse primer, 12.5 μl reaction mix, 1 μl SuperScript III RT/Platinum Taq mix and 4.5 μl RNase free water to obtain a total volume of 25 μl. In this protocol, all influenza segments are amplified simultaneously using a one-step RT-PCR and one set of primers adapted to the conserved 3′ and 5′ segment ends. Amplicon length therefore ranges from the smallest non-structural protein segment (866 nt) to the largest polybasic 2 protein segment (2316 nt), in accordance to the individual segment lengths.

Cycling conditions for the respective IAV-End-RT-PCR were conducted as described: An initial primary reverse transcription step of 30 min at 55 °C, then denaturation at 94 °C for 2 min, followed by five cycles of 94 °C for 30 s, 45 °C for 30 s and 68 °C for 3 min, then an additional 30 cycles of 94 °C for 30 s, 57 °C for 30 s and 68 °C for 3 min, and to conclude a final elongation step at 68 °C for 5 min.

After amplification, samples were purified with AMPure XP Magnetic Beads (Beckman Coulter, Fullerton, USA) in an × 0.65 sample volume to bead volume ratio. Quantification was conducted with the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific).

Sequencing of IAV-End-PCR products – IonTorrent platform

The purified avian RT-PCR amplicons were sequenced on the IonTorrent platform (Thermo Fisher Scientific) as previously described [5, 10]. Before library preparation for the respective platform, the samples were mechanically fragmented to a 500 bp size on a Covaris M220 Ultrasonicator (Covaris Ltd., Brighton, UK). The GeneRead DNA L Core Kit (Qiagen) was subsequently used for library preparation with Xpress Barcode Adapters (Qiagen). After a following size selection and clean-up step with AMPure XP Beads (Beckman Coulter), the final library was quality checked on an Agilent Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany) and quantized via qPCR with the KAPA Library Quantification Kit (Roche, Mannheim, Germany). Sequencing was conducted on the IonTorrent S5XL (Thermo Fisher Scientific) in combination with the Ion OneTouch 2 System (Thermo Fisher Scientific), encompassing twelve AIV samples per Ion 530 Chip (Thermo Fisher Scientific).

Analysis of IonTorrent sequencing data

The raw data produced was screened for adapter and primer contamination, followed by a quality trimming step. By using the Geneious Software Suite (v11.1.5; Biomatters, Auckland, New Zealand), consensus sequences were generated via a map to reference approach utilising Bowtie2 (v2.3.0; pre-set "Medium Sensitivity") [11].

Sequencing of IAV-End-PCR products – MinION sequencer

The identical purified IAV RT-PCR amplicons utilised on the IonTorrent were likewise employed for MinION sequencing along with the human strains. Following the manufacturer's instructions, the Rapid Barcoding Kit (RBK-004, ONT) was applied: This 15 min two-step method includes a transposase for simultaneous cleaving of template DNA in conjunction with attachment of twelve barcodes to the cleaved ends (step 1), followed by pooling of the barcoded samples in the desired ratio and addition of Rapid Sequencing Adapters (step 2; ONT). After library preparation, the pooled samples were loaded onto a FLO-MIN106 R9.4.1 flow cell following the manufacturer's instructions (ONT). A six-hour run was conducted with standard settings.

Table 1 Summary of MinION data from all reference samples sequenced with name, subtype and barcode. Mean coverage, mapped reads and number of nucleotide differences are shown for each individual segment. The consensus identity is calculated for all segments in %

Barcode	Sample	Subtype		PB2	PB1	PA	HA	NA	NP	MP	NS	Sum	Consensus identity %
1	R30–06	H1N1	**Mean Coverage**	749.9	188.3	700.9	2771.9	8756.7	7987.3	13,551.5	11,637.7	5793.025	99.98528113
			Mapped Reads	8102	1131	4059	8207	22,156	20,995	31,635	30,095	126,380	
			Nucleotide Differences				1	1				2	
2	R3111–07	H2N9	**Mean Coverage**	2250.2	1360.5	1001	4396.1	626.7	4582.6	8212.6	7477	3738.3375	99.99264057
			Mapped Reads	32,171	11,283	7372	11,560	2390	10,367	18,613	18,298	112,054	
			Nucleotide Differences				1					1	
3	R2555–06	H3N1	**Mean Coverage**	6373.3	2200.9	2080.6	929.1	1968	3874.8	5749.1	5016.4	3524.025	99.98528113
			Mapped Reads	40,737	14,924	14,166	4074	6248	9628	13,859	8970	112,606	
			Nucleotide Differences		1			1				2	
4	HAIV-81	H4N6	**Mean Coverage**	1462	3401.4	792.2	4161.2	8039.7	11,356.3	14,790.1	9955.9	6744.85	99.98528113
			Mapped Reads	18,920	20,238	6874	11,778	20,138	27,900	35,597	23,680	165,125	
			Nucleotide Differences	1			1					2	
5	R1612–08	H5N3	**Mean Coverage**	5033.4	3013.7	5260.7	1441.3	3776.2	1802.1	6314.7	5898.6	4067.5875	99.96320283
			Mapped Reads	35,383	19,354	31,659	8876	10,321	4821	14,220	12,908	137,542	
			Nucleotide Differences	1	1		2	1				5	
6	R617–07	H6N2	**Mean Coverage**	3933.5	2889.7	2995.6	4024	3403.6	3564.3	9820.9	5480.4	4514	99.99264057
			Mapped Reads	29,256	21,688	23,359	13,971	9179	8292	22,581	13,515	141,841	
			Nucleotide Differences				1					1	
7	R11–01	H7N7	**Mean Coverage**	5558.2	1201	1696.6	3737.4	3467.6	6623.9	7390.9	6832.7	4563.5375	99.98528113
			Mapped Reads	46,729	12,888	17,912	15,132	8502	16,267	18,781	16,333	152,544	
			Nucleotide Differences		1	1						2	
8	R249–08	H9N2	**Mean Coverage**	4445.5	3087.6	2260.4	1729.2	2652.5	2341.6	5403.8	6044.2	3495.6	99.98528113
			Mapped Reads	32,149	20,998	14,076	6316	7275	6366	12,914	13,244	113,338	
			Nucleotide Differences			1	1					2	
9	WV1677–03	H10N4	**Mean Coverage**	4296.7	1296.4	2896.6	3175.4	2728	3976.2	7689.3	5656	3964.325	99.9779217
			Mapped Reads	41,559	12,764	26,781	9445	8429	10,422	17,043	13,420	139,863	
			Nucleotide Differences			2		1				3	
10	R2675–06	H11N6	**Mean Coverage**	924.2	912.9	1378.1	1499.1	2260.8	4951.9	4345.1	3944	2527.0125	99.99264057
			Mapped Reads	14,361	12,470	13,834	5702	5402	12,233	10,004	9893	83,899	
			Nucleotide Differences	1								1	

Table 1 Summary of MinION data from all reference samples sequenced with name, subtype and barcode. Mean coverage, mapped reads and number of nucleotide differences are shown for each individual segment. The consensus identity is calculated for all segments in % *(Continued)*

Barcode	Sample	Subtype		PB2	PB1	PA	HA	NA	NP	MP	NS	Sum	Consensus identity %
11	R2613–06	H13N8	**Mean Coverage**	866.9	1040.9	1199.3	6152	5035.8	10,790.8	19,984.4	16,654.6	7715.5875	99.9779217
			Mapped Reads	9250	10,656	10,600	19,030	14,503	26,581	48,937	44,445	184,002	
			Nucleotide Differences	1	1			1				3	
12	Se-99	H16N3	**Mean Coverage**	9922	426.7	599.1	115.8	2673.5	2096	5058.4	5190.9	3260.3	99.96320283
			Mapped Reads	3843	2695	5041	393	11,992	5296	12,593	11,951	53,804	
			Nucleotide Differences	1	1		1	2				5	

Analysis of MinION sequencing data

Real time basecalling was performed with the MinIT and integrated Guppy v3.0.4 software (ONT) to produce fast5 and fastQ files. The automatic real time division into passed and failed reads by the MinIT works as a quality check, removing reads with quality scores < 7. The quality checked reads were demultiplexed and trimmed for adapters and primers using ONT Guppy Barcoding Software v3.1.5 + 781ed575, followed by mappings and a final consensus production in Geneious (v11.1.5; Biomatters) with Bowtie2 (v2.3.0; pre-set "Medium Sensitivity") [11]. Due to the segmented influenza genome and thus comparable length of ONT and IonTorrent reads, usage of an identical mapping process was possible. MinION data quality was documented with NanoPlot v1.25.0 [12].

Data availability

All sequence data (raw data, assemblies, consensus sequences) were made publicly available in the European Nucleotide Archive (ENA) under project accession PRJEB35098. Data accessions are summarised in Additional File 1, Table S2.

Results

The IAV-End-RT-PCR successfully amplified all samples and segments. In the case of the IonTorrent run, 33 h were necessary to achieve twelve full genomes with 100% coverage (Fig. 1). A sum of 11,499,351 reads were produced after quality check and removal of polyclonal reads with an average of 97% classified influenza reads, evenly distributed between the individual barcodes. Overall, 258,488 reads were without a barcode (2.2%). An average trimmed read length of 276 nt was achieved.

For MinION sequencing, starting at the RNA extraction to final consensus sequence, a total of 14 h was necessary to produce twelve complete genomes with 100% coverage (Fig. 1). In total, 2,090,778 reads were generated in the six-hour run, of which 90.43% classified as passed reads. After demultiplexing, a final count of 1,667,946 reads (79.78% of overall reads, 87.01% of passed reads) was available for further analysis leaving 248,963 passed reads (11.91% of overall reads, 12.98% of passed reads) without or unable to be allocated to a barcode. The read distribution between the twelve barcodes was roughly even, with barcodes 10 and 12 producing less than average sequence counts (Additional File 1, Table S1). Read quality was monitored by evaluating read length versus read quality per sample (Additional File 1, Figure S1) with an overall mean read quality reaching from 12.3 to 12.6, typical for MinION data. Read length ranges from 375 to 567 nt with an average of 479 nt, making the data accessible for standard mapping algorithms.

Analyses of the generated full genome sequences revealed 1,522,998 mapped MinION reads (91.30% of all passed and demultiplexed reads) and a consensus identity between the IonTorrent and MinION data of > 99.9%, with a range of one to a maximum of five nucleotide differences throughout the entire genome. Respectively, virtually all deviations were detected in homopolymer regions. Altogether, a mean coverage of 4492 reads was achieved for all MinION data (Table 1).

The additionally MinION sequenced human IAV isolates and avian swab samples passed all quality checks to produce six further complete genomes with similar read quality and high coverage. The quality check process included the primary distinction of passed (quality scores > 7) and failed reads (quality scores < 7) during live basecalling and further evaluation of the read quality by conducting NanoPlot (v1.25.0) to receive, inter alia, mean read quality and read length. Here, the cut-off value for mean read quality was set at ≥10 for further usage of the sequencing data.

Discussion

The proposed MinION workflow allows high-throughput sequencing in real time with a rapid library preparation protocol. Although MinION sequencing of IAV has previously been conducted [13], the novel combination of the IAV-End-RT-PCR with the Rapid Barcoding Kit (ONT) reduces the time for library preparation to a minimum and the analysis of low yield samples is achievable thanks to prior universal amplification. The respective RT-PCR allows for the production of less overall sequencing data due to the high proportion of viral influenza reads and a minimal host share, concurrently saving time and monetary means. The Rapid Barcoding Kit (ONT) also dramatically cuts the hands-on time needed for other NGS platforms, additionally reducing labour amount and, thus, expenditure. By utilising the MinIT (ONT), real time basecalling allows real time analysis, consequently leading to fast results, often crucial in the clinical setting.

Next-generation sequencers can produce immense amounts of data at a moderate cost, yet the application in clinical diagnostics is limited due to capital investment, complexity and time-consuming protocols. Easy, rapid and cost-efficient sequencing on the MinION platform could make sequencing accessible to a wide range of research backgrounds and might change the diagnostic process in the healthcare system. The availability of third-generation sequencers is pushing sequencing in the direction of becoming an integral part of many laboratories. The proposed IAV MinION sequencing protocol could easily be introduced into existing laboratory environments and allow direct, rapid and cost-efficient identification of diverse IAV strains. In addition,

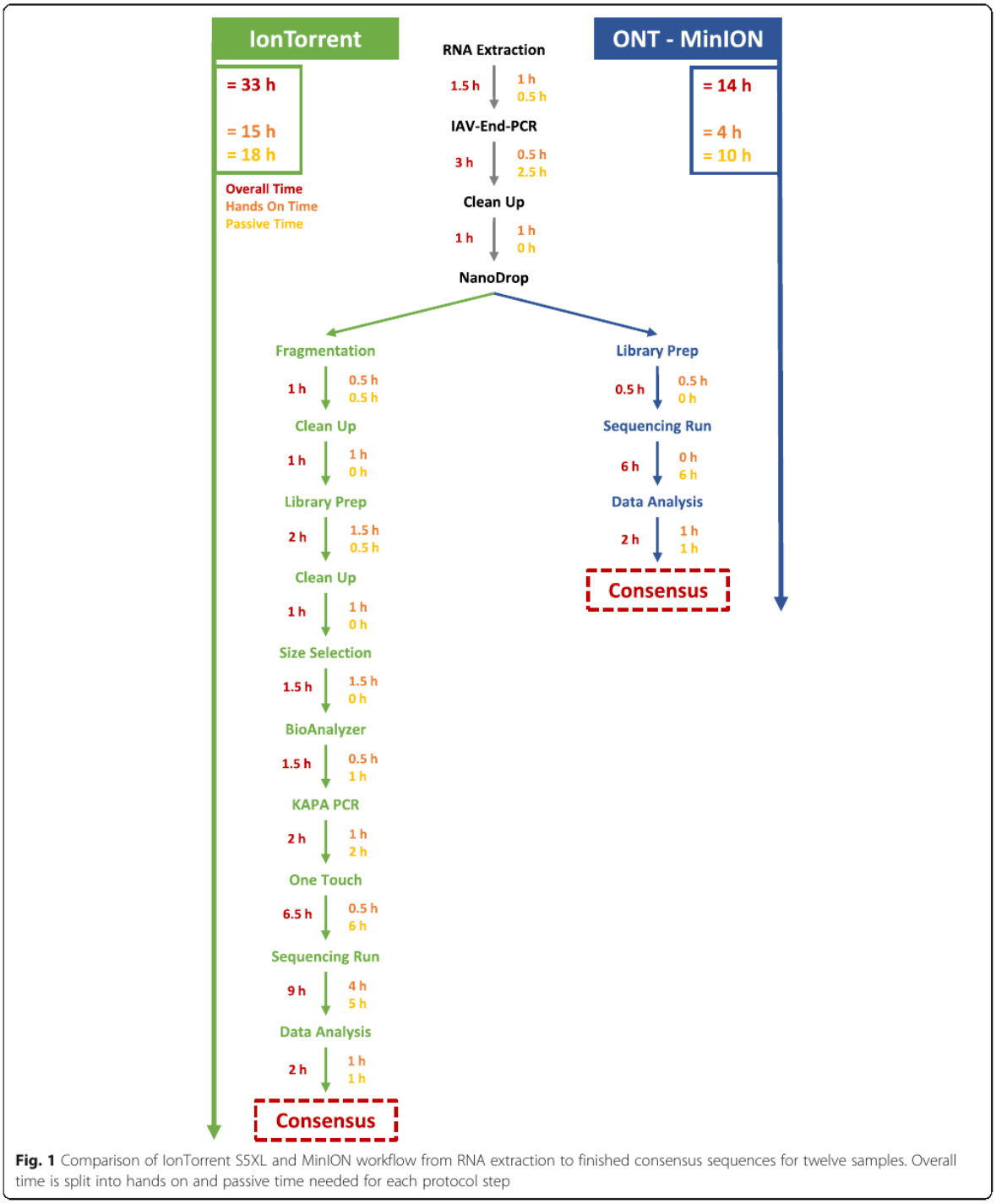

Fig. 1 Comparison of IonTorrent S5XL and MinION workflow from RNA extraction to finished consensus sequences for twelve samples. Overall time is split into hands on and passive time needed for each protocol step

this protocol allows high-throughput sequencing of, for example, AIV samples from passive surveillance studies, shown to be of great importance for the molecular epidemiology of the worldwide AIV situation [14, 15].

The suitability of sequencing approaches for field surveillance was demonstrated with swine influenza viruses and the comparison to Illumina sequencing library strategies show comparable results with longer total analysis

time and specific hands-on-time, respectively [16]. The use of multiplexing strategies is preferable due to the advantages of barcoding in higher throughput, better cost efficiency and decreased sequencing run time. The use of amplification with universal primers also allows an easier workflow and, in this study, allows better comparability of the achieved results, as the identical PCR products were sequenced on both platforms. PCR-free sequencing approaches for IAV have been described using direct RNA sequencing on the MinION platform to receive the complete coding genome of IAV [7]. This method allows avoidance of a prior PCR, and thus, the concurrent potentially resulting bias. Although this method is of great interest, the samples were high titre isolates and the limit of detection reached Ct values of 17, which lies outside the range of most clinical samples. Albeit the produced consensus sequences shared maximally 98.97% identity to the reference, the complexity and expenditure to achieve these results is not viable in the clinical setting. Multiplexing of direct RNA sequencing has yet to be produced for the MinION platform, additionally raising expenditures. In comparison, the here described MinION workflow allows superior consensus identity levels for low viral load samples at a lower cost and time consumption. In the future, direct RNA sequencing will certainly play a significant role; however, the current technological capabilities will most likely first allow the entry of third-generation sequencers into the clinical setting, in line with the aim of our proposed protocol.

Metagenomic nanopore sequencing has previously been piloted for clinical respiratory IAV samples [17]. The results of this study are promising for the combination of nanopore sequencing and metagenomics. However, although the detection of individual IAV reads was described in samples with Ct values of up to 36, whole genome sequences with the necessary coverage depth were only achieved at much lower Ct levels. Additionally, far larger datasets are needed to attain full genome coverage in comparison to the PCR-based MinION protocol. Deep sequencing and the concomitant possibility of SNP and variant detection is likewise only achievable with greater coverage depth, also attainable with the proposed protocol.

A multitude of varying bioinformatics analysis tools are available for sequencing data produced on ONT platforms, all aiming towards the improvement of the currently standing error profile. Especially in the current worldwide SARS-CoV-2 pandemic, third-generation sequencing platforms with distinct bioinformatic workflows have been implemented to obtain whole genomes [18, 19]. In the proposed protocol, the aim was to concentrate on the laboratory work and keep the implementation as accessible as possible, thus allowing better comparisons of both platforms via the utilisation of the identical annotation workflow. The comparability was shown by the generation of highly identical consensus sequences proving that the higher error rates of individual reads could be compensated by higher coverage.

The error rate of individual reads likewise affects demultiplexing of reads [20]. Misindexed reads are a known problem for nanopore sequencing, with on average 0.056% of total reads assigned to the incorrect barcode. When conducting metagenomic sequencing, a value of 0.056% misindexed reads can immensely influence the final genome construction, as often a fast majority of > 99% of the produced reads derive from the host [21, 22], leaving only few viral reads for analysis. Using the proposed method, high coverage and a large percent of viral influenza reads are expected due to the upstream IAV-End-RT-PCR (here: 91.3% of all passed and demultiplexed MinION reads were identified as influenza reads). Therefore, the fraction of misindexed reads does not affect the final consensus production and is, thus, in this case negligible.

Overall, sample processing of twelve samples can be achieved in 14 h, less than half the time required with the IonTorrent, without the need for large, expensive devices. Remarkable is the very low hands on time needed with the transposase-based rapid library MinION protocol. Although the accuracy of the MinION is known to be lower than other NGS platforms, especially struggling with homopolymer regions [23], adequate coverage leads to almost identical consensus sequences [24], as our data confirms. The MinION proved to be adaptably applicable for not only avian and human isolates, but also for representative clinical swab samples. In addition, swab samples from the recent 2020 outbreak of a novel IAV clade 2.3.4.4b H5N8 reassortant in Germany were successfully sequenced to produce full genome sequences using this method [25], demonstrating the practicality and applicability of the respective workflow.

Ongoing improvements from ONT are expected to advance basecalling accuracy with new technologies, e.g. the R10 flow cells, alongside the development of more accurate direct RNA sequencing kits to avoid PCRs and resulting biases. In addition, library preparation using automatic systems like the VolTRAX V2 (ONT) will allow even less hands-on time and reduce contamination while improving reproducibility.

Conclusions

In summary, we developed and validated a novel rapid multiplex workflow for IAV sequencing using

the MinION in combination with a one-step RT-PCR and the Rapid Barcoding Kit (ONT). This protocol is ideal for both clinical and academic settings, aiding in real time diagnostics, applicable to any IAV sample and indispensable for active outbreaks and passive surveillance.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12879-020-05367-y.

Additional file 1.

Abbreviations

IAV: Influenza A viruses; AIV: Avian influenza viruses; HPAIV: Highly pathogenic avian influenza viruses; LPAIV: Low pathogenic avian influenza viruses; ONT: Oxford Nanopore Technologies; RT-PCR: Reverse transcription polymerase chain reaction; NGS: Next-generation sequencing; WGS: Whole-genome sequencing; ENA: European Nucleotide Archive; RNA: Ribonucleic acid; Nt: Nucleotide

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Authors' contributions

JK produced, analysed and curated the sequencing data and was responsible for the first written draft of the manuscript alongside further editing and figure work. TH provided the samples for sequencing, participated in the conceptualisation and methodology and was a major contributor in the editing of the manuscript. MB participated in the conceptualisation, in funding acquisition and supervision and was a major contributor in the editing of the manuscript. AP contributed to the formal data analyses, conceptualisation of the project, funding acquisition and overall supervision alongside playing a major role in the editing process of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated and analysed in the current study are available in the European Nucleotide Archive (ENA) under project accession PRJEB35098. Data accessions are summarised in Additional File 1, Table S2.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Supplemental Material

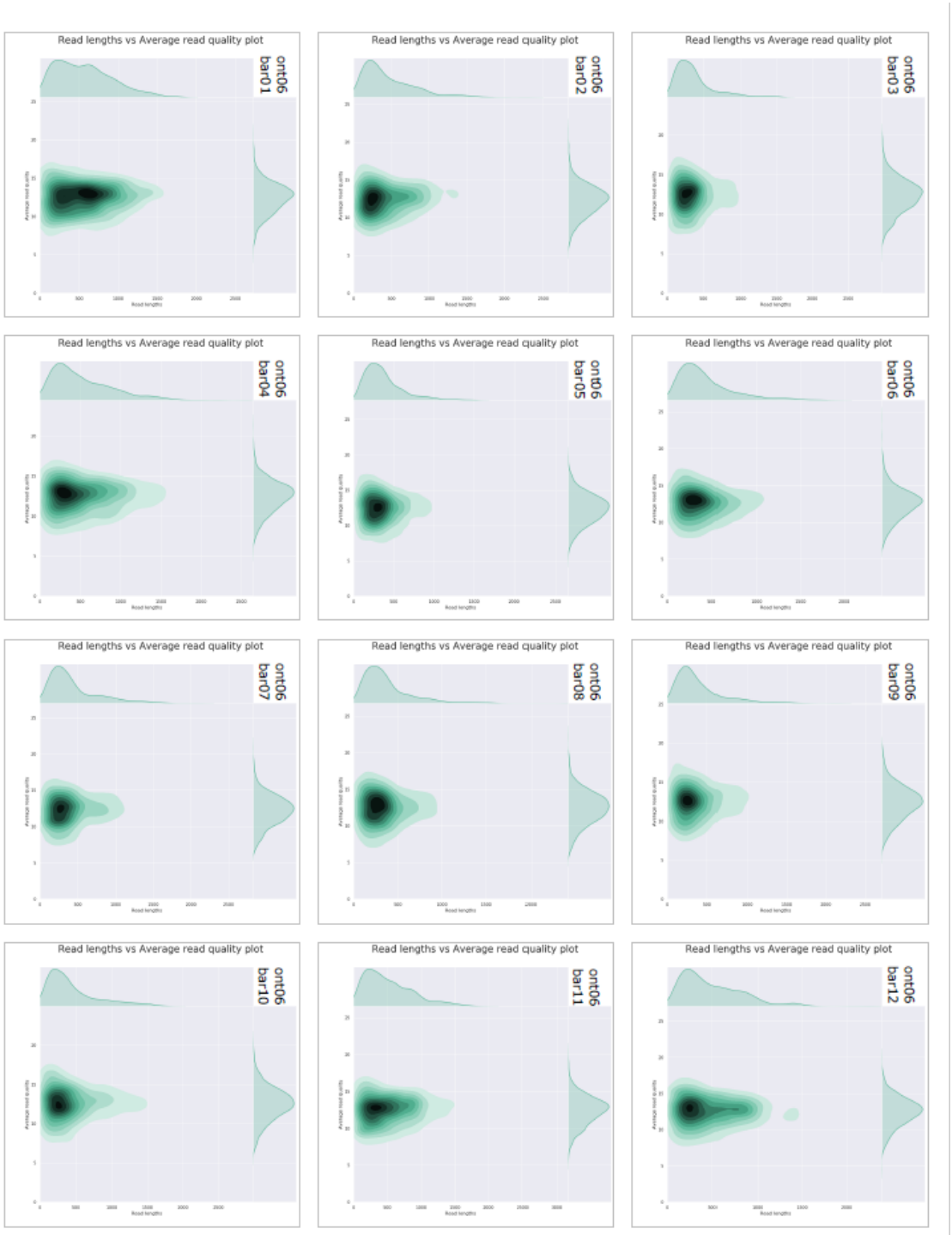
Table S1. Metrics of MinION and IonTorrent sequencing of twelve influenza reference samples, four human IAV samples and two avian IAV swab samples.

Sample information		ONT MinION					IonTorrent S5				
Sample ID	Subtype	Barcode	No. reads	No. nucleotides	Mean read length	Mean read quality	Barcode	No. reads	No. nucleotides	Mean read length	Mean read quality
R30-06	**H1N1**	RBK_01	135,319	76,779,158	567	12.4	IonXpress_001	979,254	263,435,070	269	27.5
R3111-07	**H2N9**	RBK_02	124,950	57,837,095	463	12.3	IonXpress_002	789,774	219,019,649	277	27.3
R2555-06	**H3N1**	RBK_03	127,224	47,715,460	375	12.4	IonXpress_005	896,250	249,270,903	278	26.8
HAIV-81	**H4N6**	RBK_04	184,108	97,179,395	528	12.4	IonXpress_006	945,428	255,182,523	269	27.2
R1612-08	**H5N3**	RBK_05	154,055	58,279,146	378	12.3	IonXpress_007	1,141,716	319,299,737	279	26.8
R617-07	**H6N2**	RBK_06	155,749	66,775,697	429	12.5	IonXpress_008	584,795	166,306,709	284	26.6
R11-01	**H7N7**	RBK_07	171,932	73,404,138	427	12.3	IonXpress_010	911,348	245,062,731	268	27.2
R249-08	**H9N2**	RBK_08	127,146	47,167,977	371	12.5	IonXpress_011	991,208	272,905,748	275	26.9
WV1677-03	**H10N4**	RBK_09	149,147	61,256,718	411	12.5	IonXpress_012	988,573	278,693,189	281	26.9
R2675-06	**H11N6**	RBK_10	89,162	40,107,611	450	12.6	IonXpress_013	940,860	261,449,754	277	27.1
R2613-06	**H13N8**	RBK_11	192,947	101,885,364	528	12.4	IonXpress_014	1,038,855	294,774,287	283	27.2
Se-99	**H16N3**	RBK_12	56,207	27,001,365	480	12.5	IonXpress_015	988,802	266,418,429	269	27.2
FM-47	**H1N1**	RBK_04	312,507	171,295,702	548	12.3	NA	NA	NA	NA	NA
R1541-07	**H1N1**	RBK_05	310,449	187,350,645	603	12.4	NA	NA	NA	NA	NA
H1N1-swl	**H1pdmN1**	RBK_06	308,631	197,777,138	640	12.2	NA	NA	NA	NA	NA
AR3343-17	**H1pdmN1**	RBK_07	390,993	234,193,266	599	12.5	NA	NA	NA	NA	NA
AR780-17	**H5N5**	RBK_10	15,402	9,615,096	624	12.5	IonXpress_089	426,862	127,641,921	299	27.3
AR1384-17	**H5N8**	RBK_11	79,149	48,446,910	612	12.3	IonXpress_093	426,540	134,969,178	316	27.0

Table S2. Accessions numbers (ENA) of shard data under project accession PRJEB35098.

Sample ID	Virus ID	Subtype	Sample Accession	Run Accession ONT	Run Accession Ion Torrent
R30-06	A/duck/Germany/R30/2006	H1N1	ERS4226848	ERR3822170	ERR3822188
R3111-07	A/duck/Germany/R3111/2007	H2N9	ERS4226849	ERR3822171	ERR3822189
R2555-06	A/duck/Germany/R2555/2006	H3N1	ERS4226850	ERR3822172	ERR3822190
HAIV-81	A/unknown/unknown/HAIV/1981	H4N6	ERS4226851	ERR3822173	ERR3822191
R1612-08	A/turkey/Germany//R1612/2008	H5N3	ERS4226852	ERR3822174	ERR3822192
R617-07	A/turkey/Germany/R617/2007	H6N2	ERS4226853	ERR3822175	ERR3822193
R11-01	A/turkey/Germany/R11/2007	H7N7	ERS4226854	ERR3822176	ERR3822194
R249-08	A/avian/Germany/R249/2008	H9N2	ERS4226855	ERR3822177	ERR3822195
WV1677-03	A/mallard/Germany/WV1677/2003	H10N4	ERS4226856	ERR3822178	ERR3822196
R2675-06	A/dunnock/Germany/R2675/2006	H11N6	ERS4226857	ERR3822179	ERR3822197
R2613-06	A/black-headed gull/Germany/R2613/2006	H13N8	ERS4226858	ERR3822180	ERR3822198
Se-99	A/black-headed gull/Sweden/5/1999	H16N3	ERS4226859	ERR3822181	ERR3822199
FM-47	A/Fort Mammoth/1/47	H1N1	ERS4226860	ERR3822182	NA
R1541-07	A/Niedersachsen/14/07	H1N1	ERS4226861	ERR3822183	NA
H1N1-swl	A/Regensburg/D6/09	H1pdmN1	ERS4226862	ERR3822184	NA
AR3343-17	A/Michigan/45/2015	H1pdmN1	ERS4226863	ERR3822185	NA
AR780-17	A/turkey/Germany/AR780/2017	H5N5	ERS4226864	ERR3822186	NA
AR1384-17	A/chicken/Germany/AR1384/2017	H5N8	ERS4226865	ERR3822187	NA

Figure S1. Read quality versus read length of MinION data using NanoPlot.



2. **Publication II:** Genetic Characterization and Zoonotic Potential of Highly Pathogenic Avian Influenza Virus A(H5N6/H5N5), Germany, 2017 – 2018

Publication II

Genetic Characterization and Zoonotic Potential of Highly Pathogenic Avian Influenza Virus A(H5N6/H5N5), Germany, 2017-2018

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Genetic Characterization and Zoonotic Potential of Highly Pathogenic Avian Influenza Virus A(H5N6/H5N5), Germany, 2017–2018

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We genetically characterized highly pathogenic avian influenza virus A(H5N6) clade 2.3.4.4b isolates found in Germany in 2017–2018 and assessed pathogenicity of representative H5N5 and H5N6 viruses in ferrets. These viruses had low pathogenicity; however, continued characterization of related isolates is warranted because of their high potential for reassortment.

During winter 2016–17, outbreaks of highly pathogenic avian influenza (HPAI) virus A(H5N8) clade 2.3.4.4b caused substantial losses in wild water birds and domestic poultry across Europe (*1*–*4*). This virus is related to strains from China and Mongolia and has a high potential for reassortment (*4*–*6*). Genetic and temporal analysis of these isolates revealed multiple reassortant events, indicating multiple independent entries into Europe; the outbreaks in Germany were dominated by 5 independent reassortant groups of HPAI virus H5N8 (*5*). Several outbreaks of HPAI virus H5Nx strains in wild birds confirmed the continued presence of H5 clade 2.3.4.4b in Europe well into the summer of 2017. This virus's high tendency to reassort raised concerns that further reassorted strains could dominate in HPAI outbreaks in Europe or become enzootic in wild bird populations in the future. In this study, we set out to characterize related reassortant viruses of subtype H5N6 or H5N5 isolated in Germany during 2017–2018 and delineate their zoonotic potential in ferrets.

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DOI: https://doi.org/10.3201/eid2510.181931

The Study

Starting in November 2017, H5 HPAI viruses, classified as clade 2.3.4.4b according to their hemagglutinin (HA) segments, carrying N6 segments were detected in the Netherlands (*7*), United Kingdom, Switzerland, and Germany (*8*). We used samples mostly from the outbreaks in Germany collected during December 2017–August 2018 (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/25/10/18-1931-App1.pdf). We sequenced (Appendix) and analyzed these viruses and found they carried a neuraminidase (NA) segment of subtype N6 with a high similarity to low pathogenicity avian influenza (LPAI) viruses identified in Asia during 2015–2017 (Appendix Table 2).

According to a full-genome analysis, these H5N6 viruses represent 2 mosaic reassortants of HPAI virus H5N8 found in Europe during the epizootic of 2016–17 (Figure, panel A). Reassortant group I shares all but the NA segment with viruses from the epizootic of 2016–17 (Appendix Figure 1), and because of distinct homologies in the HA, matrix, and nonstructural protein gene segments (Appendix Figure 1), these viruses were further divisible into 3 subgroups, which we designated Gre-02-17-N6, Tai-12-17-N6, and Kor-12-17-N6 (Figure, panel A). The divergence within this reassortant group might have been caused by genetic drift and would be in line with their temporal and geographic patterns of occurrence (Figure, panel B). In contrast, reassortant group II (designated Ger-12-17-N6; Figure) comprises a more homogeneous group of H5N6 viruses from Western and Central Europe. Reassortant group II is genetically distinguishable from reassortant group I by separate clustering of the polymerase acidic (PA) and polymerase basic 2 (PB2) genes (Appendix Figure 1). Group II viruses were detected in Germany during December 2017–August 2018. Their PA segment is similar to that of the HPAI virus A(H5N8) found in the Netherlands in November and December 2016, and their PB2 segment is similar to that of LPAI viruses in Europe and, to a lesser extent, HPAI H5N5 and H5N8 2.3.4.4b isolates from the epizootic of 2016–17 (Appendix Figure 1). This find-

[1]These authors contributed equally to this article.

Figure. Phylogenetic clustering and geographic distribution of highly pathogenic avian influenza A(H5N6) viruses, Europe, 2017–2018. A) Supernetwork generated by using maximum-likelihood trees of influenza virus full genomes with RAxML (https://cme.h-its.org/exelixis/web/software/raxml/index.html) and 1,000 bootstrap iterations followed by network analysis with SplitsTree4 (http://ab.inf.uni-tuebingen.de/software/splitstree4). Reassortant viruses are grouped according to their phylogenetic results. Scale bar indicates nucleotide substitutions per site. The mosaic genome structure of reassortant groups I and II is also provided. Gene segment descriptions are given in Appendix Figure 1 (https://wwwnc.cdc.gov/EID/article/25/10/18-1931-App1.pdf). B) Geographic locations of cluster isolates. Inset of cluster in the Netherlands is provided for easier visualization.

ing underscores the ability of HPAI virus clade 2.3.4.4b from the epizootic of 2016–17 to frequently reassort, probably empowered by its genome constellation, especially its HA segment.

H5N6 viruses of clades 2.3.4.4c and 2.3.4.4d and an H5 virus of clade 2.3.4.4b (A/Fujian-Sanyuan/21099/2017) have been reported in cases of human influenza; thus, concerns have been raised about these viruses' zoonotic potential (*9*). Several clade 2.3.4.4b H5 HPAI viruses isolated in South Korea (Appendix Table 3) have already been evaluated in multiple animal models and showed no zoonotic propensity in ferrets (*10*,*11*). These results concur with our previous analysis of cluster 2.3.4.4b HPAI virus H5N8 from Germany (A/tufted_duck_Germany/AR8444/2016) in human lung explants and in ferrets (*12*).

We extended the zoonotic risk assessment of these viruses by using a reassortant group II HPAI H5N6 virus (AR09/18, A/common_pochard/Germany-BY/AR09-18-L02421/2017). For comparison, we included a related reassortant HPAI H5N5 clade 2.3.4.4b virus (AR425/17, A/turkey/Germany-SH/R425/2017) with 3 genes, NA (Appendix Table 2), polymerase basic 1, and nucleoprotein, related to LPAI viruses from different countries and 4 genes, PB2, PA, matrix, and nonstructural protein,

related to HPAI viruses isolated during the epizootic of 2016–17 (*5*).

We inoculated 10 ferrets intranasally with either the H5N6 or H5N5 virus (Appendix). None of the animals displayed any respiratory signs; the only change observed was a minor, short-lived increase in body temperature. Only 1 of the 5 ferrets inoculated with H5N5 exhibited body temperatures >40°C for 3 consecutive days (5–7 days postinfection [dpi]). This particular animal also exhibited a mild gait disorder at 5 dpi, and because these atactic movements persisted (a sign qualifying for termination), the ferret was euthanized at 7 dpi. The viral RNA loads in the nasal washings of animals inoculated with H5N5 and H5N6 were low up through 7 dpi (Table), and RNA excretion ceased thereafter. However, at 7 dpi, the H5N5-inoculated ferret showing mild ataxia displayed a peak of 100 copies/μL of extracted RNA (input volume 100 μL) in the nasal washing fluid (Table).

Nucleoprotein antibody–specific seroconversion (Table) was detected in all inoculated ferrets surviving until euthanasia at 14 dpi. The serum sample of the atactic animal euthanized at 7 dpi scored reactive but not positive.

We dissected all euthanized animals and analyzed spleens, tracheas, lungs, conchae, cerebellum, and cerebrum for viral genome loads, as described previously (*12*). All organ samples taken at 14 dpi were negative; however, the cerebrum, trachea, and nasal concha of the single animal exhibiting disease euthanized at 7 dpi had a low viral load of 7–25 copies/μL of extracted RNA from ≈2 mm^3 tissue material homogenized in 1 mL of medium (input volume 100 μL).

Histopathologic workup of the sick ferret revealed mild, subacute necrotizing rhinitis; moderate, oligofocal, subacute necrotizing bronchointerstitial pneumonia; moderate, multifocal necrotizing hepatitis; severe necrotizing salpingitis; and the focal-to-multifocal intralesional presence of influenza virus matrix protein (Appendix Figure 2) consistent with systemic virus spread. Only 1 of 4 of the H5N5-infected ferrets and 2 of 5 of the H5N6-infected ferrets necropsied at 14 dpi revealed inflammatory lung lesions, yet all were negative for matrix protein by immunohistochemical staining (Appendix Table 4). Considering the low morbidity rate (10%), these H5 viruses have a mild pathogenic potential in the ferret model compared with other HPAI viruses (*13*).

Table. Viral RNA loads in nasal washing samples from ferrets infected with highly pathogenic avian influenza A(H5N6/H5N5) clade 2.3.4.4b virus isolates from Germany, 2017–2018, and seroconversion in study assessing virus zoonotic risk*

	Day postinfection, viral RNA load, genome copies/μL						
Group	0	1	3	5	7	9	Seroconversion†
Controls	–	–	–	–	–	–	–
H5N5	–	0.2	0.2	0.1	100.6	ND‡	+/–§
	–	1.2	2.8	0.1	–	–	+
	–	1.9	0.9	2.9	–	–	+
	–	1.2	–	0.1	–	–	+
	–	0.1	–	8.2	0.9	–	+
H5N6	–	–	0.8	1.3	1.3	–	+
	–	0.2	1.9	13.8	3.2	–	+
	–	0.2	–	0.2	0.2	–	+
	–	–	–	–	–	–	+
	–	0	1.3	0.2	4.2	–	+

*ND, not done; –, negative.
†Seroconversion measured with sensitive nucleoprotein ELISA (ID Screen Influenza A Antibody Competition ELISA Kit; ID.Vet, https://www.id-vet.com) with day 14 or 7 serum sample, depending on day of animal sacrifice, and compared with preinoculation serum sample.
‡Sacrificed day 7 because animal displayed neurologic symptoms.
§Reaction interpreted as reactive but not positive.

Conclusions

The genetic makeup of HPAI H5 clade 2.3.4.4b viruses fosters reassortment, which can expand their evolutionary capacity. Segment reassortment bears a concomitant danger of the emergence of strains that are more pathogenic or zoonotic or that have a higher potential to evolve to propagate in avian hosts with different migratory behaviors. H5N6 and H5N5 viruses of this clade have been continuously present in Europe since 2017, necessitating continuous surveillance and virus characterization. Our study excludes the possibility of enhanced zoonotic potential for the analyzed H5N5 and H5N6 2.3.4.4b clade viruses. Nonetheless, existing reports of clade 2.3.4.4c HPAI H5N6 virus infections in mammals and clade 2.3.4.4b-2.3.4.4d virus co-infections in humans indicate a continued risk for zoonotic events with H5Nx reassortants (*9*). Continued surveillance and characterization of these viruses is crucial to reduce the risk for outbreaks with burgeoning HPAI isolates of the goose/Guangdong lineage.

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Supplemental Material

Methods:

Sequencing and Data Evaluation

RNA of influenza-positive samples was extracted by using Trizol LS (ThermoFisher Scientific, Waltham, USA) and QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Influenza genome segments were amplified with specific primers by using Invitrogen Superscript III One-Step RT-PCR with Platinium Taq (ThermoFisher Scientific, Waltham, USA). The reverse transcription PCR (RT-PCR) amplicons were sequenced by Sanger or next-generation sequencing (NGS) as previously described (1,2). For NGS, fragmentation of the RT-PCR amplicons was done with a Covaris M220 Ultrasonicator (Covaris Ltd, Brighton, UK) applying a target size of 500 bp. The sonicated cDNA was used for library preparation by using IonTorrent Ion Xpress Barcode Adapters and GeneRead DNA Library L Core Kit (QIAGEN). Size exclusion of the library was done with AMPure XP Magnetic Beads (Beckman Coulter, Fullerton, USA). The libraries were quality checked by using High Sensitivity DNA Chips and reagents on a Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany), quantized via quantitative PCR with KAPA Library Quantification Kit Ion Torrent (Roche, Mannheim, Germany), and sequenced on an IonTorrent PGM or S5 XL (Thermo Scientific), respectively. Raw sequence data were quality-trimmed and screened for adapter and primer contamination. Consensus sequences were generated with an iterative mapping approach by using Bowtie2 (v. 2.3.0) (3) in the Geneious software suite (v. 10.2.3; Biomatters, Auckland, New Zealand). Geneious software suite was also used for quality check and automatic annotation of the sequences. Strain details and epidemiologic information of the viruses sequenced in this study are given in Appendix Table 1. For network analysis, virus sequences of German high pathogenicity avian influenza (HPAI) H5N6 viruses sequenced in this study (Appendix Table 1) were aligned by segment together with similar sequences from HPAI H5N6 strains aligned by using MAFFT (4) (scoring matrix 200PAM / k = 2, gap penalty 1.53, two iterative refinement cycles) receiving 8 alignments, 1 for each segment. Phylogenetic analyses of these alignments were done by using RAxML (5) with general time-reversible plus gamma as the substitution model applying 1,000 bootstrap replicates, resulting in 8 bootstrap-supported phylogenetic trees. All 8 trees were imported into SplitsTree4 for network generation (SuperNetwork, using mean edge weighting, including Z-rule, using Equal Angle for weighted splits transformation, Convex Hull and greedily compatibility applied) (6). Maps were created by using ArcGIS Online (www.arcgis.com). Sequences for comparison were retrieved from the Influenza Research Database (www.fludb.org) and EpiFlu™ Database (www.gisaid.org). We acknowledge the laboratories for providing sequence information via EpiFlu™ listed in Appendix Table 5. Consensus sequences were published in the EpiFlu™ Database under

accession EPI_ISL_291109, EPI_ISL_291110, EPI_ISL_305453, EPI_ISL_305454, EPI_ISL_305455, EPI_ISL_306989, EPI_ISL_313226, EPI_ISL_313227, and EPI_ISL_322179.

Dataset Selection

The sequence dataset for the phylogeny were selected to include representatives of related strains from the epizootic 2016–2017 and from European and Asian H5N6 outbreaks 2017–2018. In addition, HPAI strains used in animal trials or isolated from humans and, for PB2 phylogeny, similar sequences from low pathogenicity avian influenza viruses are included.

Animal Trial

We tested a HPAIV H5N6 virus (AR09/18; A/common pochard/Germany-BY/AR09–18-L02421/2017; AR09/18) together with a different reassortant HPAIV H5N5 (AR425/17; A/turkey/Germany-SH/R425/2017; AR425/17) in a ferret inoculation model. The animal experiments gained legal governmental approval through the ethics committee of the State Office of Agriculture, Food Safety, and Fishery in Mecklenburg-Vorpommern (LALLF M-V: LVL MV/TSD/ 7221.3–1.1–023/13). All procedures were carried out in approved biosafety level 3 facilities. We inoculated 5 ferrets (including control animals) per virus subtype intranasally using 105.5 50% tissue culture infectious dose (TCID50) per animal of H5N6 AR09/18 (total 75 µL) or 106 TCID50 per animal of H5N5 AR425/17 (total 50 µL). Nasal washes were collected every other day from all ferrets to measure virus excretion by applying 1 mL phosphate-buffered saline into each nostril. Analyses of viral RNA load from nasal washes and organ samples were performed exactly as described (7). Ferret serum samples taken preinoculation and at 14 days post infection were heat-inactivated at 56°C for 30 min and analysed by means of a commercial ELISA for the presence of antibodies against influenza A virus nucleoprotein (ID Screen Influenza A Antibody Competition ELISA Kit, ID-vet, Montpellier, France) according to the manufacturer´s instructions. Hemagglutination inhibition assays against the homologous antigens H5N5 and H5N6 were performed according to standard protocols (Commission, E. 2006/437/EC: Commission Decision of 4 August 2006 approving a Diagnostic Manual for avian influenza as provided for in Council Directive 2005/94/EC. Report No. ISSN 1725–2555, 16 [2006]).

Strain details and epidemiologic information of the viruses sequenced in this study are summarised in Appendix Table 1. Phylogenetic analyses of segments were done with similar sequences as described above. The results are given in Appendix Figure 1.

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Appendix Table 1. Strain details and epidemiologic information of the viruses sequenced in this study.

Name	Subtype	Country	Federal state	Host	Collection date	Reference
A/tufted duck/Germany/AR8444/2016*	H5N8	Germany	Schleswig-Holstein	*Aythya fuligula*	2016 Nov 7	(2)
A/turkey/Germany-SH/R425/2017*	H5N5	Germany	Schleswig-Holstein	Domestic turkey	2017 Jan 22	(1)
A/common pochard/Germany-BY/AR09–18-L02421/2017*	H5N6	Germany	Bavaria	*Aythya ferina*	2017 Dec 28	This study
A/mute swan/Switzerland/V361-L02422/2017	H5N6	Switzerland	Kanton Bern, Erlach	*Cygnus olor*	2017 Dec 18	This study
A/white stork/Germany-NI/AR251/2018	H5N6	Germany	Lower Saxony	*Ciconia ciconia*	2018 Apr	This study
A/buzzard/Germany-NRW/AR279/2018	H5N6	Germany	North Rhine-Westphalia	*Buteo*	2018 May	This study
A/chicken/Germany-SH/AR163-L02542/2018	H5N6	Germany	Schleswig-Holstein	Domestic chicken	2018 Mar 19	This study
A/chicken/Germany-SH/AR164-L02543/2018	H5N6	Germany	Schleswig-Holstein	Domestic chicken	2018 Mar 19	This study
A/duck/Germany-SH/AR165-L02544/2018	H5N6	Germany	Schleswig-Holstein	Domestic duck	2018 Mar 19	This study
A/turkey/Germany-SH/AR185-L02549/2018	H5N6	Germany	Schleswig-Holstein	Domestic turkey	2018 Mar 19	This study
A/domestic duck/Germany-MV/AR613-L02727/2018	H5N6	Germany	Mecklenburg-Vorpommern	Domestic duck	2018 Aug 31	This study

*Used in animal trial.

Appendix Table 2. NA segment similarities (protein and nucleotide) for the N6 and N5 segment of virus A/common pochard/Germany-BY/AR09–18-L02421/2017 (N6) and A/turkey/Germany-SH/R425/2017 (N5), respectively, compared with similar NA segments from low pathogenicity avian influenza viruses of different subtypes*.

Segment	Accession no.	Name	Protein identity	Nucleotide identity
N6	MF694081	A/mallard duck/Georgia/9/2016 (A/H4N6)	457/470 (97%)	1411/1441 (97%)
	MF694113	A/mallard duck/Georgia/3/2016 (A/H4N6)	457/470 (97%)	1412/1441 (97%)
	MH135674	A/duck/Bangladesh/33676/2017 (A/H4N6)	452/470 (96%)	1351/1436 (94%)
	MH071489	A/duck/Bangladesh/24268/2015 (A/H10N6)	450/470 (95%)	1363/1446 (94%)
	LC121366	A/duck/Mongolia/405/2015 (A/H3N6)	450/470 (95%)	1329/1443 (92%)
	LC121262	A/duck/Mongolia/118/2015 (A/H4N6)	450/470 (95%)	1329/1443 (92%)
	KY635728	A/duck/Bangladesh/25891/2015 (A/H4N6)	450/470 (95%)	1356/1446 (93%)
	KY635782	A/duck/Bangladesh/26920/2015 (A/H3N6)	449/470 (95%)	1358/1447 (93%)
N5	KU9629324	A/common redshank/Singapore/F83–1/2015 (A/H9N5)	468/472 (99%)	1398/1418 (98%)
	KP285887	A/migratory duck/Jiangxi/31577/2013 (A/H10N5)	467/472 (98%)	1389/1418 (97%)
	EPI866833	A/duck/Aichi/231002/2016 (A/H6N5)	465/472 (98%)	1383/1423 (97%)
	EPI866951	A/duck/Fukui/181006/2015 (A/H12N5)	462/472 (97%)	1377/1423 (96%)
	KY635758	A/black-tailed godwit/Bangladesh/24734/2015 (A/H7N5)	463/472 (98%)	1386/1423 (97%)
	MF613702	A/shorebird/Delaware Bay/327/2016 (A/H10N5)	454/472 (96%)	1295/1422 (91%)
	MF613865	A/American black duck/Alberta/274/2016 (A/H10N5)	454/472 (96%)	1296/1422 (91%)
	CY240796	A/ruddy turnstone/New Jersey/UGAI16–1448/2016 (A/H10N5)	454/472 (96%)	1299/1427 (91%)

* Typical amino acid markers that are known to support replication in mammalian hosts, for example PB2 627K and PB2 701N (8), are not present in the tested viruses and in the first clade 2.3.4.4b human isolate FuSa21099/17. The receptor binding site in the HA segments of the analysed viruses also indicated a preferred binding to α-2,3 sialic acids present in the avian respiratory tract. Furthermore, H5N8 AR8444/16 and H5N6 AR09/18 NS1 protein do not show a C-terminal PDZ binding motif, which is associated with increased virulence in mice (8). HA, hemagglutinin; NA, neuraminidase; PB2, polymerase basic protein 2.

Appendix Table 3. H5Nx-strains tested in the ferret animal model described in this study or in different animal models, and human H5Nx-strains, summarizing their abbreviation, names, and references for their evaluation of zoonotic potential with corresponding relevant genetic markers*.

Abbreviation	Name	Subtype	HA recombination binding site	PB2 627	PB2 701	NS PBM	Reference
FuSa21099/17	A/Fujian-Sanyuan/21099/2017	H5N6	QVNGQRG	E	D	GSEV	(9)
EMW541/16	A/Environment/Korea/W541/2016	H5N6	QVNGQQG	E	D	ESEV	(10)
CTW555/17	A/Common Teal/Korea/W555/2017	H5N8	QVNGQRG	E	D	GSEV	(10)
MDK16/16	A/Mandarin duck/Korea/K16–187–3/2016	H5N6	QVNGQQG	E	D	ESEV	(11)
AR8444/16	A/tufted duck/Germany/AR8444/2016	H5N8	QVNGQRG	E	D	†	(7)
AR425/17	A/turkey/Germany-SH/R425/2017	H5N5	QVNGQRG	E	D	GSEV	This study
AR09/18	A/common pochard/Germany-BY/AR09-18-L02421/2017	H5N6	QVNGQRG	E	D	†	This study

*HA, hemagglutinin; NS, non-structural protein; PB2, polymerase basic 2; PBM, PDZ binding motif.
†NS PBM not present. Truncated protein.

Appendix Table 4. Immunohistological evaluation of tested ferret tissue*.

Group	Necropsy, dpi	R.E.	O.E.	Nasolacrimal duct	Sinus maxillaris	Tonsilla palatina	Lungs	Heart	Spleen	Liver	Jejunum	Pancreas	Colon	Kidney	Cerebrum	Cerebellum	M.O.	Other
Neg. control	14	0	ND	ND	ND	0	0	0	0	0	0	0	0	0	0	0	0	
Neg. control	14	0	0	0	ND	0	0	0	0	0	0	0	ND	0	0	0	0	
H5N5	7	0	2	0	0	ND	1	0	0	2	0	0	0	0	0	0	0	1 oral mucosa, 2 ovary, 3 salpinx
H5N5	14	0	0	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	
H5N5	14	0	0	ND	ND	0	0	0	0	0	0	0	ND	0	0	0	0	
H5N5	14	0	0	ND	ND	0	0	0	0	0	0	0	ND	0	0	0	0	
H5N5	14	0	0	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	
H5N6	14	0	0	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	
H5N6	14	0	0	0	0	0	0	0	0	0	0	0	ND	0	0	0	ND	
H5N6	14	0	0	0	ND	0	0	0	0	0	0	ND	ND	0	0	0	ND	
H5N6	14	0	0	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	
H5N6	14	0	ND	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	

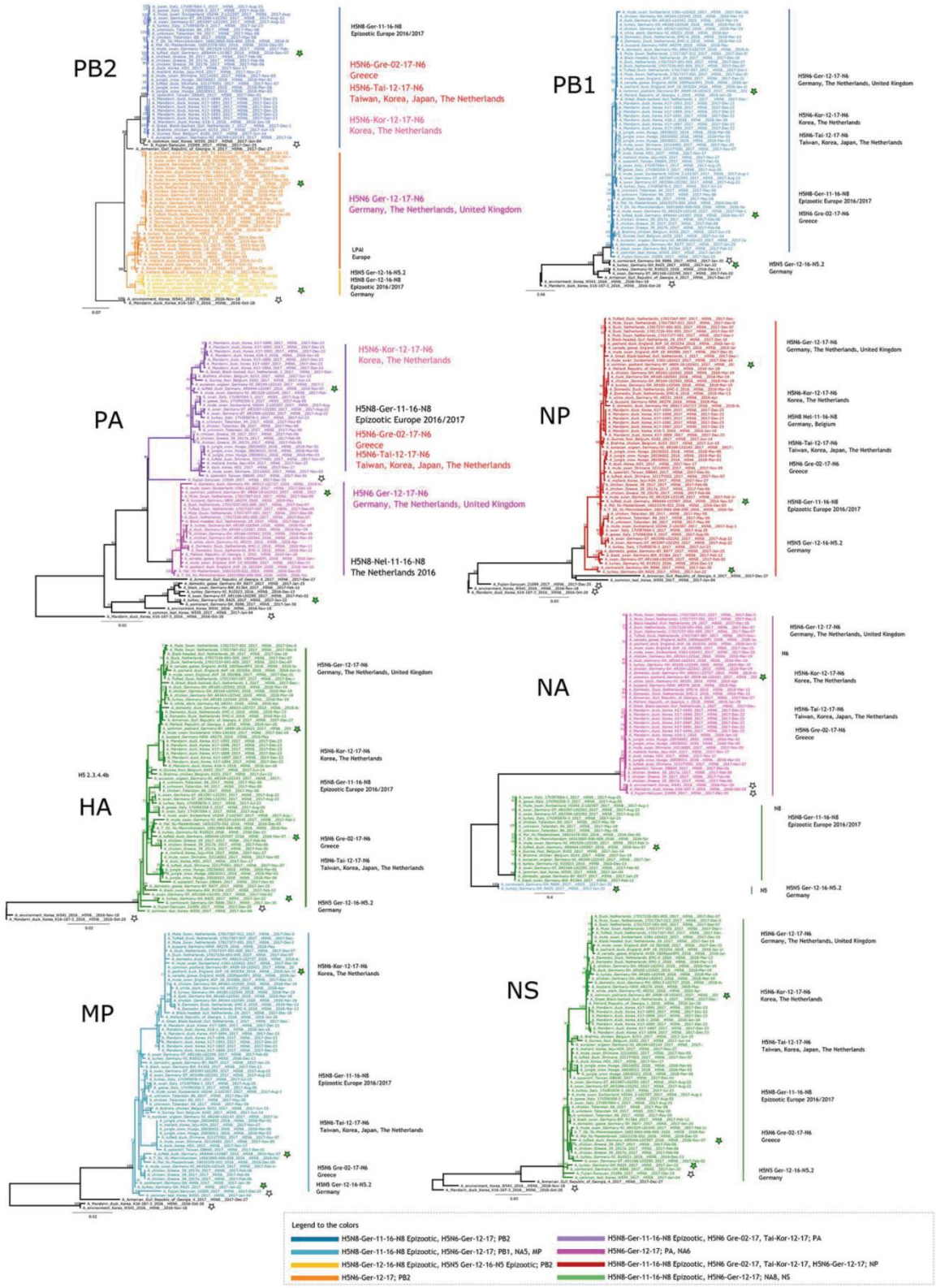
*dpi, days post infection; ND, not done; neg., negative; M.O., medulla oblongata; O.E., olfactory epithelium; R.E., respiratory epithelium. The distribution of influenza A virus matrix protein was semi-quantitatively scored as follows: 0 = no immunoreactivity; 1 = focal/oligofocal immunoreactive cells; 2 = multifocal immunoreactive cells; 3 = coalescing/diffuse immunoreactive cells.

Appendix Table 5. Acknowledgment of laboratories providing sequences in EpiFlu™ database*.

Isolate ID	Country	Isolate name	Submitting Laboratory
EPI_ISL_282143	Italy	A/goose/Italy/17VIR6358–3/2017	Istituto Zooprofilattico Sperimentale Delle Venezie
EPI_ISL_289713	The Netherlands	A/Great Black-backed Gull/Netherlands/1/2017	Erasmus Medical Center
EPI_ISL_282141	Italy	A/swan/Italy/17VIR7064–1/2017	Istituto Zooprofilattico Sperimentale Delle Venezie
EPI_ISL_292223	United Kingdom	A/mute_swan/England/AVP_18_001986/2017	APHA
EPI_ISL_288410	The Netherlands	A/Mute Swan/Netherlands/17017377–001/2017	Wageningen Bioveterinary Research
EPI_ISL_288409	The Netherlands	A/Mute Swan/Netherlands/17017367–012/2017	Wageningen Bioveterinary Research
EPI_ISL_273847	Italy	A/turkey/Italy/17VIR5878–3/2017	Istituto Zooprofilattico Sperimentale Delle Venezie
EPI_ISL_287800	Taiwan	A/spoonbill/Taiwan/DB645/2017	Animal Health Research Institute
EPI_ISL_275433	Russia	A/unknown/Tatarstan/94/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_275432	Russia	A/unknown/Tatarstan/86/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_275288	Russia	A/chicken/Tatarstan/88/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_287907	The Netherlands	A/Duck/Netherlands/17017237–001–005/2017	Wageningen Bioveterinary Research
EPI_ISL_287906	The Netherlands	A/Duck/Netherlands/17017236–001–005/2017	Wageningen Bioveterinary Research
EPI_ISL_292225	United Kingdom	A/canada_goose/England/AV58_18OPpoolEP1/2018	APHA
EPI_ISL_288412	The Netherlands	A/Tufted Duck/Netherlands/17017367–007/2017	Wageningen Bioveterinary Research
EPI_ISL_289714	The Netherlands	A/Black-headed Gull/Netherlands/29/2017	Erasmus Medical Center
EPI_ISL_297235	Russia	A/chicken/Rostov-on-Don/1598/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_297234	Russia	A/chicken/Rostov-on-Don/1321/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_291109	Germany	A/common_pochard/Germany-BY/AR09–18-L02421/2017	Friedrich-Loeffler-Institut
EPI_ISL_292224	United Kingdom	A/pochard_duck/England/AVP_18_003254/2018	APHA
EPI_ISL_312376	Georgia	A/Armenian Gull/Republic of Georgia/4/2017	National Center for Disease Control and Public Health, Georgia
EPI_ISL_289713	The Netherlands	A/Great Black-backed Gull/Netherlands/1/2017	Erasmus Medical Center
EPI_ISL_304404	China	A/Fujian-Sanyuan/21099/2017	Fujian Provincial Center for Disease Control and Prevention
EPI_ISL_288437	South Korea	A/duck/Korea/HD1/2017	Animal and Plant Quarantine Agency (O-2144)
EPI_ISL_288436	South Korea	A/mallard/Korea/Jeju-H24/2017	Animal and Plant Quarantine Agency (O-2144)
EPI_ISL_305417	The Netherlands	A/Domestic_Duck/Netherlands/EMC-6/2018	Erasmus Medical Center
EPI_ISL_305416	The Netherlands	A/Domestic_Duck/Netherlands/EMC-2/2018	Erasmus Medical Center
EPI_ISL_303520	Georgia	A/Mallard/Republic of Georgia/1/2018	National Center for Disease Control and Public Health, Georgia
EPI_ISL_268665	The Netherlands	A/Mal/NL-Mastenbroek/16015378–002/2016	Wageningen Bioveterinary Research
EPI_ISL_268669	The Netherlands	A/T_Dk/NL-Monnickendam/16013865–006–008/2016	Wageningen Bioveterinary Research
EPI_ISL_303643	South Korea	A/Mandarin_duck/Korea/K18–3/2018	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University
EPI_ISL_303642	South Korea	A/Mandarin_duck/Korea/K17–1896/2017	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University

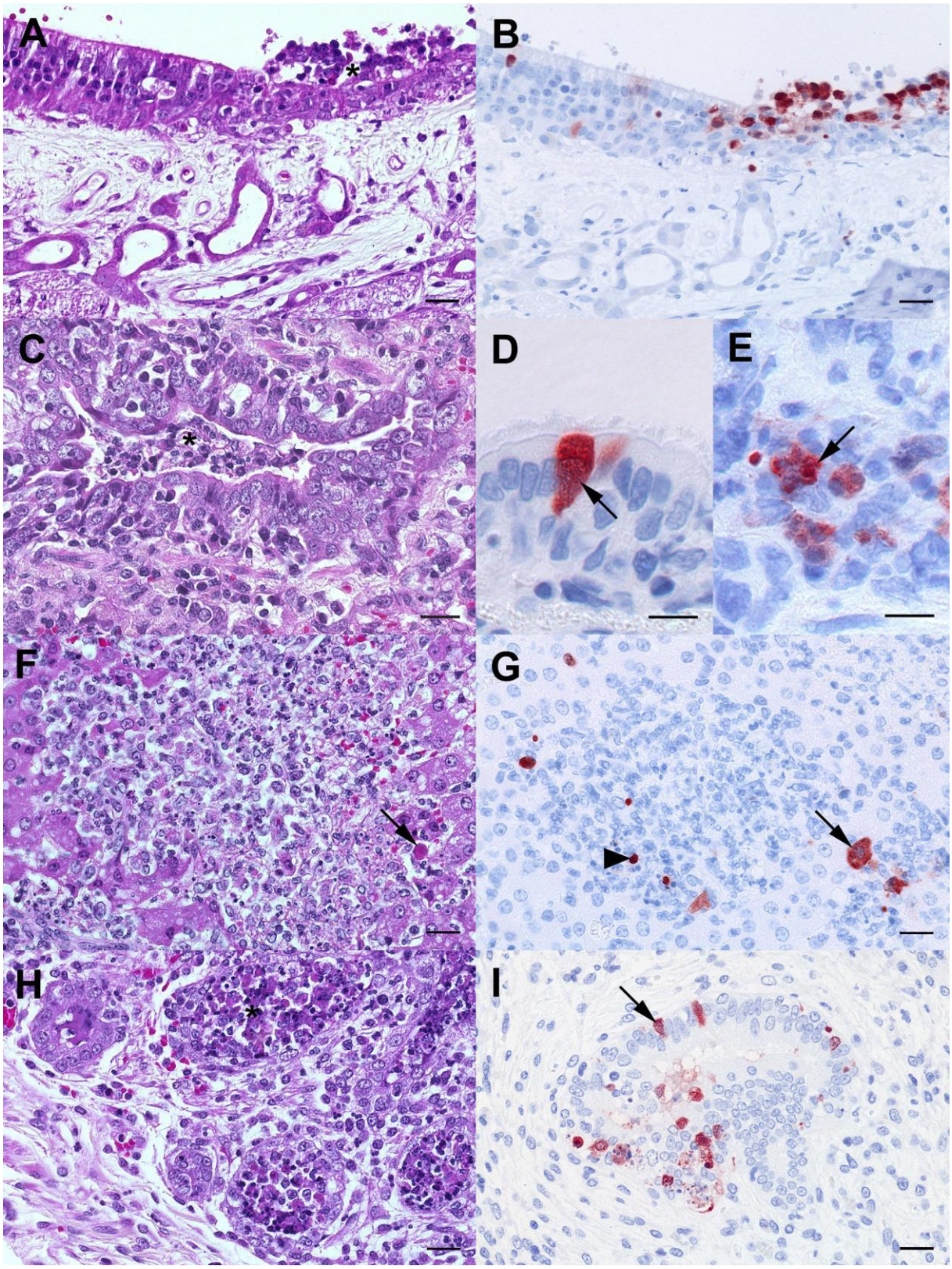
Isolate ID	Country	Isolate name	Submitting Laboratory
EPI_ISL_303641	South Korea	A/Mandarin_duck/Korea/K17–1894/2017	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University
EPI_ISL_303640	South Korea	A/Mandarin_duck/Korea/K17–1893/2017	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University
EPI_ISL_303639	South Korea	A/Mandarin_duck/Korea/K17–1891/2017	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University
EPI_ISL_303638	South Korea	A/Mandarin_duck/Korea/K17–1889/2017	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University
EPI_ISL_303637	South Korea	A/Mandarin_duck/Korea/K17–1887/2017	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University
EPI_ISL_238148	South Korea	A/Mandarin_duck/Korea/K16–187–3/2016	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University
EPI_ISL_288364	Greece	A/chicken/Greece/39_2017b/2017	TVC
EPI_ISL_288363	Greece	A/chicken/Greece/39_2017a/2017	TVC
EPI_ISL_288362	Greece	A/chicken/Greece/39_2017/2017	TVC
EPI_ISL_239393	Japan	A/duck/Aichi/231002/2016	National Institute of Animal Health
EPI_ISL_239416	Japan	A/duck/Fukui/181006/2015	National Institute of Animal Health
EPI_ISL_224753	The Netherlands	A/chicken/Netherlands/15007212/15	Wageningen Bioveterinary Research
EPI_ISL_294779	France	A/duck/France/160051/2016	ANSES
EPI_ISL_294778	France	A/duck/France/160056/2016	ANSES

*ANSES, Agence Nationale de Securite Sanitaire de l'Alimentation, de l'Environnement et du Travail; APHA, Animal and Plant Health Agency; TVC, Thessalonica Veterinary Centre.



Appendix Figure 1. Phylogenetic analyses of PB2, PB1, PA, HA, NP, NA, MP, and NS genes from H5Nx viruses done by Maximum Likelihood using RAxML. Bootstrap values of 1,000 cycles >50 are included. Scale bars indicate nucleotide substitutions per site. Reassortants are grouped according to phylogenetic results and shown to the right. Stars indicate strains tested in the ferret animal model

described in this and a previous study (green stars), strains tested in different animal models (white stars), and human strains (white stars). H5N6 viruses analysed in this study are labelled in different colours according to their clusters (see legend). The reassortants H5N8 and H5N5 Germany are described in detail in (1). HA, hemagglutinin; MP, matrix protein; NA, neuraminidase; NP, nucleoprotein; NS, non-structural protein; PA, polymerase acidic protein; PB1, polymerase basic protein 1; PB2, polymerase basic protein 2.



Appendix Figure 2. Histopathological findings in the H5N5-infected ferret necropsied at 7 days post infection (dpi). A, C, E, G) Hematoxylin eosin. B, D, F, H) Influenza A virus-matrix protein immunohistochemistry, avidin-biotin-peroxidase complex method, with a monoclonal mouse anti-influenza A virus (strain PR8, A/PR/8/34(H1N1))-matrix protein immunoglobulin G1 containing

hybridoma supernatant (clone M2–1C6–4R3, ATCC HB-64; American Type Culture Collection, Manassas, USA), 3-amino-9-ethyl-carbazol as chromogen and hematoxylin counterstain. A) Nasal cavity, olfactory mucosa. Mild, focal, subacute, necrotizing rhinitis with degeneration and loss of the olfactory epithelium (asterisk). B) Nasal cavity, olfactory mucosa. Abundant intensely influenza matrix protein immunoreactive cells and cellular debris within the lesion. C) Lung, bronchioles. Moderate, oligofocal, subacute, necrotizing bronchitis with luminal debris accumulation (asterisk). D) Lung, bronchus. A characteristic bronchial epithelial cell with intense pancellular influenza matrix protein expression (arrow). E) Lung, bronchiolus. There is intensely influenza matrix protein immunoreactive cellular debris (arrow) within the lumen of the collapsed and necrotic bronchioles. F) Liver. Moderate, multifocal, subacute, necrotizing hepatitis with infiltrating macrophages, neutrophils, and rare Councilman Corpuscles (arrow). G) Liver. Multifocal intensely influenza matrix protein immunoreactive intralesional debris (arrow) and fewer hepatocytes (arrowhead). H) Uterine tube. Severe, diffuse, acute, necrotizing salpingitis with nearly complete loss of mucosal epithelium and luminal debris accumulation (asterisk). I) Uterine tube. Multiple influenza matrix protein immunoreactive epithelial cells (arrow) and debris in luminal recesses in the lamina propria. A–C, F–I) Bar = 20 µm. D, E) Bar = 10 µm.

3. **Publication III:** Novel HPAIV H5N8 Reassortant (Clade 2.3.4.4b) Detected in Germany

Publication III

Novel HPAIV H5N8 Reassortant (Clade 2.3.4.4b) Detected in Germany

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Abstract: A novel H5N8 highly pathogenic avian influenza virus (HPAIV) was detected in a greater white-fronted goose in January 2020 in Brandenburg, Germany, and, in February 2020, in domestic chickens belonging to a smallholding in Baden-Wuerttemberg, Germany. Full-genome sequencing was conducted on the MinION platform, enabling further phylogenetic analyses. The virus of clade 2.3.4.4b holds six segments from a Eurasian/Asian/African HPAIV H5N8 reassortant and two segments from low pathogenic avian influenza H3N8 subtype viruses recently detected in wild birds in Central Russia. These new entries continue to show the reassortment potential of the clade 2.3.4.4 H5Nx viruses, underlining the necessity for full-genome sequencing and continuous surveillance.

Keywords: avian influenza viruses; HPAIV; reassortment; H5N8; third-generation sequencing; MinION

1. Introduction

The severe European epizootic of highly pathogenic avian influenza viruses (HPAIV), peaking during the winter of 2016–2017, was dominated by viral swarm incursions and frequent reassortment events [1]. All belonged to group B of clade 2.3.4.4, six reassortants classifying into three subtypes were identified in Germany from November 2016 to August 2018 [2–4]. Phylogenetic analyses of the H5N8 subtypes pointed to individual incursion events, as similar H5N8 HPAIV reassortants were found prior to the German epizootic in migratory wild water bird molting and resting areas in the regions surrounding Tartastan, Kurgan, and Lake Chany, Russian Federation [2,5]. The pronounced magnitude and economic impact of this outbreak attested to the eminent pathogenicity of clade 2.3.4.4b HPAIV [6]. Starting in December 2019, several reports of HPAI H5N8 cases in central and eastern Europe were broadcasted from the responsible national authorities (OIE; https://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2020/). Since January 2020, a clade 2.3.4.4b virus has also been detected in Germany in the form of a novel H5N8 reassortant, Ger-01-20.

2. Materials and Methods

On 16 January 2020, a greater white-fronted goose (*Anser albifrons*) was found dead close to the Polish border in the federal state of Brandenburg, Germany (Figure 1). Pathological examination determined trauma as a cause of death; nevertheless, initial testing for avian influenza virus RNA revealed very high virus loads in mixed tissue homogenates (lung and gut tissue) with RT–qPCR Cq-values of RT–qPCR H5 Cq = 16.1 and RT–qPCR N8.2 Cq = 14.1. Primary sub- and pathotyping results achieved via qPCR [7] revealed a HPAIV H5N8 of clade 2.3.4.4b (RT–qPCR H5 HP 2344b DE Cq = 14.1). Subsequently, a severe necrotizing polioencephalitis, typical of H5N8 infection in waterfowl [8], was detected by histopathology, most likely causing disorientation and predisposing the goose to the traumatic event.

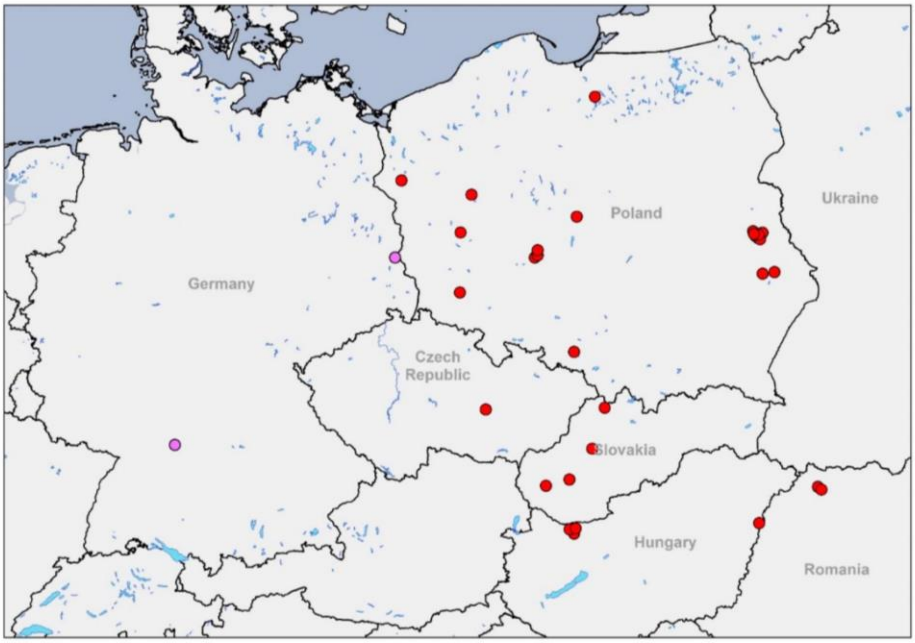

Figure 1. Geographical distribution of European HPAIV H5N8 detections from December 2019 to February 2020 (status as of 13 February 2020). The two German cases are highlighted in purple. Maps were plotted using data from the OIE website and the German animal disease notification system (TSN).

Shortly after, on 6 February 2020, chickens (*Gallus gallus domesticus*) from a small holding in the federal state of Baden-Wuerttemberg (Figure 1) also tested positive for HPAIV H5N8 of clade 2.3.4.4b (RT–qPCR H5 HP 2344b DE Cq = 22.9–25.9; RT–qPCR N8.2 Cq = 22.2–25.4) following the same testing protocol as described [7]. In this case, RNA was extracted from swab samples. During necropsy, the birds showed moderate mucous discharge in the upper respiratory tract and diarrhea. In addition, a severe acute diffuse necrotizing lymphohistiocytic enteritis and a moderate necrotizing encephalitis with perivascular cuffing and gliosis were determined as major characteristic histological lesions.

Amplification for MinION-assisted full genome sequencing of the RNA from both outbreaks was conducted prior to sequencing utilizing the Superscript III One-Step AIV-End-RT-PCR with Platinum Taq (ThermoFisher Scientific, Waltham, MA, USA) and universal AIV primers designed for the conserved ends of all segments [9]. Subsequently, after library preparation with the Rapid Barcoding Kit (RBK-004, Oxford Nanopore Technology, Oxford, UK; ONT) according to the manufacturer's instructions, full genome sequencing was performed on the MinION platform in combination with

a R9.4 flow cell (ONT), the MinIT (v19.12.1; ONT) and basecaller Guppy (v3.2.9; ONT), facilitating real-time basecalling to produce quality checked, demultiplexed, and trimmed raw data.

Consensus assembly of the sequencing data was executed with the Geneious Prime software (Biomatters, Auckland, New Zealand) in a map to reference approach, while further phylogenetic analyses were completed with RAxML [10] and SplitsTree4 [11].

Full genome sequences were deposited into the GISAID database (www.gisaid.org) under the accession numbers EPI_ISL_404993 (2020AI00018; A/white-fronted goose/Germany-BB/AI00018/2020) and EPI_ISL_410291 (2020AI00049; A/chicken/Germany-BW/AI00049/2020). Further genome sequences acquired from the GISAID database and utilized for phylogenetic analyses have been acknowledged in Table S2.

3. Results

Analyses of the full genome sequences from both outbreaks allowed for the identification of a novel reassortant, Ger-01-20, revealing a distinct segment combination that differs from reassortants described in Germany 2016/2017 and similar reassortants circulating worldwide (Figure 2).

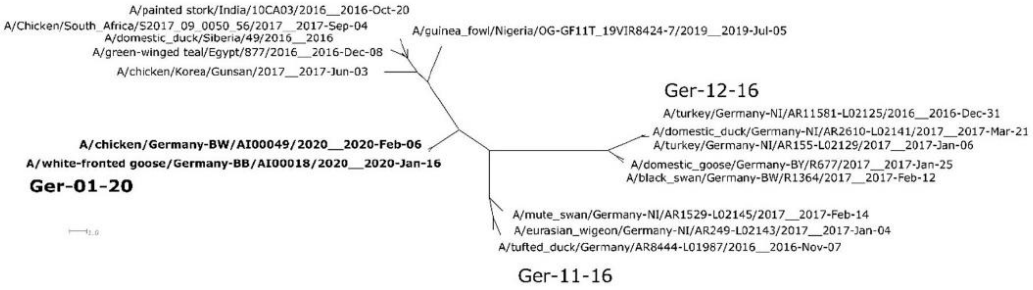

Figure 2. Supernetwork of full genomes of selected worldwide clade 2.3.4.4b H5N8 subgroups from maximum likelihood (ML) trees of PB2, PB1, PA, HA, NP, NA, MP, and NS segments. ML analyses were done using RAxML, including bootstrapping for 1000 iterations and network analyses conducted with SplitsTree4.

In comparison to the HPAIVs circulating during the 2016–2018 epizootic [2,5], the Ger-01-20 reassortant comprises of eight unique segments newly introduced to Germany (Figure 2 and Figure S1).

Six of the eight segments (1, 3, 4, 6, 7, and 8) cluster with 98% sequence identity to a previous HPAIV H5N8 reassortant found from 2016 onwards (Figure 2 and Figure S1, Table S1) [12,13]. This reassortant has been identified in areas ranging from Asia/Eurasia (India, A/painted stork/India/10CA03/2016; South Korea, A/common teal/Korea/W548_2016, A/chicken/Korea/Gunsan/2017; Central Russia, Siberia, A/domestic duck/Siberia/49/2016), Europe (Italy, A/turkey /Italy/17VIR538-1/2017) [14] to Africa (Egypt, A/green-winged teal/Egypt/877/2016; South Africa, A/Geese/South_Africa/S2017/09_0055_P1/2017) in the season 2016/2017 [12,15]. On closer examina-tion, the segments 1, 3, 4, 7, and 8 also exhibit high identity levels (98.86%–99.55%, Table S1) to a recent Nigerian HPAIV H5N8 (A/guinea fowl/Nigeria/OG-GF11T_19VIR8724-7) sampled in July 2019, while segment 6 has proven to share the highest sequence identity with HPAI H5N8 viruses circulating in Siberia, India, and Korea from 2016 to 2017 (98.56%–98.49%, Table S1).

The segments PB1 and NP, respectively, display high similarities to a low pathogenic avian influenza virus (LPAIV) of the subtype H3N8 found in wild waterbirds located in Central Russia. Segment 2 was proven to share 99.01% identity (Table S1) to a sequence from a green sandpiper from the Kurgan area of Central Russia (A/green sandpiper/Kurgan/1050/2018) sampled in late August 2018. Along these lines, segment 5 showed comparable identity levels (98.51%, Table S1) to an LPAIV from a gadwall found at Lake Chany, Central Russia (A/gadwall/Chany/893/2018), sampled shortly after in mid-October 2018 (Figure 3 and Figure S1).

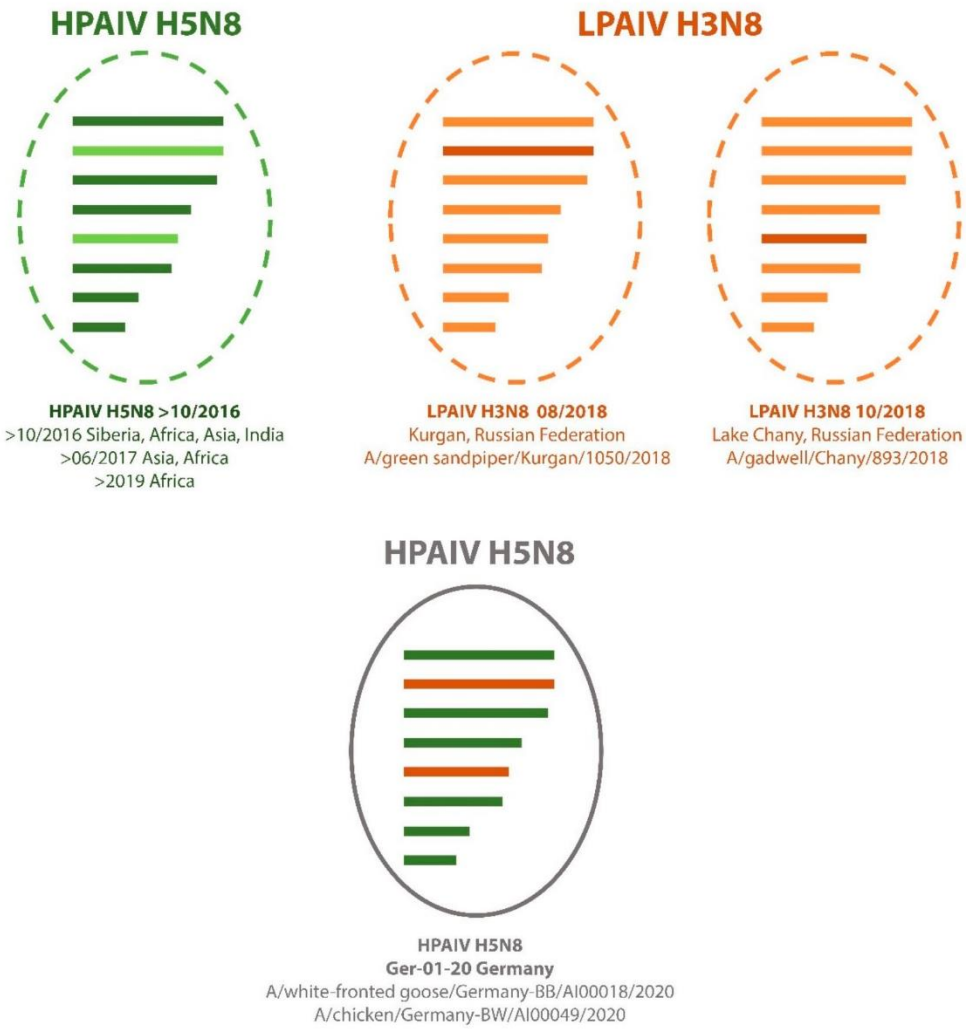

Figure 3. Schematic reassortment analyses based on full-length sequences from AIVs previously detected (upper panel) and the novel HPAIV H5N8 reassortant Ger-01-20 (lower panel). Colors are allocated according to reassortment and display the new composition of HPAIV H5N8 Ger-01-20.

Overall, the phylogenetic analyses demonstrate high similarity ranging from 99.53%–100% (Table S1) between the novel HPAIV H5N8 Ger-01-20 reassortant detected in both a wild bird and poultry and newly released sequences from Poland (A/turkey/Poland/23/2019 and A/hawk/Poland/003/2020) and the Czech Republic (A/chicken/Czech_Republic/1175-1/2020; Figure S1). Further investigation indicates a clustering of the virus from the greater white-fronted goose found in Brandenburg with both Polish sequences while the poultry outbreak in Baden-Wuerttemberg displays higher genetic similarities to the Czech Republic case (Figure S1).

4. Discussion

HP viruses of the gs/GD lineage of clade 2.3.4.4b are reportedly highly capable in attaining novel genome segments through reassortment events, a significant distinction to other gs/GD clades such as Egyptian 2.2.1.x viruses known for their genotypic stability for over more than a decade [16,17]. Promiscuity with respect to reassortment is expected to translate into potentially advantageous phenotypic features affecting viral host range and fitness. This is mirrored by the continuous and

unprecedented spread of clade 2.3.4.4 viruses represented by more than 25 reassorted genotypes across Asia, Northern America, Europe, and Africa since 2014 [16].

Based on the genetic composition of the novel HPAIV H5N8 reassortant Ger-01-20, a new incursion event into Germany is highly likely and no direct genetic correlation to German H5N8 subtypes circulating during the 2016 to 2017 epizootic was established. Currently, no distinct precursor for the Ger-01-20 reassortant has been identified. The role of migratory waterbirds in the distribution of AIVs, as a catalyst for reassortment events and as the natural reservoir for LPAIV, has been demonstrated by numerous studies [3,18–20]. Harboring of segments with sequences related to those identified in an H3N8 LPAIV in wild waterbirds from Central Russia along with segments from the HPAIV H5N8 reassortant found in Eurasia and Africa also infers a connection between the novel reassortant and migratory birds from the African Eurasian flyway as well as molting/resting grounds along the Russian–Kazakhstan/Mongolian/Chinese border [19].

The identification of the same reassortant in multiple central and eastern European countries indicates continuous circulation of the virus and demonstrates genetic connections between these cases and both German outbreaks. As a result, in combination with the notification dates and geographical locations broadcasted by the responsible national authorities (OIE; https://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2020/), it seems permissible to speculate an incursion into Germany from the East. Although genetic relations of Ger-01-20 with numerous HPAI H5N8 viruses found on the African continent (e.g., Nigeria, South Africa, Egypt) could be determined, the connection to LPAIVs found only in central Russia indicates that the African viruses are unlikely to be direct precursors, but instead suggests the circulation of similar HPAIVs on the African continent, possibly sharing the same ancestor.

Based on the limited information available at this time, no clear conclusions as to the circulation of this novel reassortant in wild birds can be drawn. The described German wild bird case is only the second of its kind in Europe in this season, with the first infection identified in a goshawk found in close proximity to an affected poultry holding in Poland. However, the identification of the novel reassortant virus in both wild birds and poultry points towards their susceptibility for infection. Thus, in addition to infected poultry, wild birds must be regarded as a reservoir and vector of this HPAIV.

5. Conclusions

The detection of the new H5N8 subtype Ger-01-20 reassortant, with its genetic backbone reverting to clade 2.3.4.4b, displays the continuing circulation of this clade and highlights its tendency for frequent reassortments and efficient long-distance transmissions. On closer inspection, the virus consists of six segments from the Eurasian/Asian/African HPAIV H5N8 reassortant and two segments from a LPAIV H3N8 subtype found in central Russia. Both German outbreaks show related genetic constellations to sequence data from Poland and the Czech Republic sampled from December 2019 to January 2020. These findings emphasize the necessity for full-genome sequencing and continuous passive surveillance in order to rapidly detect and identify novel HPAIVs, even more so due to the unprecedented genetic variety this clade entails.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/12/3/281/s1, Figure S1: Phylogenetic analyses of Ger-01-20, Table S1: Sequence identity (%), Table S2: Data acknowledgment.

Author Contributions: Conceptualization, T.H., M.B. and A.P.; outbreak investigation, C.S. (Christoph Schulze), A.E., A.H. and S.-L.L., K.R., J.S.; methodology, T.H. and A.P.; formal analysis, A.P. and J.K.; data curation, J.K.; resources, M.B.; writing—original draft preparation, J.K.; writing—review and editing, J.K., T.C.M., T.H., A.P., M.B.; outbreak visualization, C.S. (Christoph Staubach); supervision, A.P. and M.B.; project administration, A.P. and M.B.; funding acquisition, M.B. and T.C.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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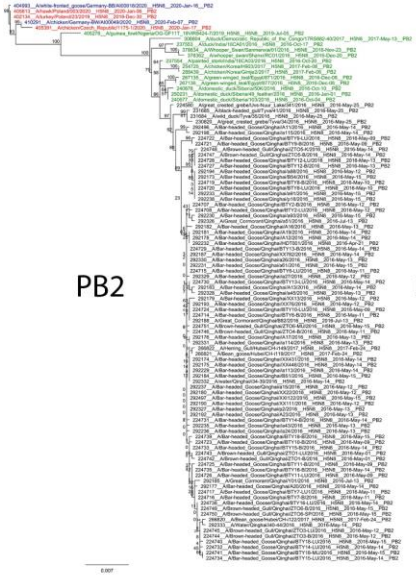
Supplemental Material

Figure S1. Phylogenetic analyses of PB2, PB1, PA, HA, NP, NA, MP and NS sequences done by Maximum Likelihood trees using RAxML with a bootstrap value of 1000 cycles. Trees include the novel reassortant Ger-01-20 (A/white-fronted goose/Germany-BB/AI00018/2020 (root) and A/chicken/Germany-BW/AI00049/2020 – both in blue lettering), and 100 closest relatives according to sequence identity. The red segments cluster with the Ger-01-20 reassortant, all originating in Eastern Europe (Poland and the Czech Republic) from December 2019 – January 2020. The dark green segments pinpoint the HPAIV H5N8 >10-2016 (Nigeria, Korea, India, South Africa) reassortant, while the orange marked sequences stand for the LPAIV H3N8 (Russia, > Aug 2017).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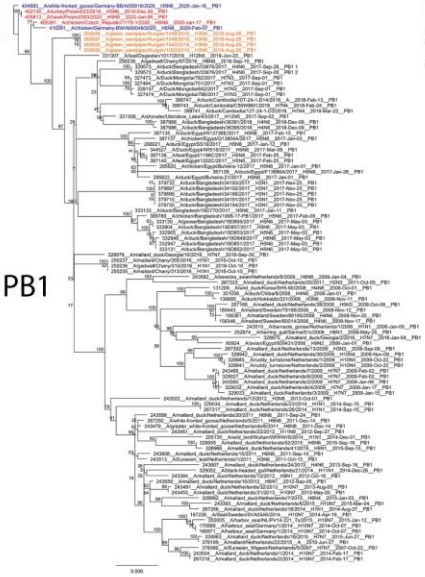
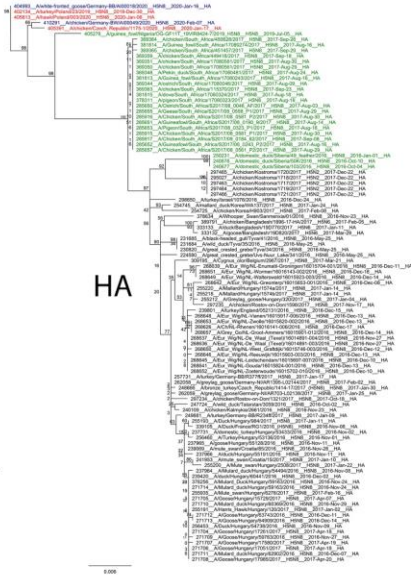

PB1


PA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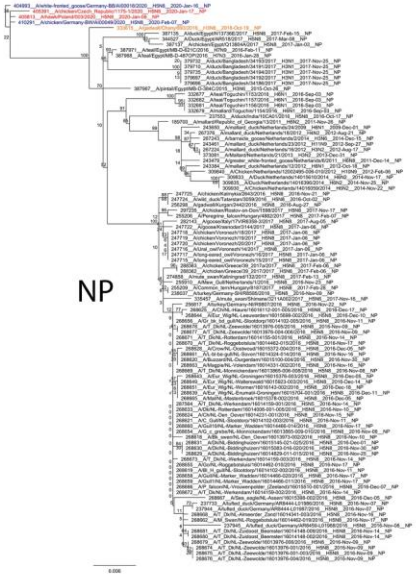
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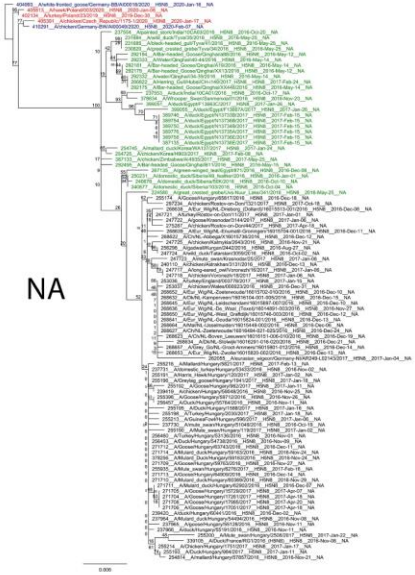
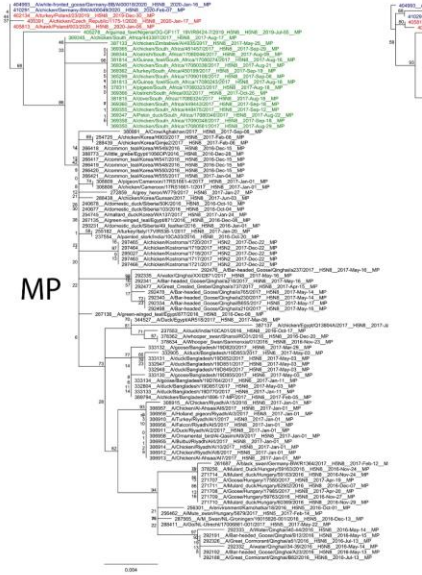

NA


MP

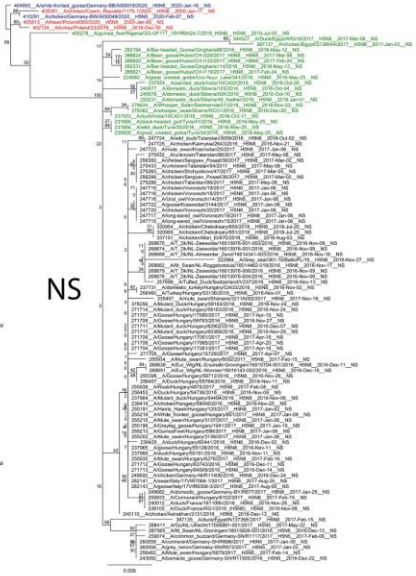

NS

Table S1. Sequence identity (%) of A/white-fronted goose/Germany-BB/AI00018/2020 in comparison to the top ten Blast results (executed on the GISAID Platform – 13.02.2020).

	ID	Name	Subtype	Collection Date	Sequence Identity %
PB2	EPI_ISL_405813	A/hawk/Poland/003/2020	H5N8	2020-Jan-06	99.96
	EPI_ISL_402134	A/turkey/Poland/23/2019	H5N8	2019-Dec-30	99.93
	EPI_ISL_410291	A/chicken/Germany-BW/AI00049/2020	H5N8	2020-Feb-07	99.78
	EPI_ISL_405391	A/chicken/Czech Republic/1175-1/2020	H5N8	2020-Jan-17	99.61
	EPI_ISL_405278	A/guinea fowl/Nigeria/OG-GF11T 19VIR8424-7/2019	H5N8	2019-Jul-05	98.86
	EPI_ISL_237554	A/painted stork/India/10CA03/2016	H5N8	2016-Oct-20	97.65
	EPI_ISL_240677	A/domestic duck/Siberia/103/2016	H5N8	2016-Oct-04	97.58
	EPI_ISL_250231	A/domestic duck/Siberia/49 feather/2016	H5N8	2016-Jan-01	97.58
	EPI_ISL_267135	A/green-winged teal/Egypt/871/2016	H5N8	2016-Dec-08	97.56
	EPI_ISL_224580	A/great crested grebe/Uvs-Nuur Lake/341/2016	H5N8	2016-May-25	97.50
	EPI_ISL_237553	A/duck/India/10CA01/2016	H5N8	2016-Oct-17	97.50
PB1	EPI_ISL_402134	A/turkey/Poland/23/2019	H5N8	2019-Dec-30	99.83
	EPI_ISL_405813	A/hawk/Poland/003/2020	H5N8	2020-Jan-06	99.82
	EPI_ISL_405391	A/chicken/Czech Republic/1175-1/2020	H5N8	2020-Jan-17	99.61
	EPI_ISL_410291	A/chicken/Germany-BW/AI00049/2020	H5N8	2020-Feb-07	99.46
	EPI_ISL_355938	A/green sandpiper/Kurgan/1050/2018	H3N8	2018-Aug-26	99.01
	EPI_ISL_331307	A/teal/Dagestan/1017/2018	H12N5	2018-Jan-23	98.72
	EPI_ISL_250237	A/mallard/Chany/355/2016	H1N1	2016-Oct-10	98.71
	EPI_ISL_250236	A/gadwall/Chany/315/2016	H1N1	2016-Oct-10	98.71
	EPI_ISL_250235	A/mallard/Chany/313/2016	H1N1	2016-Oct-10	98.71
	EPI_ISL_328976	A/mallard duck/Georgia/10/2016	H7N7	2016-Sep-30	98.66
	EPI_ISL_250238	A/gadwall/Chany/97/2016	H6N8	2016-Sep-10	98.62
PA	EPI_ISL_410291	A/chicken/Germany-BW/AI00049/2020	H5N8	2020-Feb-07	99.77
	EPI_ISL_405813	A/hawk/Poland/003/2020	H5N8	2020-Jan-06	99.72
	EPI_ISL_402134	A/turkey/Poland/23/2019	H5N8	2019-Dec-30	99.72
	EPI_ISL_405391	A/chicken/Czech Republic/1175-1/2020	H5N8	2020-Jan-17	99.55
	EPI_ISL_405278	A/guinea fowl/Nigeria/OG-GF11T 19VIR8424-7/2019	H5N8	2019-Jul-05	98.98
	EPI_ISL_378312	A/Domestic goose/South Africa/17090065/2017	H5N8	2017-Sep-04	98.51
	EPI_ISL_369355	A/chicken/South Africa/448475/2017	H5N8	2017-Sep-12	98.51
	EPI_ISL_369339	A/chicken/South Africa/440638A/2017	H5N8	2017-Aug-01	98.51
	EPI_ISL_369336	A/chicken/South Africa/Villiers/2017	H5N8	2017-Jun-19	98.51
	EPI_ISL_285657	A/Chicken/South Africa/S2017/08 0561 P2/2017	H5N8	2017-Aug-29	98.50
	EPI_ISL_285648	A/Geese/South Africa/S2017/09 0055 P1/2017	H5N8	2017-Sep-04	98.50
HA	EPI_ISL_405813	A/hawk/Poland/003/2020	H5N8	2020-Jan-06	100.00
	EPI_ISL_402134	A/turkey/Poland/23/2019	H5N8	2019-Dec-30	100.00
	EPI_ISL_410291	A/chicken/Germany-BW/AI00049/2020	H5N8	2020-Feb-07	99.60
	EPI_ISL_405391	A/chicken/Czech Republic/1175-1/2020	H5N8	2020-Jan-17	99.54
	EPI_ISL_405278	A/guinea fowl/Nigeria/OG-GF11T 19VIR8424-7/2019	H5N8	2019-Jul-05	99.06
	EPI_ISL_381815	A/dove/South Africa/17080324/2017	H5N8	2017-Aug-18	98.42
	EPI_ISL_381813	A/Guinea fowl/South Africa/17080243/2017	H5N8	2017-Aug-16	98.42
	EPI_ISL_378311	A/pigeon/South Africa/17080323/2017	H5N8	2017-Aug-18	98.42
	EPI_ISL_369359	A/chicken/South Africa/449418/2017	H5N8	2017-Sep-18	98.42
	EPI_ISL_369351	A/chicken/South Africa/17080581/2017	H5N8	2017-Aug-30	98.42
	EPI_ISL_369348	A/Pekin duck/South Africa/17080481/2017	H5N8	2017-Aug-24	98.42
NP	EPI_ISL_410291	A/chicken/Germany-BW/AI00049/2020	H5N8	2020-Feb-07	99.81
	EPI_ISL_405813	A/hawk/Poland/003/2020	H5N8	2020-Jan-06	99.79
	EPI_ISL_405391	A/chicken/Czech Republic/1175-1/2020	H5N8	2020-Jan-17	99.57
	EPI_ISL_387968	A/teal/Egypt/MB-D-487OP/2016	H7N3	2016-Jan-28	98.51
	EPI_ISL_333615	A/gadwall/Chany/893/2018	H3N8	2018-Oct-19	98.51
	EPI_ISL_387971	A/teal/Egypt/MB-D-621C/2016	H7N9	2016-Feb-11	98.44
	EPI_ISL_387967	A/pintail/Egypt/MB-D-384C/2015	H3N6	2015-Oct-26	98.25
	EPI_ISL_387135	A/duck/Egypt/N13736E/2017	H5N8	2017-Feb-15	97.99
	EPI_ISL_344527	A/Duck/Egypt/AR518/2017	H5N8	2017-Mar-08	97.99
	EPI_ISL_387137	A/chicken/Egypt/Q13804A/2017	H5N8	2017-Jan-03	97.92

	ID	Name	Subtype	Collection Date	Sequence Identity %
	EPI_ISL_189700	A/mallard/Republic of Georgia/13/2011	H6N2	2011-Nov-26	97.84
NA	EPI_ISL_402134	A/turkey/Poland/23/2019	H5N8	2019-Dec-30	99.68
	EPI_ISL_405813	A/hawk/Poland/003/2020	H5N8	2020-Jan-06	99.58
	EPI_ISL_410291	A/chicken/Germany-BW/AI00049/2020	H5N8	2020-Feb-07	99.55
	EPI_ISL_405391	A/chicken/Czech Republic/1175-1/2020	H5N8	2020-Jan-17	99.47
	EPI_ISL_250231	A/domestic duck/Siberia/49 feather/2016	H5N8	2016-Jan-01	98.56
	EPI_ISL_237554	A/painted stork/India/10CA03/2016	H5N8	2016-Oct-20	98.50
	EPI_ISL_254725	A/chicken/Korea/H903/2017	H5N8	2017-Feb-08	98.49
	EPI_ISL_387133	A/chicken/Zimbabwe/AI4935/2017	H5N8	2017-May-25	98.48
	EPI_ISL_292495	A/Bar-headed Goose/Qinghai/B11/2016	H5N8	2016-May-15	98.48
	EPI_ISL_240677	A/domestic duck/Siberia/103/2016	H5N8	2016-Oct-04	98.43
MP	EPI_ISL_410291	A/chicken/Germany-BW/AI00049/2020	H5N8	2020-Feb-07	100.00
	EPI_ISL_405813	A/hawk/Poland/003/2020	H5N8	2020-Jan-06	100.00
	EPI_ISL_402134	A/turkey/Poland/23/2019	H5N8	2019-Dec-30	100.00
	EPI_ISL_405391	A/chicken/Czech Republic/1175-1/2020	H5N8	2020-Jan-17	99.90
	EPI_ISL_369345	A/chicken/South Africa/443397/2017	H5N8	2017-Aug-17	99.59
	EPI_ISL_267135	A/green-winged teal/Egypt/871/2016	H5N8	2016-Dec-08	99.50
	EPI_ISL_250231	A/domestic duck/Siberia/49 feather/2016	H5N8	2016-Jan-01	99.50
	EPI_ISL_240677	A/domestic duck/Siberia/103/2016	H5N8	2016-Oct-04	99.50
	EPI_ISL_254745	A/mallard duck/Korea/WA137/2017	H5N8	2017-Jan-24	99.50
	EPI_ISL_388773	A/little grebe/Egypt/1056OP/2016	H5N8	2016-Dec-28	99.49
	EPI_ISL_266421	A/common teal/Korea/W555/2017	H5N8	2017-Jan-04	99.49
NS	EPI_ISL_410291	A/chicken/Germany-BW/AI00049/2020	H5N8	2020-Feb-07	99.83
	EPI_ISL_405813	A/hawk/Poland/003/2020	H5N8	2020-Jan-06	99.76
	EPI_ISL_402134	A/turkey/Poland/23/2019	H5N8	2019-Dec-30	99.64
	EPI_ISL_405391	A/chicken/Czech Republic/1175-1/2020	H5N8	2020-Jan-17	99.53
	EPI_ISL_405278	A/guinea fowl/Nigeria/OG-GF11T 19VIR8424-7/2019	H5N8	2019-Jul-05	99.16
	EPI_ISL_237553	A/duck/India/10CA01/2016	H5N8	2016-Oct-17	98.61
	EPI_ISL_231685	A/black-headed gull/Tyva/41/2016	H5N8	2016-May-25	98.61
	EPI_ISL_231684	A/wild duck/Tyva/35/2016	H5N8	2016-May-25	98.61
	EPI_ISL_230820	A/great crested grebe/Tyva/34/2016	H5N8	2016-May-25	98.61
	EPI_ISL_292194	A/Bar-headed Goose/Qinghai/a88/2016	H5N8	2016-May-12	98.60
	EPI_ISL_266820	A/Bean goose/Hubei/CH-i122/2017	H5N8	2017-Feb-24	98.60

Table S2. Data acknowledgement – We acknowledge the following laboratories for providing sequences in the EpiFlu™ Database.

Isolate ID	Isolate Name	Submitting Lab
EPI_ISL_355938	A/green sandpiper/Kurgan/1050/2018	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_355937	A/green sandpiper/Kurgan/1048/2018	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_355936	A/green sandpiper/Kurgan/1046/2018	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_355935	A/green sandpiper/Kurgan/1043/2018	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_250237	A/mallard/Chany/355/2016	Research Institute of Experimental and Clinical Medicine
EPI_ISL_250236	A/gadwall/Chany/315/2016	Research Institute of Experimental and Clinical Medicine
EPI_ISL_250235	A/mallard/Chany/313/2016	Research Institute of Experimental and Clinical Medicine
EPI_ISL_250238	A/gadwall/Chany/97/2016	Research Institute of Experimental and Clinical Medicine
EPI_ISL_331306	A/shoveler/Ubinskoe Lake/43/2017	Research Institute of Experimental and Clinical Medicine
EPI_ISL_331307	A/teal/Dagestan/1017/2018	Research Institute of Experimental and Clinical Medicine
EPI_ISL_167226	A/Seal/Sweden/SVA0546/2014	National Veterinary Institute, Uppsala, Sweden
EPI_ISL_285657	A/Chicken/South Africa/S2017/08 0561 P2/2017	National Institute for Communicable Diseases
EPI_ISL_285648	A/Geese/South Africa/S2017/09 0055 P1/2017	National Institute for Communicable Diseases
EPI_ISL_378312	A/Domestic goose/South Africa/17090065/2017	National Institute for Communicable Diseases
EPI_ISL_369355	A/chicken/South Africa/448475/2017	National Institute for Communicable Diseases
EPI_ISL_285601	A/Geese/South Africa/S2017/09 0065 P1/2017	National Institute for Communicable Diseases
EPI_ISL_285611	A/Wildbirds Guineafowl Makou Egyptian Geese/South Africa/S2017/08 0275 P1/2017	National Institute for Communicable Diseases
EPI_ISL_381814	A/Guinea fowl/South Africa/17080274/2017	National Institute for Communicable Diseases
EPI_ISL_285941	A/Guineafowl/South Africa/S2017/08 0274 P2/2017	National Institute for Communicable Diseases
EPI_ISL_369350	A/chicken/South Africa/17080561/2017	National Institute for Communicable Diseases
EPI_ISL_285607	A/Duck/South Africa/S2017/08 0340 P2/2017	National Institute for Communicable Diseases
EPI_ISL_285606	A/Duck/South Africa/S2017/08 0340 P1/2017	National Institute for Communicable Diseases
EPI_ISL_369347	A/Pekin duck/South Africa/17080340/2017	National Institute for Communicable Diseases
EPI_ISL_365299	A/chicken/South Africa/17090108/2017	National Institute for Communicable Diseases
EPI_ISL_285651	A/Guineafowl/South Africa/S2017/08 0190 9/2017	National Institute for Communicable Diseases
EPI_ISL_369349	A/Swan/South Africa/17080517/2017	National Institute for Communicable Diseases
EPI_ISL_369348	A/Pekin duck/South Africa/17080481/2017	National Institute for Communicable Diseases
EPI_ISL_369361	A/chicken/South Africa/17090335/2017	National Institute for Communicable Diseases
EPI_ISL_285610	A/Guineafowl/South Africa/S2017/08 0274 P1/2017	National Institute for Communicable Diseases
EPI_ISL_285916	A/Chicken/South Africa/S2017/08 0581 P2/2017	National Institute for Communicable Diseases
EPI_ISL_369351	A/chicken/South Africa/17080581/2017	National Institute for Communicable Diseases
EPI_ISL_369346	A/chicken/South Africa/17080336/2017	National Institute for Communicable Diseases
EPI_ISL_285624	A/Chicken/South Africa/S2017/09 0050 56/2017	National Institute for Communicable Diseases
EPI_ISL_369357	A/chicken/South Africa/449300/2017	National Institute for Communicable Diseases

Isolate ID	Isolate Name	Submitting Lab
EPI_ISL_369356	A/chicken/South Africa/17090325/2017	National Institute for Communicable Diseases
EPI_ISL_369354	A/chicken/South Africa/17090202/2017	National Institute for Communicable Diseases
EPI_ISL_369352	A/chicken/South Africa/17090050/2017	National Institute for Communicable Diseases
EPI_ISL_285652	A/Guineafowl/South Africa/S2017/08 0243 P2/2017	National Institute for Communicable Diseases
EPI_ISL_381815	A/dove/South Africa/17080324/2017	National Institute for Communicable Diseases
EPI_ISL_381813	A/Guinea fowl/South Africa/17080243/2017	National Institute for Communicable Diseases
EPI_ISL_378311	A/pigeon/South Africa/17080323/2017	National Institute for Communicable Diseases
EPI_ISL_369360	A/chicken/South Africa/449443/2017	National Institute for Communicable Diseases
EPI_ISL_369353	A/chicken/South Africa/17090100/2017	National Institute for Communicable Diseases
EPI_ISL_284004	A/Chicken/South Africa/S2017/08 0336 P3/2017	National Institute for Communicable Diseases
EPI_ISL_369364	A/chicken/South Africa/450628/2017	National Institute for Communicable Diseases
EPI_ISL_285623	A/Chicken/South Africa/S2017/08 0416 38/2017	National Institute for Communicable Diseases
EPI_ISL_369358	A/chicken/South Africa/17090348/2017	National Institute for Communicable Diseases
EPI_ISL_369365	A/chicken/South Africa/451457/2017	National Institute for Communicable Diseases
EPI_ISL_285915	A/Chicken/South Africa/S2017/08 0581 P1/2017	National Institute for Communicable Diseases
EPI_ISL_285655	A/Geese/South Africa/S2017/08 0558 P1/2017	National Institute for Communicable Diseases
EPI_ISL_369359	A/chicken/South Africa/449418/2017	National Institute for Communicable Diseases
EPI_ISL_285617	A/Guineafowl/South Africa/S2017/08 0243 P1/2017	National Institute for Communicable Diseases
EPI_ISL_285658	A/Chicken/South Africa/S2017/08 0561 P1/2017	National Institute for Communicable Diseases
EPI_ISL_285650	A/Ostrich/South Africa/S2017/08 0046 AF/2017	National Institute for Communicable Diseases
EPI_ISL_369344	A/ostrich/South Africa/17080046/2017	National Institute for Communicable Diseases
EPI_ISL_369362	A/turkey/South Africa/450199/2017	National Institute for Communicable Diseases
EPI_ISL_285649	A/Chicken/South Africa/S2017/09 0184 63/2017	National Institute for Communicable Diseases
EPI_ISL_285602	A/Geese/South Africa/S2017/09 0065 P2/2017	National Institute for Communicable Diseases
EPI_ISL_285918	A/Ostrich/South Africa/S2017/08 0362 P8 34/2017	National Institute for Communicable Diseases
EPI_ISL_285618	A/Ostrich/South Africa/S2017/08 0362 P7/2017	National Institute for Communicable Diseases
EPI_ISL_285608	A/Ostrich/South Africa/S2017/08 0362 P8 33/2017	National Institute for Communicable Diseases
EPI_ISL_369343	A/chicken/South Africa/441839/2017	National Institute for Communicable Diseases
EPI_ISL_369339	A/chicken/South Africa/440638A/2017	National Institute for Communicable Diseases
EPI_ISL_369340	A/chicken/South Africa/440638B/2017	National Institute for Communicable Diseases
EPI_ISL_369336	A/chicken/South Africa/Villiers/2017	National Institute for Communicable Diseases
EPI_ISL_369342	A/chicken/South Africa/MC002/2017	National Institute for Communicable Diseases
EPI_ISL_369338	A/chicken/South Africa/436893/2017	National Institute for Communicable Diseases
EPI_ISL_381811	A/Speckled pigeon/South Africa/08-004B/2017	National Institute for Communicable Diseases
EPI_ISL_369363	A/chicken/South Africa/115370/2017	National Institute for Communicable Diseases
EPI_ISL_285653	A/Pigeon/South Africa/S2017/08 0323 P1/2017	National Institute for Communicable Diseases
EPI_ISL_285604	A/Ostrich/South Africa/S2017/08 0046 P3/2017	National Institute for Communicable Diseases

Isolate ID	Isolate Name	Submitting Lab
EPI_ISL_285917	A/Chicken/South Africa/S2017/09 0184 62/2017	National Institute for Communicable Diseases
EPI_ISL_285625	A/Duck/South Africa/S2017/08 0481 P2/2017	National Institute for Communicable Diseases
EPI_ISL_285944	A/Chicken/South Africa/S2017/08 0336 P2/2017	National Institute for Communicable Diseases
EPI_ISL_285480	A/Ostrich/South Africa/S2017/08 0362 P10/2017	National Institute for Communicable Diseases
EPI_ISL_285512	A/Chicken/South Africa/S2017/08 0336 P1/2017	National Institute for Communicable Diseases
EPI_ISL_285616	A/Ostrich/South Africa/S2017/08 0161 P8/2017	National Institute for Communicable Diseases
EPI_ISL_369345	A/chicken/South Africa/443397/2017	National Institute for Communicable Diseases
EPI_ISL_237554	A/painted stork/India/10CA03/2016	ICAR-National Institute of High Security Animal Diseases
EPI_ISL_240677	A/domestic duck/Siberia/103/2016	Research Institute of Experimental and Clinical Medicine
EPI_ISL_250231	A/domestic duck/Siberia/49 feather/2016	Research Institute of Experimental and Clinical Medicine
EPI_ISL_240678	A/domestic duck/Siberia/50K/2016	Research Institute of Experimental and Clinical Medicine
EPI_ISL_297466	A/chicken/Kostroma/1721/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_297465	A/chicken/Kostroma/1720/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_297464	A/chicken/Kostroma/1719/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_297463	A/chicken/Kostroma/1717/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_295027	A/chicken/Kostroma/1718/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_254745	A/mallard duck/Korea/WA137/2017	Animal and Plant Quarantine Agency (S-2026)
EPI_ISL_254725	A/chicken/Korea/H903/2017	Animal and Plant Quarantine Agency (S-2026)
EPI_ISL_292498	A/Bar-headed Goose/Qinghai/a210/2017	Wuhan Institute of Virology
EPI_ISL_292479	A/Bar-headed Goose/Qinghai/a893/2017	Wuhan Institute of Virology
EPI_ISL_292478	A/Bar-headed Goose/Qinghai/a765/2017	Wuhan Institute of Virology
EPI_ISL_292477	A/Great Crested Grebe/Qinghai/a737/2017	Wuhan Institute of Virology
EPI_ISL_292476	A/Bar-headed Goose/Qinghai/a237/2017	Wuhan Institute of Virology
EPI_ISL_292335	A/water/Qinghai/XXII2871/2017	Wuhan Institute of Virology
EPI_ISL_292334	A/Bar-headed Goose/Qinghai/B655/2017	Wuhan Institute of Virology
EPI_ISL_292341	A/Bar-headed Goose/Qinghai/a218/2017	Wuhan Institute of Virology
EPI_ISL_292340	A/Bar-headed Goose/Qinghai/a230/2017	Wuhan Institute of Virology
EPI_ISL_306958	A/Ornamental bird/Al-Qasim/AI9/2017	Wuhan Institute of Virology
EPI_ISL_306956	A/Falcon/Riyadh/AI5/2017	Center of Influenza Research, University of Hong Kong
EPI_ISL_306955	A/Bulbul/Riyadh/AI4/2017	Center of Influenza Research, University of Hong Kong
EPI_ISL_306915	A/Chicken/Riyadh/A15/2018	Center of Influenza Research, University of Hong Kong
EPI_ISL_306912	A/Chicken/Riyadh/AI6/2017	Center of Influenza Research, University of Hong Kong
EPI_ISL_306910	A/Turkey/Riyadh/AI1/2017	Center of Influenza Research, University of Hong Kong
EPI_ISL_306914	A/Chicken/Riyadh/AI10/2017	Center of Influenza Research, University of Hong Kong
EPI_ISL_306959	A/Holland pigeon/Riyadh/AI3/2017	Center of Influenza Research, University of Hong Kong
EPI_ISL_306957	A/Chicken/Al-Ahsaa/AI8/2017	Center of Influenza Research, University of Hong Kong
EPI_ISL_306913	A/Chicken/Al-Ahsaa/AI7/2017	Center of Influenza Research, University of Hong Kong

Isolate ID	Isolate Name	Submitting Lab
EPI_ISL_306911	A/Duck/Riyadh/AI2/2017	Center of Influenza Research, University of Hong Kong
EPI_ISL_231685	A/black-headed gull/Tyva/41/2016	WHO National Influenza Centre Russian Federation
EPI_ISL_231684	A/wild duck/Tyva/35/2016	WHO National Influenza Centre Russian Federation
EPI_ISL_230820	A/great crested grebe/Tyva/34/2016	WHO National Influenza Centre Russian Federation
EPI_ISL_240109	A/chicken/Kalmykia/2661/2016	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_248666	A/bronze turkey/Czech Republic/1414-17/2017	State Veterinary Institute Prague
EPI_ISL_247724	A/wild duck/Tatarstan/3059/2016	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_297234	A/chicken/Rostov-on-Don/1321/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_237965	A/goose/Hungary/55128/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_239069	A/mute swan/Croatia/85/2016	Croatian Veterinary Institute
EPI_ISL_237731	A/domestic turkey/Hungary/53433/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_256460	A/Turkey/Hungary/53136/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_238197	A/mute swan/Croatia/78/2016	Croatian Veterinary Institute
EPI_ISL_255193	A/Duck/Hungary/984/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_378256	A/Mulard Duck/Hungary/59163/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_271714	A/Mulard duck/Hungary/59163/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255935	A/Mute swan/Hungary/6276/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_239420	A/duck/Hungary/60441/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_271711	A/Mulard duck/Hungary/62902/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_271710	A/Mulard duck/Hungary/60369/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_271708	A/Goose/Hungary/17985/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_271706	A/Goose/Hungary/17051/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_271705	A/Goose/Hungary/15729/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_271704	A/Goose/Hungary/17261/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_256453	A/Duck/Hungary/54738/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255191	A/Harris Hawk/Hungary/120/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_237964	A/Mulard duck/Hungary/54494/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_271713	A/Goose/Hungary/64909/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_271712	A/Goose/Hungary/63743/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_271709	A/Goose/Hungary/59763/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_271707	A/Goose/Hungary/17580/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255937	A/Pheasant/Hungary/7685/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255936	A/Pheasant/Hungary/6553/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_237966	A/duck/Hungary/55191/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_239419	A/chicken/Hungary/59048/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255938	A/Rook/Hungary/4975/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_256457	A/Duck/Hungary/55764/2016	Central Agricultural Office Veterinary Diagnostic Directorate

Isolate ID	Isolate Name	Submitting Lab
EPI_ISL_255398	A/Goose/Hungary/59712/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_237730	A/mute swan/Hungary/51049/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255212	A/Greylag goose/Hungary/320/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255220	A/Mallard/Hungary/1574a/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255218	A/Mallard/Hungary/1574b/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_297235	A/chicken/Rostov-on-Don/1598/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_268652	A/Eur Wig/NL-Zoeterwoude/16015702-010/2016	Wageningen Bioveterinary Research
EPI_ISL_268645	A/Eur Wig/NL-Leidschendam/16015697-007/2016	Wageningen Bioveterinary Research
EPI_ISL_268641	A/Eur Wig/NL-Gouda/16015824-001/2016	Wageningen Bioveterinary Research
EPI_ISL_268637	A/Eur Wig/NL-De Waal (Texel)/16014891-004/2016	Wageningen Bioveterinary Research
EPI_ISL_268636	A/Eur Wig/NL-De Waal (Texel)/16014891-003/2016	Wageningen Bioveterinary Research
EPI_ISL_268657	A/Grey Go/NL-Groot-Ammers/16015901-012/2016	Wageningen Bioveterinary Research
EPI_ISL_268653	A/Eur Wig/NL-Zwolle/16015820-002/2016	Wageningen Bioveterinary Research
EPI_ISL_268626	A/Ch/NL-Rhenen/16016141-006/2016	Wageningen Bioveterinary Research
EPI_ISL_268650	A/Eur Wig/NL-West Graftdijk/16015746-003/2016	Wageningen Bioveterinary Research
EPI_ISL_268648	A/Eur Wig/NL-Vianen/16015917-006/2016	Wageningen Bioveterinary Research
EPI_ISL_268646	A/Eur Wig/NL-Reeuwijk/16015903-003/2016	Wageningen Bioveterinary Research
EPI_ISL_268632	A/Dk/NL-Kamperveen/16016104-001-005/2016	Wageningen Bioveterinary Research
EPI_ISL_239801	A/turkey/England/052131/2016	Animal and Plant Health Agency (APHA)
EPI_ISL_268627	A/Ch/NL-Zoeterwoude/16016484-021-025/2016	Wageningen Bioveterinary Research
EPI_ISL_268623	A/Ch/NL-Boven Leeuwen/16016151-006-010/2016	Wageningen Bioveterinary Research
EPI_ISL_268642	A/Eur Wig/NL-Greonterp/16015653-001/2016	Wageningen Bioveterinary Research
EPI_ISL_275287	A/chicken/Rostov-on-Don/44/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_247723	A/mute swan/Krasnodar/25/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_268651	A/Eur Wig/NL-Wormer/16016143-002/2016	Wageningen Bioveterinary Research
EPI_ISL_268649	A/Eur Wig/NL-Walterswald/16015923-003/2016	Wageningen Bioveterinary Research
EPI_ISL_268638	A/Eur Wig/NL-Drieborg (Dollard)/16015513-001/2016	Wageningen Bioveterinary Research
EPI_ISL_339105	A/Duck/France/RG1/2016 (H5N8)	Ecole Veterinaire de Toulouse
EPI_ISL_255934	A/Mute swan/Hungary/6092/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255200	A/Mute swan/Hungary/2508/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_224580	A/great crested grebe/Uvs-Nuur Lake/341/2016	Research Institute of Experimental and Clinical Medicine
EPI_ISL_268625	A/Ch/NL-Hiaure/16016112-001-005/2016	Wageningen Bioveterinary Research
EPI_ISL_333615	A/gadwall/Chany/893/2018	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_287564	A/T Dk/NL-Werkendam/16014159-001/2016	Wageningen Bioveterinary Research
EPI_ISL_268672	A/T Dk/NL-Werkendam/16014159-002/2016	Wageningen Bioveterinary Research
EPI_ISL_268671	A/T Dk/NL-Rotterdam/16014155-001/2016	Wageningen Bioveterinary Research
EPI_ISL_268670	A/T Dk/NL-Roggebotsluis/16014462-015/2016	Wageningen Bioveterinary Research

Isolate ID	Isolate Name	Submitting Lab
EPI_ISL_268669	A/T Dk/NL-Monnickendam/16013865-006-008/2016	Wageningen Bioveterinary Research
EPI_ISL_268666	A/P falcon/NL-Vrouwenpolder (Zeeland)/16015510-001/2016	Wageningen Bioveterinary Research
EPI_ISL_268660	A/Gull10/NL-Marker Wadden/16014466-014/2016	Wageningen Bioveterinary Research
EPI_ISL_268659	A/Gull1/NL-Marker Wadden/16014466-011/2016	Wageningen Bioveterinary Research
EPI_ISL_268658	A/Gull/NL-Marker Wadden/16014466-020/2016	Wageningen Bioveterinary Research
EPI_ISL_268656	A/Gr bk bd gull/NL-Slootdorp/16014102-005/2016	Wageningen Bioveterinary Research
EPI_ISL_268655	A/Go/NL-Roggebotsluis/16014462-010/2016	Wageningen Bioveterinary Research
EPI_ISL_268654	A/G c grebe/NL-Monnickendam/16013865-009-010/2016	Wageningen Bioveterinary Research
EPI_ISL_268633	A/Dk/NL-Rotterdam/16014008-001-005/2016	Wageningen Bioveterinary Research
EPI_ISL_268624	A/Ch/NL-Den Oever/16014231-001/2016	Wageningen Bioveterinary Research
EPI_ISL_268621	A/C Gull/NL-Slootdorp/16014102-003/2016	Wageningen Bioveterinary Research
EPI_ISL_268619	A/Bl H gull/NL-Slootdorp/16014102-002/2016	Wageningen Bioveterinary Research
EPI_ISL_274858	A/mute swan/Kaliningrad/132/2017	Wageningen Bioveterinary Research
EPI_ISL_268678	A/T Dk/NL-Zeewolde/16013976-005/2016	Wageningen Bioveterinary Research
EPI_ISL_268677	A/T Dk/NL-Zeewolde/16013976-004-006/2016	Wageningen Bioveterinary Research
EPI_ISL_268665	A/Mal/NL-Mastenbroek/16015378-002/2016	Wageningen Bioveterinary Research
EPI_ISL_268639	A/Eur Wig/NL-Enumatil-Groningen/16015704-001/2016	Wageningen Bioveterinary Research
EPI_ISL_268643	A/Eur Wig/NL-Groningen/16015376-003/2016	Wageningen Bioveterinary Research
EPI_ISL_268663	A/Magpie/NL-Volendam/16014331-002/2016	Wageningen Bioveterinary Research
EPI_ISL_268661	A/L-bl-ba-gull/NL-Sovon/16014324-014/2016	Wageningen Bioveterinary Research
EPI_ISL_268620	A/Buzzard/NL-Durgerdam/16015100-004/2016	Wageningen Bioveterinary Research
EPI_ISL_268628	A/Crow/NL-Oostwoud/16015372-004/2016	Wageningen Bioveterinary Research
EPI_ISL_268662	A/M Swan/NL-Roggebotsluis/16014462-019/2016	Wageningen Bioveterinary Research
EPI_ISL_255209	A/Common tern/Hungary/8187/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_268681	A/T Dk/NL-Zuidoost Beemster/16014148-009/2016	Wageningen Bioveterinary Research
EPI_ISL_268680	A/T Dk/NL-Zuidoost Beemster/16014148-002/2016	Wageningen Bioveterinary Research
EPI_ISL_268668	A/T Dk/NL-Almeerder Zand/16014341-003/2016	Wageningen Bioveterinary Research
EPI_ISL_268679	A/T Dk/NL-Zeewolde/16013976-006/2016	Wageningen Bioveterinary Research
EPI_ISL_268676	A/T Dk/NL-Zeewolde/16013976-004/2016	Wageningen Bioveterinary Research
EPI_ISL_268675	A/T Dk/NL-Zeewolde/16013976-001-003/2016	Wageningen Bioveterinary Research
EPI_ISL_268674	A/T Dk/NL-Zeewolde/16013976-001/2016	Wageningen Bioveterinary Research
EPI_ISL_268673	A/T Dk/NL-Werkendam/16014159-003/2016	Wageningen Bioveterinary Research
EPI_ISL_268618	A/Bk swan/NL-Den Oever/16013973-002/2016	Wageningen Bioveterinary Research
EPI_ISL_255910	A/Mew Gull/Netherlands/1/2016	Erasmus Medical Center
EPI_ISL_268631	A/Dk/NL-Biddinghuizen/16015145-021-025/2016	Wageningen Bioveterinary Research
EPI_ISL_268630	A/Dk/NL-Biddinghuizen/16015083-016-020/2016	Wageningen Bioveterinary Research
EPI_ISL_268629	A/Dk/NL-Biddinghuizen/16014829-011-015/2016	Wageningen Bioveterinary Research

Isolate ID	Isolate Name	Submitting Lab
EPI_ISL_268644	A/Eur Wig/NL-Leeuwarden/16015699-002/2016	Wageningen Bioveterinary Research
EPI_ISL_268682	A/Teal/NL-Ferwert/16015273-013/2016	Wageningen Bioveterinary Research
EPI_ISL_288363	A/chicken/Greece/39 2017a/2017	Thessalonica Veterinary Centre
EPI_ISL_288362	A/chicken/Greece/39 2017/2017	Thessalonica Veterinary Centre
EPI_ISL_256298	A/gadwall/Kurgan/2442/2016	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_247725	A/chicken/Kalmykia/2643/2016	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_247720	A/chicken/Voronezh/20/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_247719	A/chicken/Voronezh/19/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_247718	A/chicken/Voronezh/18/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_247717	A/long-eared owl/Voronezh/16/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_247716	A/Ural owl/Voronezh/14/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_247715	A/long-eared owl/Voronezh/15/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_282143	A/goose/Italy/17VIR6358-3/2017	Istituto Zooprofilattico Sperimentale Delle Venezie
EPI_ISL_282141	A/swan/Italy/17VIR7064-1/2017	Istituto Zooprofilattico Sperimentale Delle Venezie
EPI_ISL_247722	A/goose/Krasnodar/3144/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_255206	A/Peregrine falcon/Hungary/4882/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_335457	A/mute swan/Shimane/3211A002/2017	National Institute of Animal Health
EPI_ISL_268667	A/Sea eagle/NL-Assen/16015398-002/2016	Wageningen Bioveterinary Research
EPI_ISL_267376	A/mallard duck/Netherlands/18/2012	Erasmus Medical Center
EPI_ISL_373081	A/Mallard/Netherlands/21/2013	Erasmus Medical Center
EPI_ISL_267224	A/mallard duck/Netherlands/16/2012	Erasmus Medical Center
EPI_ISL_267243	A/barnacle goose/Netherlands/2/2014	Erasmus Medical Center
EPI_ISL_243650	A/mallard duck/Netherlands/24/2009	Erasmus Medical Center
EPI_ISL_309840	A/Chicken/Netherlands/12002495-006-010/2012	Wageningen Bioveterinary Research
EPI_ISL_309930	A/Chicken/Netherlands/14016059/2014	Wageningen Bioveterinary Research
EPI_ISL_309835	A/Duck/Netherlands/14016396/2014	Wageningen Bioveterinary Research
EPI_ISL_309833	A/Duck/Netherlands/14015610/2014	Wageningen Bioveterinary Research
EPI_ISL_332682	A/teal/Toguchin/1157/2016	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_332681	A/teal/Toguchin/1156/2016	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_332679	A/mallard/Toguchin/1154/2016	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_332677	A/teal/Toguchin/1153/2016	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_237553	A/duck/India/10CA01/2016	ICAR-National Institute of High Security Animal Diseases
EPI_ISL_292194	A/Bar-headed Goose/Qinghai/a88/2016	Wuhan Institute of Virology
EPI_ISL_292331	A/Bar-headed Goose/Qinghai/a114/2016	Wuhan Institute of Virology
EPI_ISL_266820	A/Bean goose/Hubei/CH-i122/2017 H5N8	Wuhan Institute of Virology
EPI_ISL_266824	A/Bean goose/Hubei/CH-i320/2017 H5N8	Wuhan Institute of Virology
EPI_ISL_266821	A/Bean goose/Hubei/CH-i119/2017	Wuhan Institute of Virology

Isolate ID	Isolate Name	Submitting Lab
EPI_ISL_240110	A/chicken/Astrakhan/3131/2016	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_255219	A/White fronted goose/Hungary/801/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255215	A/Mute swan/Hungary/3137/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255213	A/GuineaFowl/Hungary/596/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255202	A/Mute swan/Hungary/3139/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255196	A/Greylag goose/Hungary/1941/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_275433	A/unknown/Tatarstan/94/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_275288	A/chicken/Tatarstan/88/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_275283	A/chicken/Shchyolkovo/47/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_256300	A/chicken/Sergiyev Posad/39/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_256299	A/chicken/Sergiyev Posad/38/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_275432	A/unknown/Tatarstan/86/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_322984	A/Grey seal/361-10/BalticPL/16	Stiftung Tierärztliche Hochschule Hannover
EPI_ISL_257699	A/Tufted Duck/Switzerland/V237/2016	Faculty of Veterinary Medicine at the University of Bern
EPI_ISL_240012	A/duck/France/161108h/2016	Agence Nationale De Securite Sanitaire De L'alimentation
EPI_ISL_255933	A/Cormorant/Hungary/6102/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_256462	A/Mute swan/Hungary/5879/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_288411	A/Go/NL-Utrecht/17006881-001/2017	Wageningen Bioveterinary Research
EPI_ISL_287565	A/M Swan/NL-Groningen/16015826-001/2016	Wageningen Bioveterinary Research
EPI_ISL_337151	A/chicken/Mari El/870/2018	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_320955	A/chicken/Cheboksary/851/2018	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_320954	A/chicken/Cheboksary/850/2018	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_320953	A/chicken/Cheboksary/849/2018	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_320685	A/chicken/Samara/679/2018	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_320684	A/goose/Samara/675/2018	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_320683	A/goose/Samara/673/2018	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_320682	A/chicken/Kursk/762/2018	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_288439	A/chicken/Korea/Gimje2/2017	Animal and Plant Quarantine Agency (S-2145)
EPI_ISL_292187	A/Bar-headed Goose/Qinghai/XX782/2016	Wuhan Institute of Virology
EPI_ISL_292170	A/Bar-headed Goose/Qinghai/A22/2016	Wuhan Institute of Virology
EPI_ISL_292495	A/Bar-headed Goose/Qinghai/B11/2016	Wuhan Institute of Virology
EPI_ISL_292181	A/Bar-headed Goose/Qinghai/A19/2016	Wuhan Institute of Virology
EPI_ISL_292179	A/Bar-headed Goose/Qinghai/XX13/2016	Wuhan Institute of Virology
EPI_ISL_292332	A/water/Qinghai/i34-39/2016	Wuhan Institute of Virology
EPI_ISL_292175	A/Bar-headed Goose/Qinghai/XX446/2016	Wuhan Institute of Virology
EPI_ISL_266822	A/Herring Gull/Hubei/CH-i149/2017 H5N8	Wuhan Institute of Virology
EPI_ISL_292333	A/Water/Qinghai/i40-44/2016	Wuhan Institute of Virology

Isolate ID	Isolate Name	Submitting Lab
EPI_ISL_255214	A/Chicken/Hungary/1751/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_254814	A/mallard/Hungary/57857/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255216	A/Mallard/Hungary/5821/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255192	A/Goose/Hungary/982/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255198	A/Turkey/Hungary/2030/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255190	A/Mute swan/Hungary/119/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255195	A/Duck/Hungary/1588/2017	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_255174	A/Goose/Hungary/65817/2016	Central Agricultural Office Veterinary Diagnostic Directorate
EPI_ISL_247721	A/turkey/Rostov-on-Don/11/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_268622	A/Ch/NL-Abbega/X16015736/2016	Wageningen Bioveterinary Research
EPI_ISL_268664	A/Mal/NL-IJsselmuiden/16015448-002/2016	Wageningen Bioveterinary Research
EPI_ISL_253036	A/turkey/England/003778/2017	Animal and Plant Health Agency (APHA)
EPI_ISL_253037	A/chicken/Wales/000023/2016	Animal and Plant Health Agency (APHA)
EPI_ISL_268634	A/Dk/NL-Stolwijk/16016291-016-020/2016	Wageningen Bioveterinary Research
EPI_ISL_255182	A/turkey/Italy/17VIR538-1/2017	Istituto Zooprofilattico Sperimentale Delle Venezie
EPI_ISL_288438	A/chicken/Korea/Gunsan/2017	Animal and Plant Quarantine Agency (S-2145)
EPI_ISL_333629	A/Environment/Fujiansanyuan/08/2017	Fujian Center for Disease Control and Prevention
EPI_ISL_256301	A/environment/Kamchatka/18/2016	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_292326	A/Great Cormorant/Qinghai/a51/2016	Wuhan Institute of Virology
EPI_ISL_292192	A/Bar-headed Goose/Qinghai/A23/2016	Wuhan Institute of Virology
EPI_ISL_292191	A/Bar-headed Goose/Qinghai/B12/2016	Wuhan Institute of Virology
EPI_ISL_292188	A/Great Cormorant/Qinghai/B82/2016	Wuhan Institute of Virology
EPI_ISL_224744	A/Brown-headed Gull/Qinghai/ZTO3-B/2016	Wuhan Institute of Virology
EPI_ISL_224743	A/Brown-headed Gull/Qinghai/ZTO1-LU/2016	Wuhan Institute of Virology
EPI_ISL_224734	A/Bar-headed Goose/Qinghai/BTY15-LU/2016	Wuhan Institute of Virology
EPI_ISL_224726	A/Bar-headed Goose/Qinghai/BTY11-LU/2016	Wuhan Institute of Virology
EPI_ISL_224717	A/Bar-headed Goose/Qinghai/BTY7-LU1/2016	Wuhan Institute of Virology
EPI_ISL_288364	A/chicken/Greece/39 2017b/2017	Thessalonica Veterinary Centre (TVC)
EPI_ISL_292497	A/Bar-headed Goose/Qinghai/XXI122/2016	Wuhan Institute of Virology
EPI_ISL_292330	A/Bar-headed Goose/Qinghai/a26/2016	Wuhan Institute of Virology
EPI_ISL_292327	A/Bar-headed Goose/Qinghai/p2/2016	Wuhan Institute of Virology
EPI_ISL_292237	A/Bar-headed Goose/Qinghai/a15/2016	Wuhan Institute of Virology
EPI_ISL_292231	A/Bar-headed Goose/Qinghai/a61/2016	Wuhan Institute of Virology
EPI_ISL_292190	A/Bar-headed Goose/Qinghai/XX111/2016	Wuhan Institute of Virology
EPI_ISL_292180	A/Bar-headed Goose/Qinghai/XX22/2016	Wuhan Institute of Virology
EPI_ISL_224729	A/Bar-headed Goose/Qinghai/BTY13-B/2016	Wuhan Institute of Virology
EPI_ISL_224715	A/Bar-headed Goose/Qinghai/BTY6-LU/2016	Wuhan Institute of Virology

Isolate ID	Isolate Name	Submitting Lab
EPI_ISL_292229	A/Bar-headed Goose/Qinghai/a113/2016	Wuhan Institute of Virology
EPI_ISL_292184	A/Bar-headed Goose/Qinghai/B51/2016	Wuhan Institute of Virology
EPI_ISL_292174	A/Bar-headed Goose/Qinghai/XX431/2016	Wuhan Institute of Virology
EPI_ISL_292176	A/Bar-headed Goose/Qinghai/A17/2016	Wuhan Institute of Virology
EPI_ISL_292235	A/Bar-headed Goose/Qinghai/a43/2016	Wuhan Institute of Virology
EPI_ISL_292232	A/Bar-headed Goose/Qinghai/HDT001/2016	Wuhan Institute of Virology
EPI_ISL_292178	A/Bar-headed Goose/Qinghai/A12/2016	Wuhan Institute of Virology
EPI_ISL_224751	A/Brown-headed Gull/Qinghai/ZTO6-MU/2016	Wuhan Institute of Virology
EPI_ISL_224746	A/Brown-headed Gull/Qinghai/ZTO4-B/2016	Wuhan Institute of Virology
EPI_ISL_224733	A/Bar-headed Goose/Qinghai/BTY15-B/2016	Wuhan Institute of Virology
EPI_ISL_224731	A/Bar-headed Goose/Qinghai/BTY14-B/2016	Wuhan Institute of Virology
EPI_ISL_224730	A/Bar-headed Goose/Qinghai/BTY13-LU/2016	Wuhan Institute of Virology
EPI_ISL_224716	A/Bar-headed Goose/Qinghai/BTY7-B/2016	Wuhan Institute of Virology
EPI_ISL_224725	A/Bar-headed Goose/Qinghai/BTY11-B/2016	Wuhan Institute of Virology
EPI_ISL_224735	A/Bar-headed Goose/Qinghai/BTY16-B/2016	Wuhan Institute of Virology
EPI_ISL_224742	A/Brown-headed Gull/Qinghai/ZTO1-B/2016	Wuhan Institute of Virology
EPI_ISL_291952	A/Bar-headed Goose/Qinghai/B44/2016	Wuhan Institute of Virology
EPI_ISL_292183	A/Bar-headed Goose/Qinghai/A13/2016	Wuhan Institute of Virology
EPI_ISL_292328	A/Bar-headed Goose/Qinghai/a45/2016	Wuhan Institute of Virology
EPI_ISL_292329	A/Bar-headed Goose/Qinghai/a27/2016	Wuhan Institute of Virology
EPI_ISL_292236	A/Bar-headed Goose/Qinghai/a24/2016	Wuhan Institute of Virology
EPI_ISL_292193	A/Bar-headed Goose/Qinghai/XX76/2016	Wuhan Institute of Virology
EPI_ISL_224724	A/Bar-headed Goose/Qinghai/BTY10-LU/2016	Wuhan Institute of Virology
EPI_ISL_224714	A/Bar-headed Goose/Qinghai/BTY6-B/2016	Wuhan Institute of Virology
EPI_ISL_224707	A/Bar-headed Goose/Qinghai/BTY2-B/2016	Wuhan Institute of Virology
EPI_ISL_224708	A/Bar-headed Goose/Qinghai/BTY2-LU/2016	Wuhan Institute of Virology
EPI_ISL_292230	A/Bar-headed Goose/Qinghai/a93/2016	Wuhan Institute of Virology
EPI_ISL_224745	A/Brown-headed Gull/Qinghai/ZTO3-LU/2016	Wuhan Institute of Virology
EPI_ISL_224750	A/Brown-headed Gull/Qinghai/ZTO6-SP/2016	Wuhan Institute of Virology
EPI_ISL_224749	A/Brown-headed Gull/Qinghai/ZTO6-B/2016	Wuhan Institute of Virology
EPI_ISL_224736	A/Bar-headed Goose/Qinghai/BTY16-LU/2016	Wuhan Institute of Virology
EPI_ISL_224741	A/Bar-headed Goose/Qinghai/BTY18-MU/2016	Wuhan Institute of Virology
EPI_ISL_224732	A/Bar-headed Goose/Qinghai/BTY14-LU/2016	Wuhan Institute of Virology
EPI_ISL_224740	A/Bar-headed Goose/Qinghai/BTY18-LU/2016	Wuhan Institute of Virology
EPI_ISL_224739	A/Bar-headed Goose/Qinghai/BTY18-B/2016	Wuhan Institute of Virology
EPI_ISL_224723	A/Bar-headed Goose/Qinghai/BTY10-B/2016	Wuhan Institute of Virology
EPI_ISL_292177	A/Bar-headed Goose/Qinghai/A20/2016	Wuhan Institute of Virology

Isolate ID	Isolate Name	Submitting Lab
EPI_ISL_292182	A/Bar-headed Goose/Qinghai/A16/2016	Wuhan Institute of Virology
EPI_ISL_292185	A/Great Cormorant/Qinghai/Y01/2016	Wuhan Institute of Virology
EPI_ISL_224718	A/Bar-headed Goose/Qinghai/BTY7-LU2/2016	Wuhan Institute of Virology
EPI_ISL_224747	A/Brown-headed Gull/Qinghai/ZTO5-B/2016	Wuhan Institute of Virology
EPI_ISL_224728	A/Bar-headed Goose/Qinghai/BTY12-LU/2016	Wuhan Institute of Virology
EPI_ISL_224727	A/Bar-headed Goose/Qinghai/BTY12-B/2016	Wuhan Institute of Virology
EPI_ISL_292238	A/Bar-headed Goose/Qinghai/p18/2016	Wuhan Institute of Virology
EPI_ISL_292233	A/Bar-headed Goose/Qinghai/a91/2016	Wuhan Institute of Virology
EPI_ISL_292173	A/Bar-headed Goose/Qinghai/B54/2016	Wuhan Institute of Virology
EPI_ISL_224720	A/Bar-headed Goose/Qinghai/BTY8-LU/2016	Wuhan Institute of Virology
EPI_ISL_224719	A/Bar-headed Goose/Qinghai/BTY8-B/2016	Wuhan Institute of Virology
EPI_ISL_224712	A/Bar-headed Goose/Qinghai/BTY4-LU/2016	Wuhan Institute of Virology
EPI_ISL_224704	A/Bar-headed Goose/Qinghai/BTY1-B/2016	Wuhan Institute of Virology
EPI_ISL_224748	A/Brown-headed Gull/Qinghai/ZTO5-K/2016	Wuhan Institute of Virology
EPI_ISL_224721	A/Bar-headed Goose/Qinghai/BTY9-B/2016	Wuhan Institute of Virology
EPI_ISL_224722	A/Bar-headed Goose/Qinghai/BTY9-LU/2016	Wuhan Institute of Virology
EPI_ISL_292496	A/Bar-headed Goose/Qinghai/A11/2016	Wuhan Institute of Virology
EPI_ISL_292198	A/Bar-headed Goose/Qinghai/a115/2016	Wuhan Institute of Virology
EPI_ISL_404993	A/white-fronted Goose/Germany-BB/AI00018/2020	Friedrich-Loeffler-Institute
EPI_ISL_410291	A/chicken/Germany-BW/AI00049/2020	Friedrich-Loeffler-Institute
EPI_ISL_405813	A/hawk/Poland/003/2020	National Veterinary Research Institut Poland
EPI_ISL_402134	A/turkey/Poland/23/2019	National Veterinary Research Institut Poland
EPI_ISL_405391	A/chicken/Czech Republic/1175-1/2020	State Veterinary Institute Prague
EPI_ISL_405278	A/guinea fowl/Nigeria/OG-GF11T 19VIR8424-7/2019	Istituto Zooprofilattico Sperimentale Delle Venezie

4. **Publication IV:** Novel Reassortant Highly Pathogenic Avian Influenza A(H5N2) Virus in Broiler Chickens, Egypt

Publication IV

Novel Reassortant Highly Pathogenic Avian Influenza A(H5N2) Virus in Broiler Chickens, Egypt

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Novel Reassortant Highly Pathogenic Avian Influenza A(H5N2) Virus in Broiler Chickens, Egypt

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We detected a novel reassortant highly pathogenic avian influenza A(H5N2) virus in 3 poultry farms in Egypt. The virus carried genome segments of a pigeon H9N2 influenza virus detected in 2014, a nucleoprotein segment of contemporary chicken H9N2 viruses from Egypt, and hemagglutinin derived from the 2.3.4.4b H5N8 virus clade.

Since 2006, Egypt's poultry industry has been plagued by endemic infections with highly pathogenic avian influenza (HPAI) virus, subtype H5N1, clade 2.2.1, of the goose/Guangdong (gs/GD) lineage (*1*). In addition, low pathogenicity avian influenza (LPAI) virus of subtype H9N2, G1 lineage, introduced in 2011 (*2*), and HPAI H5N8 (gs/GD clade 2.3.4.4b) introduced in 2016, have become entrenched in local poultry populations (*3*). Despite ongoing control measures, respiratory disease with increased mortality rates is endemic in poultry farms in Egypt.

The zoonotic nature of HPAI H5N1 2.2.1 viruses has caused in Egypt the highest number of human infection cases per country worldwide; a low level of sporadic benign human cases of H9N2 viral infection has also been reported from Egypt (*4*). Continued adaptation by point mutations, but not reassortment, to enhance replication in mammalian hosts has been repeatedly reported in avian influenza in Egypt (*5*). Here, we describe the detection of a new reassortant HPAI virus in commercial chicken holdings in Egypt. This virus carries the hemagglutinin (HA) gene of HPAI clade 2.3.4.4b H5N8 virus and 7 genome segments derived from Egyptian H9N2 viruses (*6*).

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DOI: https://doi.org/10.3201/eid2601.190570

The Study

During January–April 2019, we examined samples from 11 commercial broiler farms reporting respiratory clinical signs among chickens by using the Riems Influenza A Typing Assay (*7*). We detected co-presence of avian influenza viruses subtypes H5 and H9 with N2 (8 farms) as well as H5N2 only (3 farms).

We selected 8 samples representing H5N8, H9N2, and H5N1 from 2017–2018, plus 1 positive H5N2 sample from 2019, for full-genome sequencing (Table 1; Appendix 1 Table, https://wwwnc.cdc.gov/EID/article/26/1/19-0570-App1.pdf). Sanger- and next-generation sequencing results identified various reassortants new to Egypt (Figure 1). All H5 HA segments encoded a polybasic cleavage site, PLREKRRKR-GLF (H5 clade 2.3.4.4b) or PQGEKRRKKR-GLF (H5 clade 2.2.1.2), thus classifying those viruses as highly pathogenic. We identified the closest related sequences by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) homology searches in the GISAID (http://platform.gisaid.org) and International Nucleotide Sequence Data Collaboration (http://www.insdc.org) databases. Phylogenetic analyses of each genome segment aided in clustering sequences (Appendix 1 Figure). We delineated the putative origin of each of the genome segments (Figure 1). The HA segment of the novel HPAI H5N2 reassortant virus was derived from clade 2.3.4.4b viruses with closest homology to viruses circulating in ducks in Egypt in 2017 (Figure 2), whereas ≥4 additional genome segments (polymerase basic 1, polymerase basic 2, polymerase, and nonstructural protein) originated from novel reassortant H9N2 viruses first detected in pigeons

Table. Characteristics of avian influenza viruses in samples from diseased poultry, Egypt*

Strain	Subtype	Collection date	Host species	Governorate	Flock size	Mortality rate, %	Genome sequence	Clade
A/duck/Egypt/AR518/2017	H5N8	2017 Mar 15	Duck	Giza	3,000	40	Full	2.3.4.4b
A/duck/Egypt/AR560/2018	H5N8	2018 May 20	Duck	Giza	5,000	35	Full	2.3.4.4b
A/turkey/Egypt/AR550/2018	H5N8	2018 Mar 2	Turkey	Beni-Suef	5,000	100	Full	2.3.4.4b
A/duck/Egypt/AR526/2017	H5N1	2017 Mar 20	Duck	Beni-Suef	3,000	15	Full	2.2.1.2
A/chicken/Egypt/AR528/2017	H5N1	2017 Mar 22	Chicken, layer	Beni-Suef	5,000	30	Full	2.2.1.2
A/chicken/Egypt/AR544/2018	H9N2	2018 Jan 20	Chicken, broiler	Giza	10,000	25	Full	G1.B
A/chicken/Egypt/AR545/2018	H9N2	2018 Mar 25	Chicken, broiler	Qualiobia	5,000	25	Full	G1.B
A/chicken/Egypt/AR546/2018	H9N2	2018 Mar 22	Chicken, broiler	El-Menia	8,000	10	Full	G1.B
A/chicken/Egypt/AI00994/2019	H5N2	2019 Jan 19	Chicken, broiler	Beheira	17,000	47	Full	2.3.4.4b
A/chicken/Egypt/AI00986/2019	H5N2	2019 Jan 5	Chicken, broiler	Fayoum	10,000	5	HA (partial)	2.3.4.4b
A/chicken/Egypt/AI00987/2019	H5, H9, N2	2019 Jan 9	Chicken, broiler	Beheira	7,000	15	HA (partial)	2.3.4.4b
A/chicken/Egypt/AI00988/2019	H5, H9, N2	2019 Jan 19	Chicken, broiler	Beheira	8,000	20	HA (partial)	2.3.4.4b
A/chicken/Egypt/AI00989/2019	H5, H9, N2	2019 Jan 27	Chicken, broiler	El-Menia	6,000	14	HA (partial)	2.3.4.4b
A/chicken/Egypt/AI00991/2019	H5, H9, N2	2019 Feb 16	Chicken, broiler	Beni-Suef	74,000	16	NA*	NA
A/chicken/Egypt/AI00992/2019	H5N2	2019 Mar 3	Chicken, broiler	Beheira	5,000	15	HA (partial)	2.3.4.4b
A/duck/Egypt/AI00993/2019	H5, N8, N2	2019 Jan 14	Chicken, broiler	Giza	4,000	15	NA	NA
A/chicken/Egypt/AI00995/2019	H5, H9, N2	2019 Jan 14	Chicken, broiler	Beheira	33,000	33.3	NA	NA
A/chicken/Egypt/AI00996/2019	H5, H9, N2	2019 Jan 15	Chicken, broiler	Beheira	10,000	60	NA	NA
A/chicken/Egypt/AI00997/2019	H5, N8, H9, N2	2019 Mar 9	Chicken, broiler	Dakhalia	40,000	7.5	NA	NA

*HA, hemagglutinin; NA, not available.

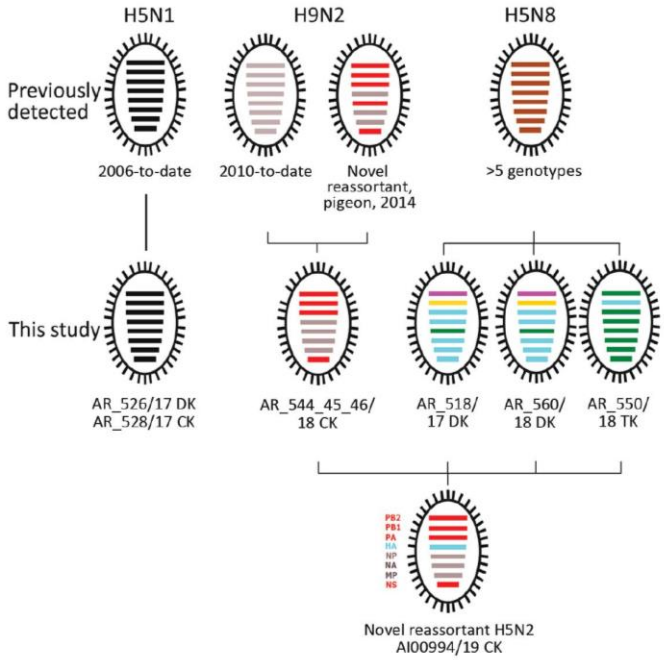

Figure 1. Genotype and reassortment analyses based on full-length genome sequences of avian influenza viruses in Egypt previously detected and those identified in this study. Colors indicate grouping of segment origin according to phylogenetic analyses (Appendix 1 Figure, https://wwwnc.cdc.gov/EID/article/26/1/19-0570-App1.pdf): highly pathogenic avian influenza (HPAI) H5N1 2.2.1.2 virus from Egypt (black); H9N2 subtype from Egypt circulating in chickens since 2010 (gray); H9N2 subtype from Egypt first detected in pigeons in 2014 (red); HPAI H5N8 viruses previously detected and circulating in Egypt (brown; different genotypes); Polymerase basic (PB) 2 segment most closely related to an H3N6 virus from Bangladesh (purple); PB1 segment most closely related to an H7N7 virus from Georgia (yellow); segments most closely related to H5N8 viruses from China (blue) or Russia (green).

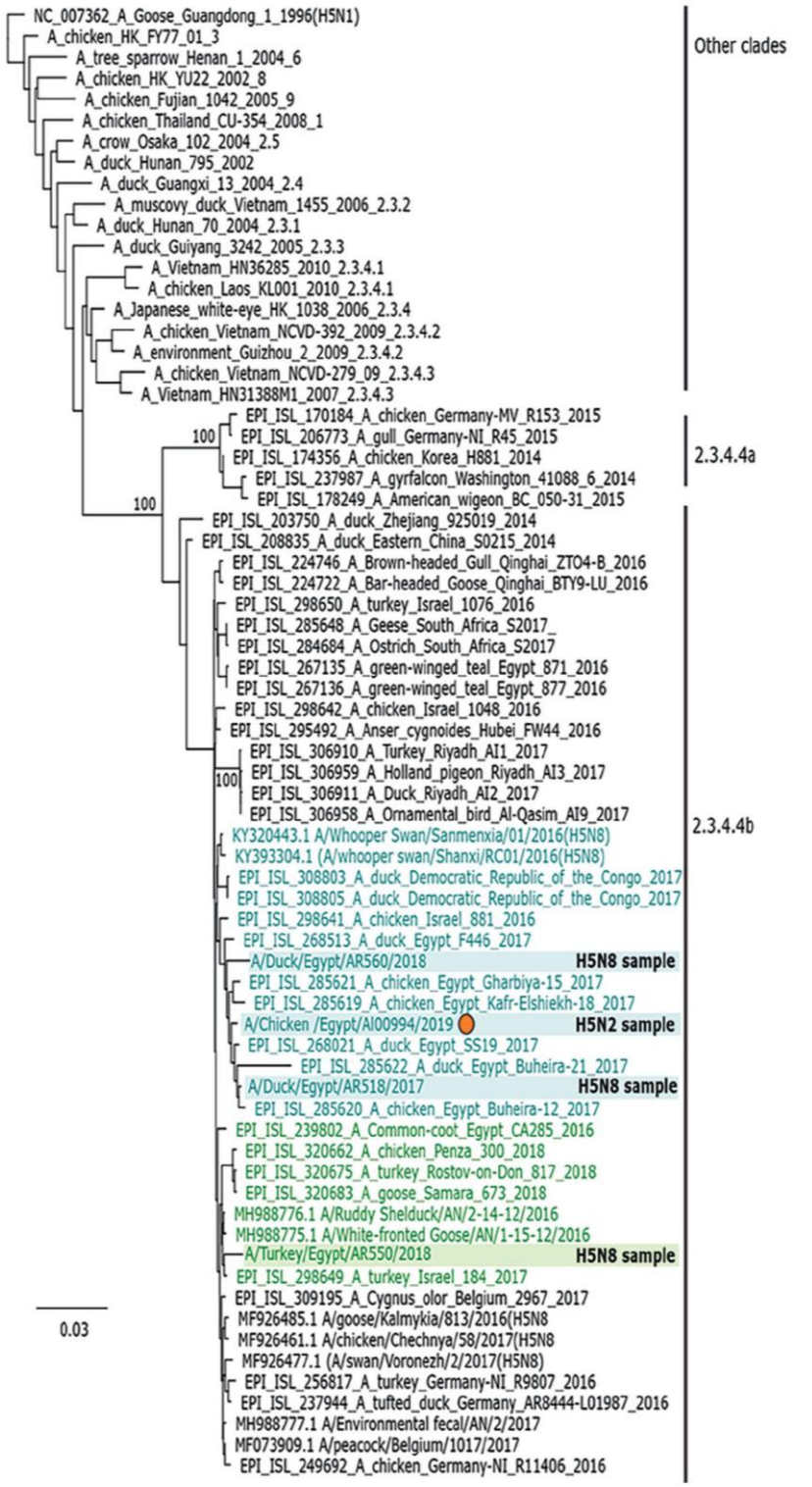

Figure 2. Phylogenetic analysis of the hemagglutinin segments of reassortant highly pathogenic avian influenza H5N2 and H5N8 viruses belonging to clade 2.3.3.4b from Egypt and reference viruses. Sequence analysis was based on alignment analyses by MAFFT version 7.450 embedded in the Geneious software suite, version 11.1.7 (https://www.geneious.com) with manual editing. We performed maximum-likelihood calculations using PhyML version 3.0 (http://www.atgc-montpellier.fr/phyml); we chose the best-fit model according to the Bayesian selection criterion using Model Finder embedded in Geneious. Colors indicate grouping of segment origin and match those shown in Figure 1: blue, most closely related to H5N8 viruses from China; green, most closely related to H5N8 viruses from Russia and Europe. GenBank or GISAID accession numbers (http://www.gisaid.org) are provided for reference sequences. Scale bar indicates nucleotide substitutions per site.

in Egypt during 2014 (*6*). The nucleoprotein segment and perhaps others were acquired from H9N2 viruses circulating in chickens in Egypt since 2010. Matrix and neuraminidase segments are identical in the pigeon and chicken H9N2 viruses. We identified no new mutations in the genome of reassortant H5N2 that would suggest increased adaptation to mammalian hosts. In addition, we observed 2 previously undescribed genotypes of HPAI H5N8 with distinct polymerase basic 1 and 2 segment origins (Figure 1). The composition of HPAI H5N1 viruses phylogenetically assigned to clade 2.2.1.2 (Figure 1; Appendix 1 Figure) was unaltered compared with other HPAI H5N1 viruses isolated since 2015.

Natural reassortants between H5 HPAI of the gs/GD lineage and H9N2 viruses, including subtype H5N2, have repeatedly emerged in Southeast Asia (*8*). So far, both the HPAI H5N1 2.2.1.2 and the co-circulating H9N2-G1 viruses appeared to be genotypically stable in poultry in Egypt. Successful forced reassortment of these viruses by co-cultivation and serum selection in embryonated chicken eggs ruled out a principal incompatibility between their genome segments (*5*); however, Naguib et al. did not rescue an H5N2 reassortant.

We and others have shown that HPAI H5 viruses of clade 2.3.4.4 have a high tendency to reassort with various influenza A viruses of wild birds or poultry (*9*). Thus, the incursion of clade 2.3.4.4b viruses into Egypt in 2016 not only added another antigenically distinct HPAI virus, but also signaled an increased reassortment risk. In fact, the 2.3.4.4b H5N8 virus proved to be a parent of the newly emerged H5N2 reassortant. Likewise, the second parental virus, an influenza A(H9N2) virus first detected in pigeons, was not described in Egypt before 2014. Genotypically, this H9N2 virus is distinct from the third parental virus, that is, the original H9N2 virus introduced to poultry in Egypt in 2010 (Figure 1). Infection of pigeons with clade 2.3.4.4b H5N8 HPAI virus has been described in Egypt, although pigeons are believed to be less susceptible to avian influenza infections (*10*). Although we cannot attribute the origin of the current HPAI H5N2 reassortant to a single host species, we cannot exclude pigeons as a possible host.

In March 2019, Egypt's Ministry of Agriculture announced the detection of a new influenza A(H5N2) virus from seemingly healthy ducks in the Dakahlia governorate (*11*); recently published information on this reassortant indicated the presence of a neuraminidase N2 segment of chicken H9N2 viruses in the background of an HPAI H5 clade 2.3.4.4b virus (*11*). Our data confirm the presence of a different H5N2 reassortant and its occurrence in chickens in different geographic regions of Egypt (Table 1). We detected the current reassortant HPAI H5N2 viruses in 2 different broiler farms in Beheira (January and March 2019) and 1 broiler farm in Fayoum (January 2019) governorates (Table 1). The HA amino acid sequence of these reassortants does not signal antigenic variation compared with parent HPAI H5 subtype of clade 2.3.4.4b. Antigenic and further phenotypic properties, such as host specificity, require investigation as soon as isolates are available. For the H5N2-positive samples, only FTA card material was available at Friedrich-Loeffler-Institut. However, H5N2 isolates were successfully generated at the Beni-Suef University, Egypt, but were currently not available for further antigenic and phenotypic analyses (M. El-Kady, unpub. data).

Intensified targeted surveillance in poultry and pigeons is urgently required and may lead to detection of additional reassortants. However, co-detection in a sample of H5N8 and H9N2 subtypes by reverse transcription quantitative PCR may blur the identification of H5N2 reassortants; plaque purification of such samples would aid in separating subtypes but cannot currently be used in routine diagnostics.

There is a risk for transboundary spread of HPAI A(H5N2) virus in northern Africa and the Middle East, and similar reassortment events are to be expected in regions where clade 2.3.4.4 HPAI and H9N2 viruses are co-circulating. Long-term solutions in combating avian influenza virus infections in poultry are sorely needed and would help to lower risks of human exposure to zoonotic avian influenza viruses such as the highly zoonotic H7N9 viruses in China that carry a full set of internal genes of an H9N2-G1–like avian influenza virus (*12*).

Acknowledgments

We acknowledge the originating and submitting laboratories that provided sequences available in the EpiFlu database (http://www.gisaid.org) (Appendix 2, https://wwwnc.cdc.gov/EID/26/1/19-0570-App2.xlsx).

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Supplemental Material

Appendix Table 1. Avian influenza sequences included in the study of highly pathogenic avian influenza virus A(H5N2), Egypt.

AIV designation	AIV subtype	GISAID Sequence Accession No.							
		PB2	PB1	PA	HA	NP	NA	M	NS
A/Chicken/Egypt/Al00994/2019	H5N2	EPI1420452	EPI1420451	EPI1420416	EPI14204383	EPI1420434	EPI1420450	EPI1420384	EPI1420397
A/Duck/Egypt/AR518/2017	H5N8	EPI1420351	EPI1420350	EPI1420349	EPI1381391	EPI1420346	EPI1420345	EPI1420344	EPI1420347
A/Duck/Egypt/AR560/2018	H5N8	EPI1420334	EPI1420333	EPI1420332	EPI1381402	EPI1420330	EPI1420329	EPI1420328	EPI1420331
A/Turkey/Egypt/AR550/2018	H5N8	EPI1420343	EPI1420342	EPI1420341	EPI1381395	EPI1420339	EPI1420338	EPI1420336	EPI1420340
A/Duck/Egypt/AR526/2017	H5N1	EPI1420366	EPI1420364	EPI1420363	EPI1381393	EPI1420358	EPI1420357	EPI1420355	EPI1420362
A/Chicken/Egypt/AR528/2017	H5N1	EPI1420377	EPI1420376	EPI1420375	EPI1381432	EPI1420372	EPI14203571	EPI1420369	EPI1420373
A/Chicken/Egypt/AR544/2018	H9N2	EPI1420308	EPI1420307	EPI1420306	EPI1381412	EPI1420304	EPI1381411	EPI142035301	EPI1420305
A/Chicken/Egypt/AR545/2018	H9N2	EPI1420327	EPI1420326	EPI1420325	EPI131413	EPI1420323	EPI14203322	EPI1420321	EPI1420324
A/Chicken/Egypt/AR546/2018	H9N2	EPI1420318	EPI1420317	EPI1420316	EPI1381414	EPI1420314	EPI1420313	EPI1420312	EPI1420315
A/Chicken/Egypt/AI00986/2019	**H5**N2				EPI1542792				
A/Chicken/Egypt/AI00987/2019	**H5**, H9, N2				EPI1542793				
A/Chicken/Egypt/AI00988/2019	**H5**, H9, N2				EPI1542794				
A/Chicken/Egypt/AI00989/2019	**H5**, H9, N2				EPI1542795				
A/Chicken/Egypt/AI00990/2019	**H5**, H9, N2				EPI1542796				
A/Chicken/Egypt/AI00992/2019	**H5**N2				EPI1542798				

*Bold text indicates the hemagglutinin subtype sequenced in samples in which >1 HA subtype was detected. AIV, Avian influenza virus; HA, hemagglutinin, M, matrix; NA, neuraminidase; NP, nucleoprotein; NSP, non-structural protein; PA, polymerase; PB, polymerase basic.


A
Influenza A virus (A/turkey/Wisconsin/1/1966(H9N2)
Influenza A virus (A/Chicken/Shanghai/F/98(H9N2)
Influenza A virus (A/Chicken/Beijing/1/94(H9N2)
Influenza A virus (A/duck/Hong Kong/Y439/1997(H9N2)
100
A/Duck/Egypt/AR526/2017
A/Chicken/Egypt/AR528/2017
H5N1 samples
Influenza A virus (A/duck/Giza/15292S/2015(H5N1)
Influenza A virus (A/chicken/Egypt/A10351A/2014(H5N1)
Influenza A virus (A/Egypt/MOH-NRC-8434/2014(H5N1)
Influenza A virus (A/chicken/Sharkeya/14209SS/2014(H5N1)
Influenza A virus (A/Egypt/MOH-NRC-7271/2014(H5N1)
Influenza A virus (A/Egypt/MOH-NRC-7305/2014(H5N1)
Influenza A virus (A/chicken/Italy/1875/2000(H7N1)
EPI_ISL_268511_A/Common-coot/Egypt/CA285/2016 (H5N8)
Influenza A virus (A/teal/Italy/3812/2005(H5N3)
Influenza A virus (A/spot-billed duck/Korea/KNU SYG06/2006(H5N3)
Influenza A virus (A/aquatic bird/Korea/w113/2006(H5N2)
Influenza A virus (A/chicken/Hubei/2856/2007(H5N1)
Influenza A virus (A/long-tailed shrike/Hong Kong/2762/2007(H5N1)
Influenza A virus (A/chicken/Hunan/3157/2006(H5N1)
Influenza A virus (A/bar-headed goose/Qinghai/F/2007(H5N1)
Influenza A virus (A/lesser kestrel/Heilongjiang/194/2007(H5N1)
Influenza A virus (A/duck/France/150233/2015(H5N2)
Influenza A virus (A/mallard duck/Georgia/11/2011(H1N1)
Influenza A virus (A/shelduck/Yasnopolyanske/4-08-02/2011(H1N1)
Influenza A virus (A/white-fronted goose/Netherlands/5/2011(H6N8)
Influenza A virus (A/turkey/Poland/08/2014(H9N2)
Influenza A virus (A/duck/France/150236/2015(H5N9)
Influenza A virus (A/Mallard/Netherlands/21/2013(H3N2)
Influenza A virus (A/chicken/France/150169a/2015(H5N1)
Influenza A virus (A/Mallard/Netherlands/33/2015(H4N6)
100
Influenza A virus (A/mallard/France/090360/2009(H9N2)
Influenza A virus (A/duck/Moscow/4652/2011(H4N6)
Influenza A virus (A/mallard/Republic of Georgia/13/2011(H6N2)
Influenza A virus (A/common Teal/Republic of Georgia/1/2011(H3N8)
Influenza A virus (A/black-headed gull/Netherlands/21/2014(H11N1)
Influenza A virus (A/mallard/Netherlands/29/2013(H3N8)
Influenza A virus (A/pigeon/Egypt/S10409A/2014(H9N2)
Influenza A virus (A/pigeon/Egypt/S10408B/2014(H9N2)
A/Chicken/Egypt/AR544/2018
A/Chicken /Egypt/AI00994/2019
H5N2 and H9N2 samples
A/Chicken/Egypt/AR546/2018
A/Chicken/Egypt/AR545/2018
Influenza A virus (A/chicken/Egypt/ABD1/2015(H9N2)
Influenza A virus (A/spot-billed duck/Shanghai/SH12/2014(H4N6)
Influenza A virus (A/duck/Zhejiang/6D10/2013(H2N8)
Influenza A virus (A/duck/Bangladesh/26980/2015(H7N9)
EPI_ISL_267135_A/green-winged_teal/Egypt/871/2016 (H5N8)
EPI_ISL_267136_A/green-winged_teal/Egypt/877/2016 (H5N8)
Influenza A virus (A/whooper swan/Shanxi/RC01/2016(H5N8)
Influenza A virus (A/duck/Democratic Republic of the Congo/17RS882-33/2017(H5N8)
Influenza A virus (A/Muscovy duck/Democratic Republic of the Congo/TCH4_PB2/2017(H5N8)
Influenza A virus (A/chicken/Uganda/17RS115-15/2017(H5N8)
Influenza A virus (A/white_winged_black_tern/Uganda/17RS115-3/2017(H5N8)
Influenza A virus (A/duck/Mongolia/521/2015(H3N8)
Influenza A virus (A/duck/Mongolia/30/2015(H3N8)
Influenza A virus (A/duck/Mongolia/482/2015(H2N3)
Influenza A virus (A/duck/Bangladesh/25891/2015(H4N6)
Influenza A virus (A/duck/Bangladesh/26918/2015(H3N6)
Influenza A virus (A/duck/Bangladesh/33137/2017(H3N2)
EPI_ISL_285622_A/duck/Egypt/Buheira-21/2017 (H5N8)
EPI_ISL_268021_A/duck/Egypt/SS19/2017 (H5N8)
EPI_ISL_285620_A/chicken/Egypt/Buheira-12/2017 (H5N8)
A/Duck/Egypt/AR518/2017
A/Duck/Egypt/AR560/2018
H5N8 samples
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Influenza A virus (A/chicken/Chechnya/58/2017(H5N8)
Influenza A virus (A/swan/Krasnodar/44/2017(H5N8)
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Influenza A virus (A/jungle crow/Hyogo/2803E011/2018(H5N6)
Influenza A virus (A/goose/Kalmykia/813/2016(H5N8)
Influenza A virus (A/Brahma chicken/Belgium/6153/2017(H5N8)
Influenza A virus (A/Guinea fowl/Belgium/6102/2017(H5N8)
Influenza A virus (A/swan/Voronezh/2/2017(H5N8)
Influenza A virus (A/Ruddy Shelduck/AN/2-14-12/2016(H5N8)
A/Turkey/Egypt/AR550/2018
H5N8 sample
Influenza A virus (A/White-fronted Goose/AN/1-15-12/2016(H5N8)
EPI_ISL_268513_A/duck/Egypt/F446/2017 (H5N8)
EPI_ISL_285621_A/chicken/Egypt/Gharbiya-15/2017 (H5N8)
0.03


B
(A/turkey/Wisconsin/1/1966(H9N2)
(A/Chicken/Beijing/1/94(H9N2)
(A/duck/Hong Kong/Y439/1997(H9N2)
(A/Chicken/Shanghai/F/98(H9N2)
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A/Chicken/Egypt/AR528/2017
H5N1 samples
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(A/Egypt/MOH-NRC-7305/2014(H5N1)
(A/duck/France/05057b/2005(H5N2)
(A/mallard duck/Netherlands/63/2006(H5N2)
(A/mallard duck/Netherlands/34/2006(H3N8)
(A/mallard/Sweden/5814/2005(H4N6)
(A/black-headed gull/Netherlands/1/2008(H1N3)
(A/mallard/Sweden/99785/2009(H9N2)
(A/mallard duck/Netherlands/86/2008(H7N1)
(A/bewicks swan/Netherlands/6/2006(H1N1)
(A/greater white-fronted goose/Netherlands/1/2007(H1N1)
(A/mallard duck/Netherlands/35/2015(H4N6)
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H5N2 and H9N2 samples
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A/Turkey/Egypt/AR550/2018
H5N8 sample
100
EPI_ISL_267136_A/green-winged_teal/Egypt/877/2016 (H5N8)
EPI_ISL_267135_A/green-winged_teal/Egypt/871/2016(H5N8)
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(A/Anser cygnoides/Hubei/FW44/2016(H5N8)
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(A/Environmental Fecal/AN/2/2017(H5N8)
(A/swan/Voronezh/2/2017(H5N8)
EPI_ISL_268511_A/Common-coot/Egypt/CA285/2016 (H5N8)
EPI_ISL_285619_A/chicken/Egypt/Kafr-Elshiekh-18/2017 (H5N8)
EPI_ISL_268513_A/duck/Egypt/F446/2017 (H5N8)
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(A/Ruddy Shelduck/AN/2-14-12/2016(H5N8)
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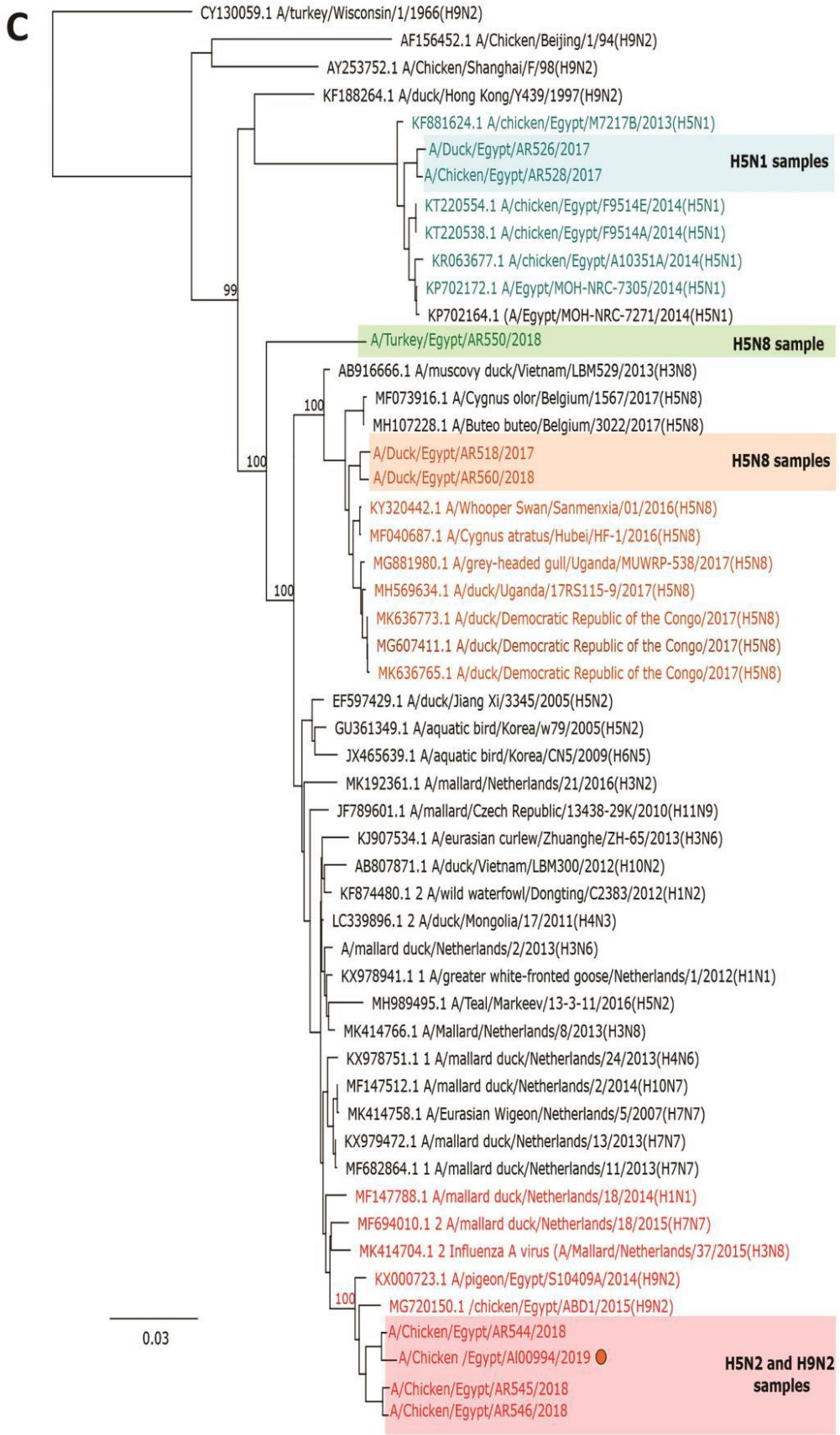
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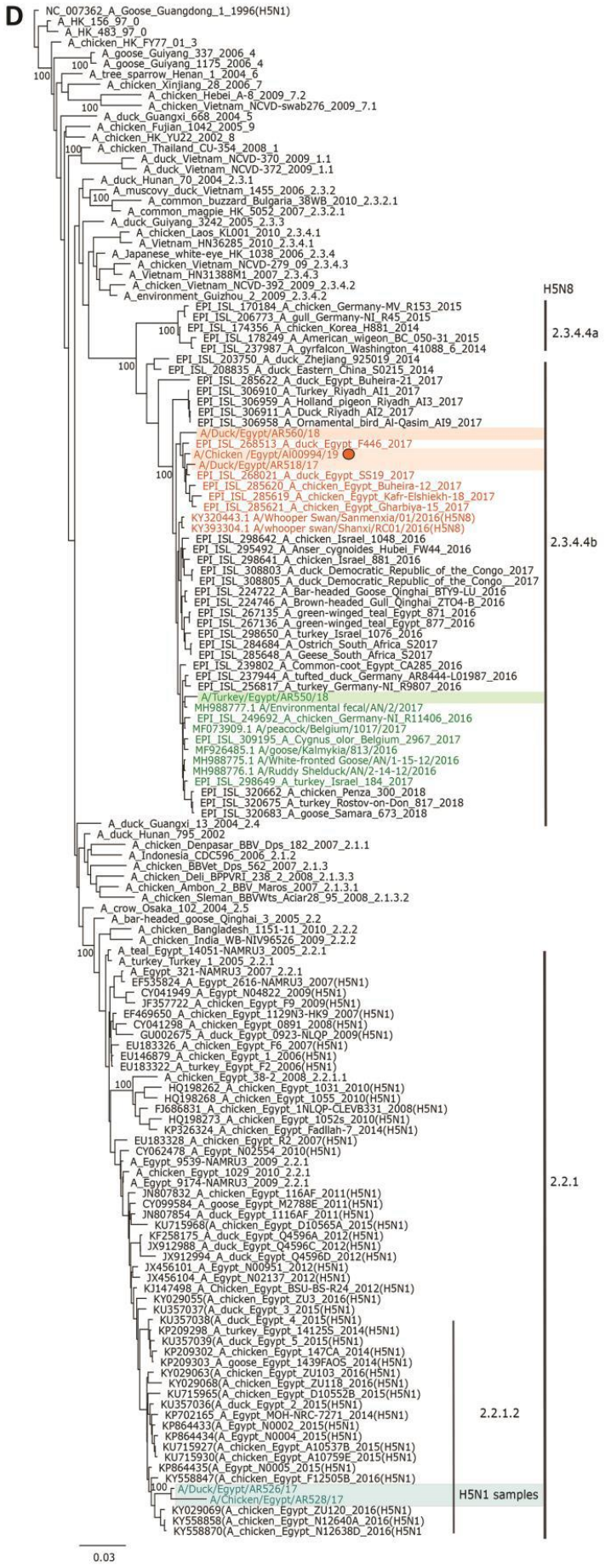
AY253752.1 A/Chicken/Shanghai/F/98(H9N2)
KF188264.1 A/duck/Hong Kong/Y439/1997(H9N2)
KF881624.1 A/chicken/Egypt/M7217B/2013(H5N1)
A/Duck/Egypt/AR526/2017
A/Chicken/Egypt/AR528/2017
H5N1 samples
KT220554.1 A/chicken/Egypt/F9514E/2014(H5N1)
KT220538.1 A/chicken/Egypt/F9514A/2014(H5N1)
KR063677.1 A/chicken/Egypt/A10351A/2014(H5N1)
KP702172.1 A/Egypt/MOH-NRC-7305/2014(H5N1)
KP702164.1 (A/Egypt/MOH-NRC-7271/2014(H5N1)
99
A/Turkey/Egypt/AR550/2018
H5N8 sample
AB916666.1 A/muscovy duck/Vietnam/LBM529/2013(H3N8)
MF073916.1 A/Cygnus olor/Belgium/1567/2017(H5N8)
MH107228.1 A/Buteo buteo/Belgium/3022/2017(H5N8)
100
100
A/Duck/Egypt/AR518/2017
A/Duck/Egypt/AR560/2018
H5N8 samples
KY320442.1 A/Whooper Swan/Sanmenxia/01/2016(H5N8)
MF040687.1 A/Cygnus atratus/Hubei/HF-1/2016(H5N8)
MG881980.1 A/grey-headed gull/Uganda/MUWRP-538/2017(H5N8)
100
MH569634.1 A/duck/Uganda/17RS115-9/2017(H5N8)
MK636773.1 A/duck/Democratic Republic of the Congo/2017(H5N8)
MG607411.1 A/duck/Democratic Republic of the Congo/2017(H5N8)
MK636765.1 A/duck/Democratic Republic of the Congo/2017(H5N8)
EF597429.1 A/duck/Jiang Xi/3345/2005(H5N2)
GU361349.1 A/aquatic bird/Korea/w79/2005(H5N2)
JX465639.1 A/aquatic bird/Korea/CN5/2009(H6N5)
MK192361.1 A/mallard/Netherlands/21/2016(H3N2)
JF789601.1 A/mallard/Czech Republic/13438-29K/2010(H11N9)
KJ907534.1 A/eurasian curlew/Zhuanghe/ZH-65/2013(H3N6)
AB807871.1 A/duck/Vietnam/LBM300/2012(H10N2)
KF874480.1 2 A/wild waterfowl/Dongting/C2383/2012(H1N2)
LC339896.1 2 A/duck/Mongolia/17/2011(H4N3)
A/mallard duck/Netherlands/2/2013(H3N6)
KX978941.1 1 A/greater white-fronted goose/Netherlands/1/2012(H1N1)
MH989495.1 A/Teal/Markeev/13-3-11/2016(H5N2)
MK414766.1 A/Mallard/Netherlands/8/2013(H3N8)
KX978751.1 1 A/mallard duck/Netherlands/24/2013(H4N6)
MF147512.1 A/mallard duck/Netherlands/2/2014(H10N7)
MK414758.1 A/Eurasian Wigeon/Netherlands/5/2007(H7N7)
KX979472.1 A/mallard duck/Netherlands/13/2013(H7N7)
MF682864.1 1 A/mallard duck/Netherlands/11/2013(H7N7)
MF147788.1 A/mallard duck/Netherlands/18/2014(H1N1)
MF694010.1 2 A/mallard duck/Netherlands/18/2015(H7N7)
MK414704.1 2 Influenza A virus (A/Mallard/Netherlands/37/2015(H3N8)
KX000723.1 A/pigeon/Egypt/S10409A/2014(H9N2)
100
MG720150.1 /chicken/Egypt/ABD1/2015(H9N2)
A/Chicken/Egypt/AR544/2018
A/Chicken /Egypt/AI00994/2019
A/Chicken/Egypt/AR545/2018
A/Chicken/Egypt/AR546/2018
H5N2 and H9N2 samples
0.03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A_chicken_HK_FY77_01_3
A_goose_Guiyang_337_2006_4
A_goose_Guiyang_1175_2006_4
A_tree_sparrow_Henan_1_2004_6
A_chicken_Xinjiang_28_2006_7
A_chicken_Hebei_A-8_2009_7.2
A_chicken_Vietnam_NCVD-swab276_2009_7.1
A_duck_Guangxi_668_2004_5
A_chicken_Fujian_1042_2005_9
A_chicken_HK_YU22_2002_8
A_chicken_Thailand_CU-354_2008_1
A_duck_Vietnam_NCVD-370_2009_1.1
A_duck_Vietnam_NCVD-372_2009_1.1
A_duck_Hunan_70_2004_2.3.1
A_muscovy_duck_Vietnam_1455_2006_2.3.2
A_common_buzzard_Bulgaria_38WB_2010_2.3.2.1
A_common_magpie_HK_5052_2007_2.3.2.1
A_duck_Guiyang_3242_2005_2.3.3
A_chicken_Laos_KL001_2010_2.3.4.1
A_Vietnam_HN36285_2010_2.3.4.1
A_Japanese_white-eye_HK_1038_2006_2.3.4
A_chicken_Vietnam_NCVD-279_09_2.3.4.3
A_Vietnam_HN31388M1_2007_2.3.4.3
A_chicken_Vietnam_NCVD-392_2009_2.3.4.2
A_environment_Guizhou_2_2009_2.3.4.2
EPI_ISL_170184_A_chicken_Germany-MV_R153_2015
EPI_ISL_206773_A_gull_Germany-NI_R45_2015
EPI_ISL_174356_A_chicken_Korea_H881_2014
EPI_ISL_178249_A_American_wigeon_BC_050-31_2015
EPI_ISL_237987_A_gyrfalcon_Washington_41088_6_2014
EPI_ISL_203750_A_duck_Zhejiang_925019_2014
EPI_ISL_208835_A_duck_Eastern_China_S0215_2014
EPI_ISL_285622_A_duck_Egypt_Buheira-21_2017
EPI_ISL_306910_A_Turkey_Riyadh_AI1_2017
EPI_ISL_306959_A_Holland_pigeon_Riyadh_AI3_2017
EPI_ISL_306911_A_Duck_Riyadh_AI2_2017
EPI_ISL_306958_A_Ornamental_bird_Al-Qasim_AI9_2017
A/Duck/Egypt/AR550/18
EPI_ISL_268513_A_duck_Egypt_F446_2017
A/Chicken /Egypt/AI00994/19
A/Duck/Egypt/AR518/17
EPI_ISL_268021_A_duck_Egypt_SS19_2017
EPI_ISL_285620_A_chicken_Egypt_Buheira-12_2017
EPI_ISL_285619_A_chicken_Egypt_Kafr-Elshiekh-18_2017
EPI_ISL_285621_A_chicken_Egypt_Gharbiya-15_2017
KY320443.1 A/Whooper Swan/Sanmenxia/01/2016(H5N8)
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EPI_ISL_298642_A_chicken_Israel_1048_2016
EPI_ISL_295492_A_Anser_cygnoides_Hubei_FW44_2016
EPI_ISL_298641_A_chicken_Israel_881_2016
EPI_ISL_308803_A_duck_Democratic_Republic_of_the_Congo_2017
EPI_ISL_308805_A_duck_Democratic_Republic_of_the_Congo__2017
EPI_ISL_224722_A_Bar-headed_Goose_Qinghai_BTY9-LU_2016
EPI_ISL_224746_A_Brown-headed_Gull_Qinghai_ZTO4-B_2016
EPI_ISL_267135_A_green-winged_teal_Egypt_871_2016
EPI_ISL_267136_A_green-winged_teal_Egypt_877_2016
EPI_ISL_298650_A_turkey_Israel_1076_2016
EPI_ISL_284684_A_Ostrich_South_Africa_S2017
EPI_ISL_285648_A_Geese_South_Africa_S2017
EPI_ISL_239802_A_Common-coot_Egypt_CA285_2016
EPI_ISL_237944_A_tufted_duck_Germany_AR8444-L01987_2016
EPI_ISL_256817_A_turkey_Germany-NI_R9807_2016
A/Turkey/Egypt/AR550/18
MH988777.1 A/Environmental fecal/AN/2/2017
EPI_ISL_249692_A_chicken_Germany-NI_R11406_2016
MF073909.1 A/peacock/Belgium/1017/2017
EPI_ISL_309195_A_Cygnus_olor_Belgium_2967_2017
MF926485.1 A/goose/Kalmykia/813/2016
MH988775.1 A/White-fronted Goose/AN/1-15-12/2016
MH988776.1 A/Ruddy Shelduck/AN/2-14-12/2016
EPI_ISL_298649_A_turkey_Israel_184_2017
EPI_ISL_320662_A_chicken_Penza_300_2018
EPI_ISL_320675_A_turkey_Rostov-on-Don_817_2018
EPI_ISL_320683_A_goose_Samara_673_2018
A_duck_Guangxi_13_2004_2.4
A_duck_Hunan_795_2002
A_chicken_Denpasar_BBV_Dps_182_2007_2.1.1
A_Indonesia_CDC596_2006_2.1.2
A_chicken_BBVet_Dps_562_2007_2.1.3
A_chicken_Deli_BPPVRI_238_2_2008_2.1.3.3
A_chicken_Ambon_2_BBV_Maros_2007_2.1.3.1
A_chicken_Sleman_BBVWts_Aciar28_95_2008_2.1.3.2
A_crow_Osaka_102_2004_2.5
A_bar-headed_goose_Qinghai_3_2005_2.2
A_chicken_Bangladesh_1151-11_2010_2.2.2
A_chicken_India_WB-NIV96526_2009_2.2.2
A_teal_Egypt_14051-NAMRU3_2005_2.2.1
A_turkey_Turkey_1_2005_2.2.1
A_Egypt_321-NAMRU3_2007_2.2.1
EF535824_A_Egypt_2616-NAMRU3_2007(H5N1)
CY041949_A_Egypt_N04822_2009(H5N1)
JF357722_A_chicken_Egypt_F9_2009(H5N1)
EF469650_A_chicken_Egypt_1129N3-HK9_2007(H5N1)
CY041298_A_chicken_Egypt_0891_2008(H5N1)
GU002675_A_duck_Egypt_0923-NLQP_2009(H5N1)
EU183326_A_chicken_Egypt_F6_2007(H5N1)
EU146879_A_chicken_Egypt_1_2006(H5N1)
EU183322_A_turkey_Egypt_F2_2006(H5N1)
A_chicken_Egypt_38-2_2008_2.2.1.1
HQ198262_A_chicken_Egypt_1031_2010(H5N1)
HQ198268_A_chicken_Egypt_1055_2010(H5N1)
FJ686831_A_chicken_Egypt_1NLQP-CLEVB331_2008(H5N1)
HQ198273_A_chicken_Egypt_1052s_2010(H5N1)
KP326324_A_chicken_Egypt_Fadllah-7_2014(H5N1)
EU183328_A_chicken_Egypt_R2_2007(H5N1)
CY062478_A_Egypt_N02554_2010(H5N1)
A_Egypt_9539-NAMRU3_2009_2.2.1
A_chicken_Egypt_1029_2010_2.2.1
A_Egypt_9174-NAMRU3_2009_2.2.1
JN807832_A_chicken_Egypt_116AF_2011(H5N1)
CY099584_A_goose_Egypt_M2788E_2011(H5N1)
JN807854_A_duck_Egypt_1116AF_2011(H5N1)
KU715968(A_chicken_Egypt_D10565A_2015(H5N1)
KF258175_A_duck_Egypt_Q4596A_2012(H5N1)
JX912988_A_duck_Egypt_Q4596C_2012(H5N1)
JX912994_A_duck_Egypt_Q4596D_2012(H5N1)
JX456101_A_Egypt_N00951_2012(H5N1)
JX456104_A_Egypt_N02137_2012(H5N1)
KJ147498_A_Chicken_Egypt_BSU-BS-R24_2012(H5N1)
KY029055(A_chicken_Egypt_ZU3_2016(H5N1)
KU357037(A_duck_Egypt_3_2015(H5N1)
KU357038(A_duck_Egypt_4_2015(H5N1)
KP209298_A_turkey_Egypt_14125S_2014(H5N1)
KU357039(A_duck_Egypt_5_2015(H5N1)
KP209302_A_chicken_Egypt_147CA_2014(H5N1)
KP209303_A_goose_Egypt_1439FAOS_2014(H5N1)
KY029063(A_chicken_Egypt_ZU103_2016(H5N1)
KY029068(A_chicken_Egypt_ZU118_2016(H5N1)
KU715965(A_chicken_Egypt_D10552B_2015(H5N1)
KU357036(A_duck_Egypt_2_2015(H5N1)
KP702165_A_Egypt_MOH-NRC-7271_2014(H5N1)
KP864433(A_Egypt_N0002_2015(H5N1)
KP864434(A_Egypt_N0004_2015(H5N1)
KU715927(A_chicken_Egypt_A10537B_2015(H5N1)
KU715930(A_chicken_Egypt_A10759E_2015(H5N1)
KP864435(A_Egypt_N0005_2015(H5N1)
KY558847(A_chicken_Egypt_F12505B_2016(H5N1)
A/Duck/Egypt/AR526/17
A/Chicken/Egypt/AR528/17
KY029069(A_chicken_Egypt_ZU120_2016(H5N1)
KY558858(A_chicken_Egypt_N12640A_2016(H5N1)
KY558870(A_chicken_Egypt_N12638D_2016(H5N1
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2.3.4.4b
2.2.1
2.2.1.2
H5N1 samples
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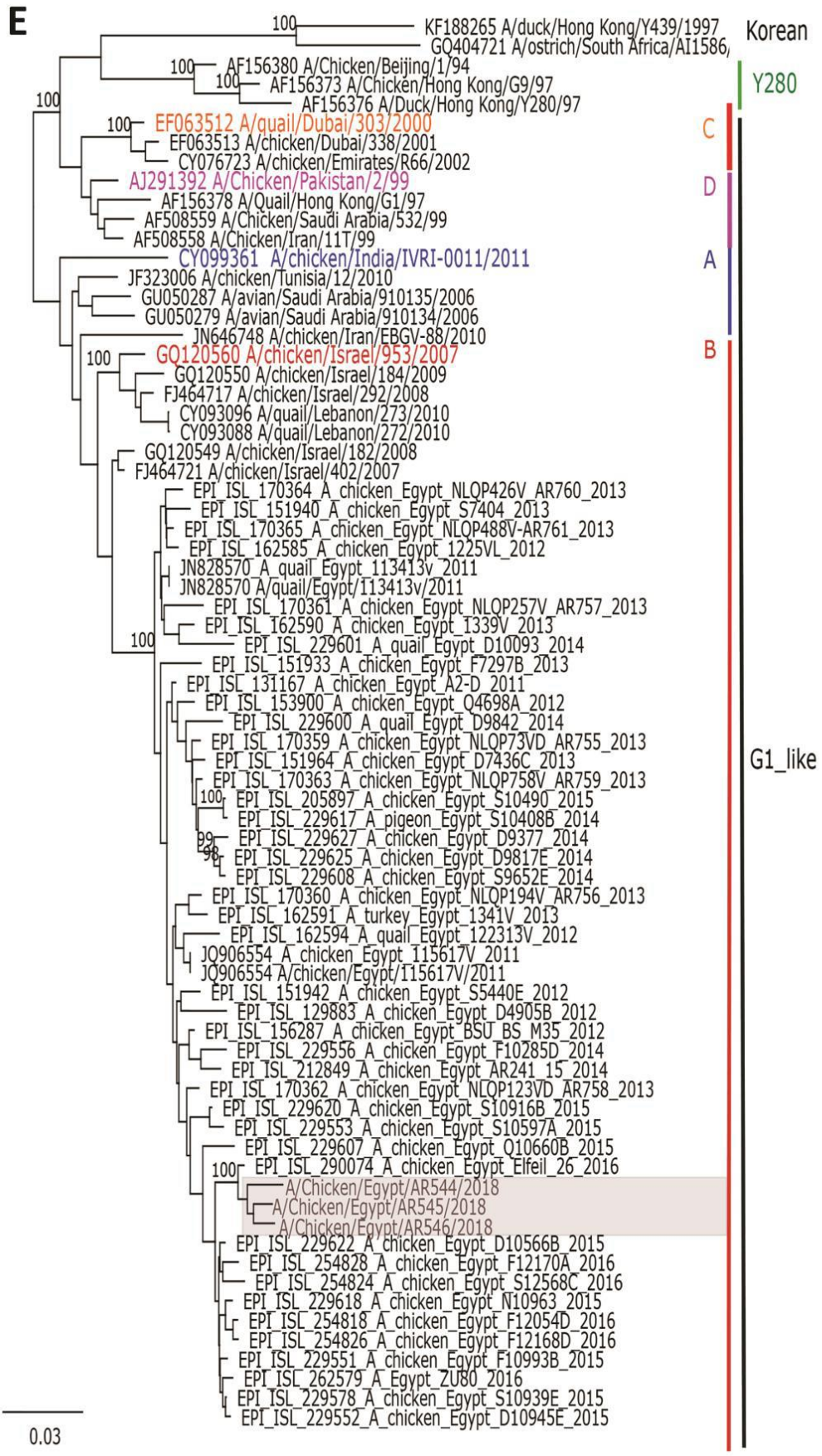
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GQ404721 A/ostrich/South Africa/AI1586/
Korean
AF156380 A/Chicken/Beijing/1/94
AF156373 A/Chicken/Hong Kong/G9/97
AF156376 A/Duck/Hong Kong/Y280/97
Y280
EF063512 A/quail/Dubai/303/2000
EF063513 A/chicken/Dubai/338/2001
CY076723 A/chicken/Emirates/R66/2002
C
AJ291392 A/Chicken/Pakistan/2/99
AF156378 A/Quail/Hong Kong/G1/97
AF508559 A/Chicken/Saudi Arabia/532/99
AF508558 A/Chicken/Iran/11T/99
D
CY099361 A/chicken/India/IVRI-0011/2011
JF323006 A/chicken/Tunisia/12/2010
GU050287 A/avian/Saudi Arabia/910135/2006
GU050279 A/avian/Saudi Arabia/910134/2006
JN646748 A/chicken/Iran/EBGV-88/2010
A
GQ120560 A/chicken/Israel/953/2007
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FJ464717 A/chicken/Israel/292/2008
CY093096 A/quail/Lebanon/273/2010
CY093088 A/quail/Lebanon/272/2010
GQ120549 A/chicken/Israel/182/2008
FJ464721 A/chicken/Israel/402/2007
B
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EPI_ISL_170365_A_chicken_Egypt_NLQP488V-AR761_2013
EPI_ISL_162585_A_chicken_Egypt_1225VL_2012
JN828570_A_quail_Egypt_113413v_2011
JN828570 A/quail/Egypt/113413v/2011
EPI_ISL_170361_A_chicken_Egypt_NLQP257V_AR757_2013
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EPI_ISL_229601_A_quail_Egypt_D10093_2014
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EPI_ISL_131167_A_chicken_Egypt_A2-D_2011
EPI_ISL_153900_A_chicken_Egypt_Q4698A_2012
EPI_ISL_229600_A_quail_Egypt_D9842_2014
EPI_ISL_170359_A_chicken_Egypt_NLQP73VD_AR755_2013
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EPI_ISL_229627_A_chicken_Egypt_D9377_2014
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EPI_ISL_162591_A_turkey_Egypt_1341V_2013
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EPI_ISL_129883_A_chicken_Egypt_D4905B_2012
EPI_ISL_156287_A_chicken_Egypt_BSU_BS_M35_2012
EPI_ISL_229556_A_chicken_Egypt_F10285D_2014
EPI_ISL_212849_A_chicken_Egypt_AR241_15_2014
EPI_ISL_170362_A_chicken_Egypt_NLQP123VD_AR758_2013
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EPI_ISL_229607_A_chicken_Egypt_Q10660B_2015
EPI_ISL_290074_A_chicken_Egypt_Elfeil_26_2016
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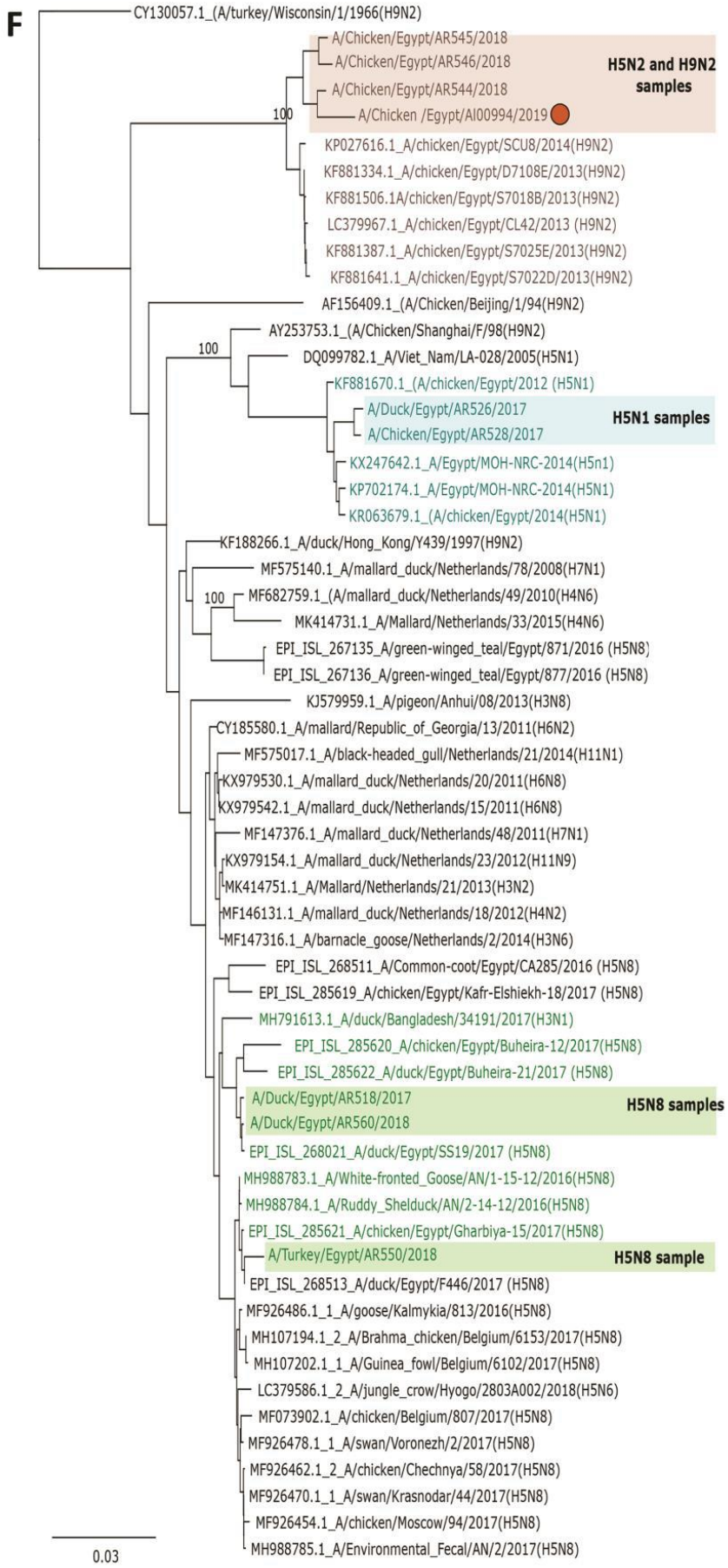
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A/Chicken /Egypt/AI00994/2019
H5N2 and H9N2 samples
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KF881506.1A/chicken/Egypt/S7018B/2013(H9N2)
LC379967.1_A/chicken/Egypt/CL42/2013 (H9N2)
KF881387.1_A/chicken/Egypt/S7025E/2013(H9N2)
KF881641.1_A/chicken/Egypt/S7022D/2013(H9N2)
AF156409.1_(A/Chicken/Beijing/1/94(H9N2)
AY253753.1_(A/Chicken/Shanghai/F/98(H9N2)
100
DQ099782.1_A/Viet_Nam/LA-028/2005(H5N1)
KF881670.1_(A/chicken/Egypt/2012 (H5N1)
A/Duck/Egypt/AR526/2017
A/Chicken/Egypt/AR528/2017
H5N1 samples
KX247642.1_A/Egypt/MOH-NRC-2014(H5n1)
KP702174.1_A/Egypt/MOH-NRC-2014(H5N1)
KR063679.1_(A/chicken/Egypt/2014(H5N1)
KF188266.1_A/duck/Hong_Kong/Y439/1997(H9N2)
MF575140.1_A/mallard_duck/Netherlands/78/2008(H7N1)
100
MF682759.1_(A/mallard_duck/Netherlands/49/2010(H4N6)
MK414731.1_A/Mallard/Netherlands/33/2015(H4N6)
EPI_ISL_267135_A/green-winged_teal/Egypt/871/2016 (H5N8)
EPI_ISL_267136_A/green-winged_teal/Egypt/877/2016 (H5N8)
KJ579959.1_A/pigeon/Anhui/08/2013(H3N8)
CY185580.1_A/mallard/Republic_of_Georgia/13/2011(H6N2)
MF575017.1_A/black-headed_gull/Netherlands/21/2014(H11N1)
KX979530.1_A/mallard_duck/Netherlands/20/2011(H6N8)
KX979542.1_A/mallard_duck/Netherlands/15/2011(H6N8)
MF147376.1_A/mallard_duck/Netherlands/48/2011(H7N1)
KX979154.1_A/mallard_duck/Netherlands/23/2012(H11N9)
MK414751.1_A/Mallard/Netherlands/21/2013(H3N2)
MF146131.1_A/mallard_duck/Netherlands/18/2012(H4N2)
MF147316.1_A/barnacle_goose/Netherlands/2/2014(H3N6)
EPI_ISL_268511_A/Common-coot/Egypt/CA285/2016 (H5N8)
EPI_ISL_285619_A/chicken/Egypt/Kafr-Elshiekh-18/2017 (H5N8)
MH791613.1_A/duck/Bangladesh/34191/2017(H3N1)
EPI_ISL_285620_A/chicken/Egypt/Buheira-12/2017(H5N8)
EPI_ISL_285622_A/duck/Egypt/Buheira-21/2017 (H5N8)
A/Duck/Egypt/AR518/2017
A/Duck/Egypt/AR560/2018
H5N8 samples
EPI_ISL_268021_A/duck/Egypt/SS19/2017 (H5N8)
MH988783.1_A/White-fronted_Goose/AN/1-15-12/2016(H5N8)
MH988784.1_A/Ruddy_Shelduck/AN/2-14-12/2016(H5N8)
EPI_ISL_285621_A/chicken/Egypt/Gharbiya-15/2017(H5N8)
A/Turkey/Egypt/AR550/2018
H5N8 sample
EPI_ISL_268513_A/duck/Egypt/F446/2017 (H5N8)
MF926486.1_1_A/goose/Kalmykia/813/2016(H5N8)
MH107194.1_2_A/Brahma_chicken/Belgium/6153/2017(H5N8)
MH107202.1_1_A/Guinea_fowl/Belgium/6102/2017(H5N8)
LC379586.1_2_A/jungle_crow/Hyogo/2803A002/2018(H5N6)
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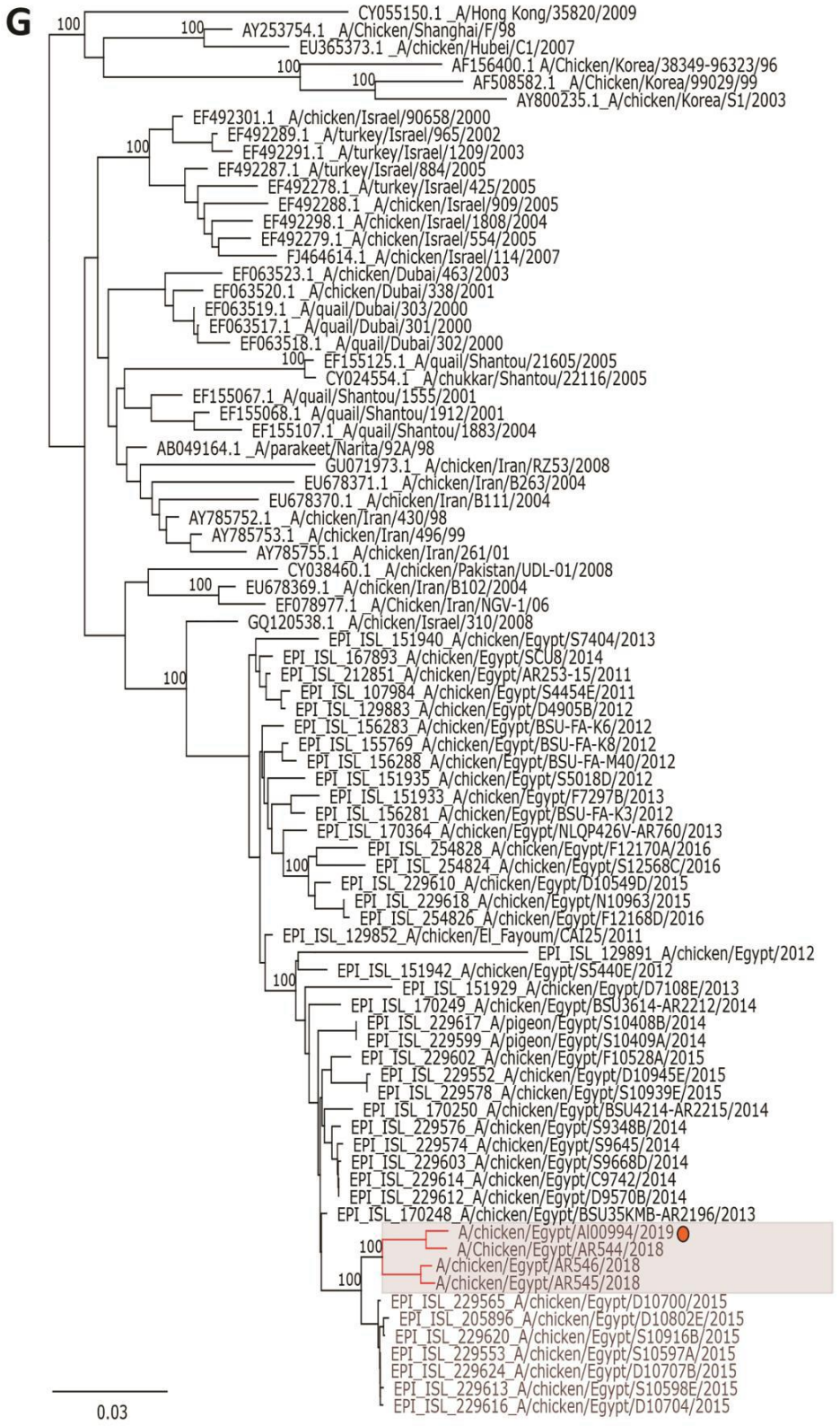
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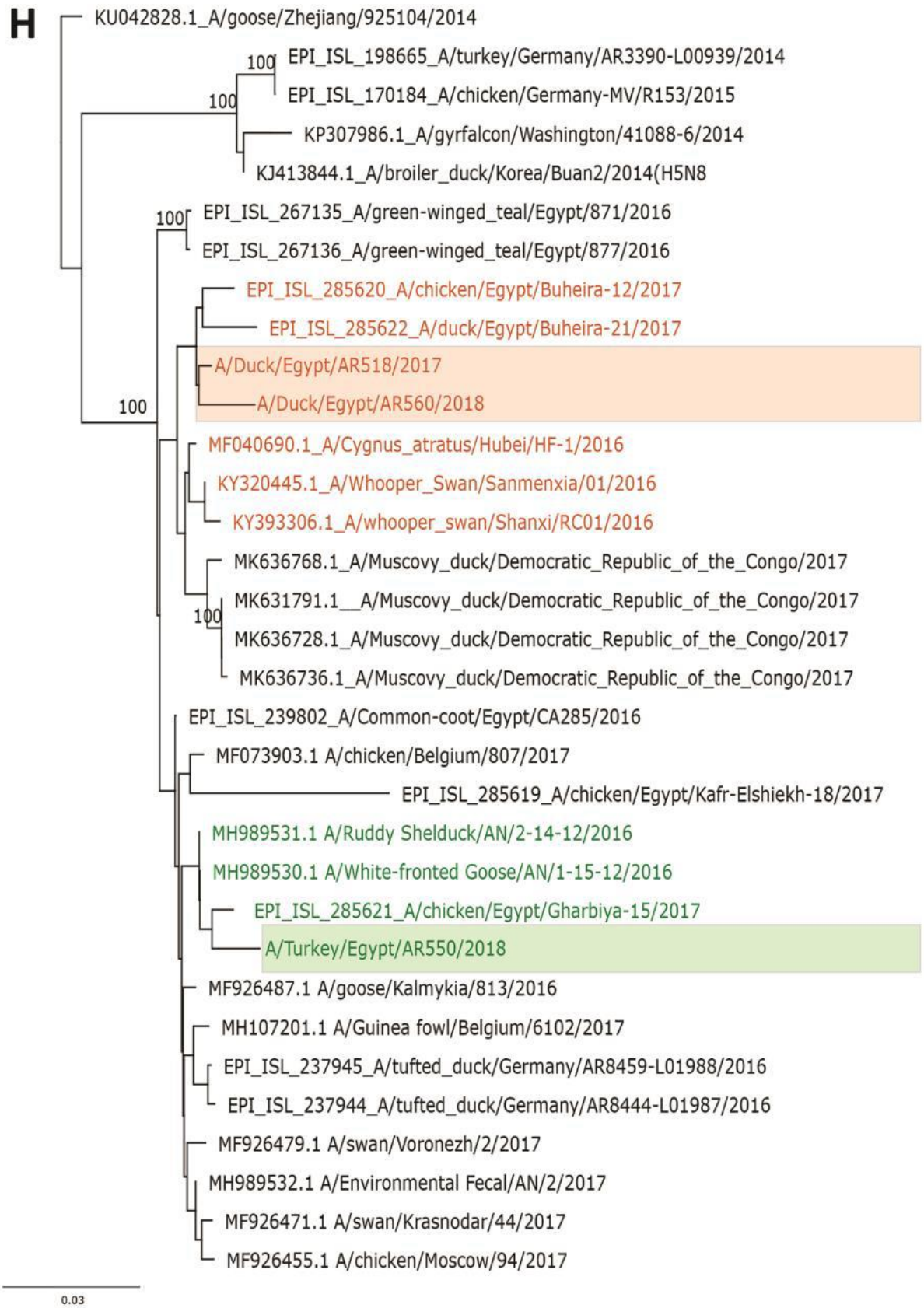
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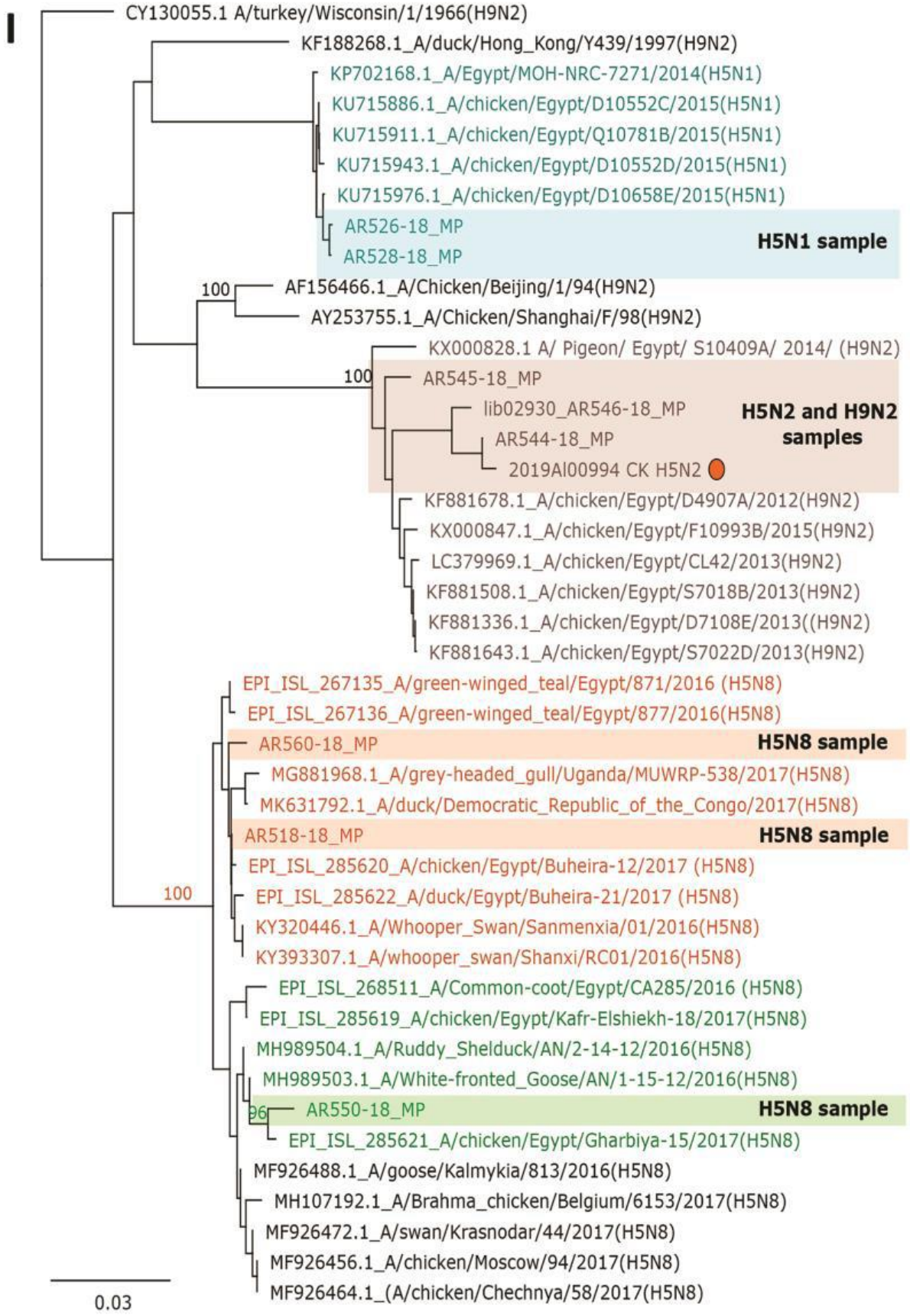
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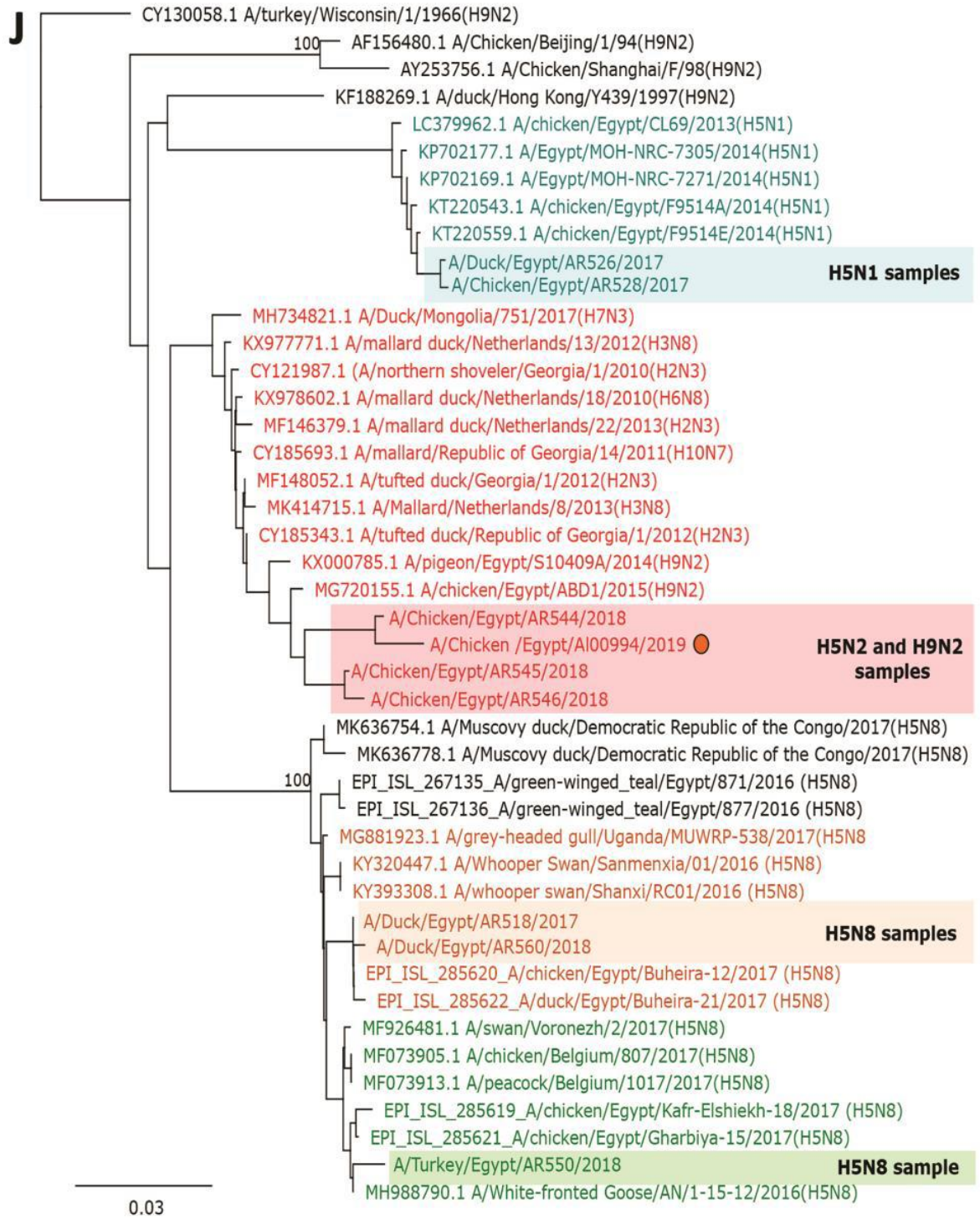
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H5N8 sample
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Appendix Figure. Phylogenetic analysis of segments of highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI). Sequence analysis was based on alignment analyses by MAFFTv7.450 embedded in the Geneious software suite, version 11.1.7 (https://www.geneious.com) with manual editing. We performed maximum likelihood calculations using PhyML version 3.0 (http://www.atgc-montpellier.fr/phyml), we chose the best-fit model according to the Bayesian selection criterion using Model Finder embedded in the Geneious software suite, version 11.1.7, https://www.geneious.com). Colours indicate grouping of segment origin according to phylogenetic analyses: highly pathogenic avian influenza virus (HPAIV) H5N1 2.2.1.2 from Egypt (cyan); H9N2 subtype from Egypt circulating in chickens since 2010 (grey); H9N2 subtype from Egypt first detected in pigeons in 2014 (red); PB2 segment most closely related to an H3N6 virus from Bangladesh (purple); PB1 segment most closely related to an H7N7 virus from Georgia (blue); segments most closely related to H5N8 viruses from China (orange) or Russia (green). Acknowledgments for sequences shared via EpiFlu™ are given in Appendix Table 2. A) Polymerase basic protein 2 (PB2) of HPAI H5N8, H5N1, and reassortant H5N2, and LPAI H9N2. B) Polymerase basic protein 1 (PB1) of HPAI H5N8, H5N1, and reassortant H5N2 and LPAI H9N2. C) Polymerase acidic protein (PA) of HPAI H5N8, H5N1, and reassortant H5N2, and LPAI H9N2. E) Hemagglutinin (HA _9) of LPAI H9N2. F) Nucleoprotein (NP) of HPAI H5N8, H5N1, and reassortant H5N2, and LPAI H9N2. G) Neuraminidase protein (NA_2) of HPAI reassortant H5N2 and LPAI H9N2. H) Neuraminidase protein (NA_8) of HPAI H5N8. I) Matrix protein (M) of HPAI H5N8, H5N1, and reassortant H5N2, and LPAI H9N2. J) Non-structural protein (NS) of HPAI H5N8, H5N1, and reassortant H5N2, and LPAI H9N2.

CHAPTER V: DISCUSSION

V. DISCUSSION

Over the past decade, four major human pandemics and numerous sporadic and enzootic outbreaks caused by IAV have affected the (veterinary) public health sector and instigated vast (economic) losses in the livestock industry and wild bird population. The rapid evolution and subsequent constant emergence of novel (zoonotic) IAV strains alongside the worldwide spread of the gs/GD HPAIV H5 lineage calls for dedicated AIV surveillance to allow timely detection and expedient prevention. Whole-genome evaluation by second-generation sequencing (SGS) or third-generation sequencing (TGS) has become the gold standard for surveillance studies and outbreak investigations, attributable to the expedited identification of all harboured IAV segments. This permits precise genetic characterisation and additional in-depth analysis of the outbreak dynamics. As curtailing of novel pandemic threats is believed to be best achieved by effectively controlling AIV in poultry, exact understanding of outbreak scenarios by phylogenetic analysis can aid in the establishment of viable prevention measures.
Therefore, this thesis aims to portray the possibilities and importance of rapid, real-time, and accessible WGS as the foundation for quick detection of novel IAV strains and advanced, in-depth genetic analysis and molecular epidemiology studies. In addition, insights into outbreak scenarios can permit improved prevention to evade novel epidemic or even pandemic threats.

Objective I: Establishment and application of a rapid, real-time MinION nanopore sequencing workflow for IAV
Publication I, III

Due to the mutable nature of IAV, the application of WGS in the clinical setting, in outbreak scenarios and in surveillance studies is indispensable. Although high-throughput second-generation sequencers have drastically improved the time, cost and quality factor involved in full genome evaluation, further advancements are required to enable universal employment of WGS. The recent TGS movement has started to fill this gap, portrayed here by the described MinION nanopore sequencing workflow for IAV: A rapid, real-time, long-read, multiplexing protocol suited for all types of (clinical and low yield) IAV samples (Publication I).

Comparison of high quality IonTorrent and lesser quality MinION reads of identical samples demonstrated consensus identity levels of >99.9%. Although the quality of ONT reads is lacking, sufficient coverage depth can almost fully minimise consensus errors with most divergences in homopolymer regions. The upstream IAV-End-real-time (RT)-PCR aids in the production of less data by minimal host share and high viral proportion while permitting WGS of low yield samples. This, alongside the application of a rapid transposase-based multiplexing kit, enables significant cost and time reduction (Publication I). The real-time availability of sequencing data permits rapid analysis of

IAV samples, crucial for clinical and outbreak scenarios to aid in disease prevention and control, as demonstrated in Publication III.

PCR-free sequencing of IAV on the MinION platform has been previously conducted, utilising a metagenomic (135) or direct RNA approach (136). The potential bias introduced by PCR has been thoroughly investigated in previous studies (137-141). Metagenomic sequencing permits the detection of not only IAV, but also other, potentially novel pathogens. The respective study struggled with full genome coverage of low yield samples, and far larger datasets were needed to assemble whole genome sequences with adequate coverage (135). Likewise, direct RNA sequencing of IAV permitted the avoidance of a upstream PCR with promising results, however, the high error rate of the available ONT Direct RNA Sequencing Kit and need for high titre IAV isolates to achieve full genome coverage makes its application unsuitable for clinical and outbreak samples (136). For rapid and inexpensive MinION IAV WGS, inclusion of the universal IAV-End-RT-PCR permits the production of smaller datasets with deep coverage, thus drastically reducing costs and time consumption.

Further reduction of expenditures can be achieved by multiplexing. The Rapid Barcoding Kit by ONT can sequence up to 12 samples at once. For SGS, multiplexing is a standard procedure and incorrect assignment of barcodes seldom. Due to the high error rate of ONT reads the process of demultiplexing can be negatively affected, resulting in “misindexed” reads. On average, 0.056% of total MinION reads are assigned to the incorrect barcode (142). For metagenomic studies, this value can obscure final consensus production. Combination with the prior IAV-End-RT-PCR evades this problem by amplification of the viral nucleic acid, thus the fraction of misindexed reads has a negligible impact on consensus construction.

The suitability of MinION sequencing for field surveillance has previously been demonstrated for swIAV (71) and EBOV (73, 74). This highlights the potential for universal employment of ONT sequencing. Alike the findings in Publication I, comparable results in consensus similarities and low hands-on-time were described. The overall low capital cost, small footprint and lack of maintenance expenses makes the MinION device ideal for on-site sequencing and pushes WGS to become an integral part of many laboratories.

Bioinformatics play a major role in WGS. The high error profile of TGS calls for specifically adapted workflows to handle TGS data (66, 143). Basecalling of raw data is where most errors occur. Here, the most current basecallers (e.g. Guppy by ONT) divide the raw signal into blocks termed “events”. Each event is translated into a “most-likely” set of bases and ideally, every event should differ by one base. Because the speed of DNA translocation through the nanopore is not stable, events can often not be clearly divided and result in deletions. Additionally, individual bases in homopolymer regions can often

not be distinguished. Seeing as correctly basecalled data forms the foundation for all following analyses, improvement of basecalling is of utmost importance to decrease the error rate (144-146). Due to the novelty of ONT, ongoing improvements of the chemistry (e.g. introduction of the dual-headed R10 nanopore flow cell) and succeeding bioinformatic analysis, especially basecalling, will continue to lower the standing error profile.

Objective II: Genetic characterisation, outbreak dynamics and molecular epidemiology of clade 2.3.4.4b HPAIV subtype H5 in Germany, 2016 – 2020

Publication II, III

By utilisation of WGS, dissection of clade 2.3.4.4b HPAIV H5 outbreak dynamics in terms of genetic characterisation and molecular epidemiology is possible. The unprecedented tendency for reassortment of the respective clade calls for full genome analysis to allow precise identification of novel sub- and genotypes. In-depth understanding of outbreaks allows the establishment of expedient preventive measures to avoid future epidemics.

In Germany, clade 2.3.4.4b HPAIV H5 strains have been abundantly reported since the first introduction in 2016 (17-19, 21, 22). Reassortant designation by WGS has permitted the identification of three subtypes (H5N8, H5N5 and H5N6) encompassing seven distinct genotypes (Ger-11-16, Ger-12-16.1 and Ger-12-16.2, Ger-12-16-N5.1 and Ger-12-16-N5.2, Ger-12-17-N6 and Ger-01-20) to date (Publication II, III). Full genome evaluation together with molecular epidemiology has revealed different outbreak dynamics of each reassortant, comprising wild bird-mediated introduction and transmission, farm-to-farm poultry transmission with cluster development and reassortment within a poultry holding after wild bird-mediated introduction. The zoonotic potential of H5N5 and especially H5N6 clade 2.3.4.4b strains could be delineated in ferrets, excluding enhanced zoonotic propensity (Publication II).

A major characteristic of the gs/GD lineage clade 2.3.4.4b is the frequent reassortment and advanced capability in attaining novel genome segments (147). In contrast, Egyptian 2.2.1.x viruses have shown genotypic stability for over a decade (148). Reassortment can potentially translate into advantageous phenotypic features for the virus affecting viral host range and fitness. Europe, including Germany, experienced the most severe epidemic to date during the winter of 2016/2017, driven by multiple HPAIV H5 reassortants (22, 149-152).

Enabled by WGS, a clade 2.3.4.4 virus identified at the Russia-Mongolia border was uniformly detected as the backbone for all German clade 2.3.4.4b HPAIV reassortants. Migratory birds play a central role in the transcontinental spread of AIV and act as a catalyst for reassortment events. Moulting and

breeding grounds of migratory waterfowl in Central Asia provide countless opportunities for transmission and reassortment and previous studies have demonstrated the potential sources and spread of AIV in spatio-temporal analysis of the main clade 2.3.4.4b reassortants (153, 154). For example, Ger-11-16, Ger-12-16 and Ger-01-20 carry different LPAIV segments detected in Central Asia. In line with the European study by Lycett et al. (153), the geographic distribution of the reassortants in Germany concurred with these findings. Distinction of reassortants by WGS allows the exact genetic dissection, but also enables the identification of multiple individual incursions into Germany. The most recent Ger-01-20 reassortant could therefore be proven a novel introduction into the country instead of the result of continuous local HPAIV circulation (Publication III).

Due to reports of human influenza cases by clade 2.3.4.4c, 2.3.4.4d and, in one case, clade 2.3.4.4b (155), concerns about the zoonotic potential of the clade 2.3.4.4b HPAIV H5N6 reassortant identified in Germany in 2017 (Publication III). Previous studies evaluated several clade 2.3.4.4b HPAIV H5 isolates from South Korea and the Ger-11-16 reassortant, resulting in no zoonotic propensity in ferrets (156-158). Frequent reassortment comes with the concomitant danger of the emergence of adapted, zoonotic or more pathogenic strains. Thus, continuous surveillance and virus characterisation is indispensable to reduce the risk of endemicity and zoonosis.

Ongoing surveillance is of utmost importance to aid in the genetic characterisation of novel strains. The importance of migratory wild birds in the dissemination of AIV has been previously described and analysed (14). Precise dissection and establishment of relations to progeny viruses involved in the reassortment of new (HP)AIV can only be achieved if wide-ranging surveillance of the LPAIV wild bird reservoir is conducted. Thus, the identification and evaluation of reassortment events is highly dependent on an up-to-date AIV genome sequencing and upload to publicly available databases.

Rapid and precise WGS of LPAIV and HPAIV is essential for the detection of novel reassortant viruses and their characterisation. Avoidance of enzootic virus circulation, epidemics or pandemics is greatly aided by genetic evaluation to allow appropriate response and prevention measures. The gs/GD H5 lineage, especially clade 2.3.4.4b, needs continuous surveillance to reduce the risk of major outbreaks in the wild bird and poultry population. In addition, WGS allows in-depth understanding of outbreak dynamics and interactions between wild birds and poultry. This knowledge builds the foundation for essential biosecurity measures in commercial poultry holdings to minimise economic losses and the risk of zoonotic emergence.

Objective III: Surveillance for the identification and genetic characterisation of novel reasssortant HPAIV subtype H5 circulating in Egypt, 2019

Publication IV

Since the introduction of the endemic HPAIV subtype H5N1 (gs/GD lineage, clade 2.2.1) in 2006, LPAIV subtype H9N2 (G1 lineage) in 2011, and more recently, HPAIV subtype H5N8 (gs/GD lineage, clade 2.3.4.4b) in 2016, the Egyptian poultry population has become entrenched with AIV (124, 134, 159). Co-circulation of the respective H5N1 and H9N2 viruses in Egypt so far appeared to be genotypically stable with no reassortment events (132). In contrast, clade 2.3.4.4b HPAI H5N8 viruses have shown high reassortment frequency in multiple outbreaks (4). Thus, the incursion of the respective HPAIV H5N8 strain into Egypt called for wide-ranging surveillance of the poultry population to detect potentially novel reassortants.

Publication IV describes the identification of a novel H5N2 reassortant after respiratory symptoms were reported in multiple commercial Egyptian poultry holdings. WGS revealed the HA segment to be derived from clade 2.3.4.4b HPAI H5N8 viruses. All other segments shared the closest homology to LPAI H9N2 viruses circulating in chickens since 2010 or a novel reassortant H9N2 virus identified in pigeons in 2014.

In Southeast Asia, natural reassortment of HPAI H5 viruses of the gs/GD lineage and LPAI H9N2 viruses has been reported, resulting, *inter alia*, in the emergence of novel H5N2 reassortants (160). The genotypic stability of the respective lineages despite co-circulation in Egypt saw gradual evolution by point mutations, but no reassortment events. Naguib et al. successfully forced virus reassortment by co-cultivation in embryonated chicken eggs, excluding principal incompatibility (148). The incursion of clade 2.3.4.4b HPAI H5N8 viruses beckoned an increased reassortment risk due to the previously observed characteristics of the respective clade, as shown by the novel H5N2 reassortant. In addition to the described HPAI H5N2 virus in Publication IV, a second novel H5N2 reassortant was described by Hagag et al. in March 2019 (161). In contrast, only the NA segment derived from endemic G1 lineage H9N2 viruses while all other segments had their origin in clade 2.3.4.4b HPAI H5N8 viruses.

The endemic circulation of various AIV strains in Egypt has been aided by multiple factors. First, Egypt is located at the crossing of the East Africa/East Asia and Mediterranean/Black Sea flyway (162), resulting in the passing of many, potentially AIV infected, migratory birds. Second, the forms of poultry farming are highly diverse in Egypt, including high-density commercial farming and free-range backyard or rooftop rearing (163). Trading usually commences via live bird-poultry markets, presenting a perfect habitat for AIV (164). Third, close contact between humans and birds and the lack of biosecurity measures enables AIV dissemination and trans-species transmissions (27, 165). Thus, the probability of further novel reassortant viruses is highly likely.

Due to the endemic co-circulation of H9N2 and H5N8 viruses in Egypt, detection and surveillance by quantitative PCR (qPCR) may hinder the identification of the novel H5N2 reassortant. In comparison, active surveillance with WGS can help to detect the described H5N2 virus, whilst allowing the identification of other novel reassortants. The risk of transboundary spread of the novel HPAI H5N2 virus in the Middle East and (northern) Africa could result in further reassortment events and cause great economic losses in the poultry production sector. Long-term solutions to stop the spread of AIV in Egypt by improved biosecurity measures are sorely needed, as the ongoing evolution of AIV can potentially result in zoonotic strains.

CHAPTER VI: SUMMARY

VI. SUMMARY

The constant evolution and pandemic potential of influenza A viruses (IAV) makes them one of the most complex and precarious viral pathogens globally. Alongside the impact on animal health and the livestock industry, some strains can carry zoonotic potential with extensive public health consequences. Classified depending on the host of origin, IAV are termed avian influenza viruses (AIV), swine influenza A viruses (swIAV), or otherwise in line with the host. Additionally, AIV are further divided into highly pathogenic AIV (HPAIV) or low pathogenic AIV (LPAIV) according to their level of pathogenicity. Characteristic for IAV is the extraordinary rate of evolution and rapid adaptation to host or environmental changes by point mutations, segment reassortment or, in rare cases, ribonucleic acid (RNA) recombination. This results in the unceasing risk of newly emerging and potentially zoonotic IAV strains, making rapid and continuous surveillance with full genomic evaluation of IAV indispensable.

This thesis presents the development and validation of a rapid, multiplex and portable third-generation nanopore sequencing protocol for whole-genome sequencing (WGS) of IAV. In comparison to large, expensive and laborious second-generation sequencers, the proposed nanopore workflow allows a great reduction in time and cost by utilising the “size-of-a-chocolate-bar” MinION sequencer (Oxford Nanopore Technologies) with multiplexing and rapid, transposase-based library preparation. Only full genome characterisation can permit detailed understanding of outbreak dynamics and molecular epidemiology while aiding in rapid outbreak response and the establishment of appropriate prevention strategies.

Since the emergence of the goose/Guangdong (gs/GD) lineage in 1996, specifically gs/GD clade 2.3.4.4 HPAI H5 viruses have shown an unprecedented tendency for reassortment and high mortality among the wild bird population and poultry industry. Here, the application of WGS is of utmost importance to identify new reassortants in a timely manner. After the first introduction of clade 2.3.4.4b HPAI H5 viruses to Germany in 2016, three subtypes (H5N8, H5N5 and H5N6) carrying seven reassortants (Ger-11-16, Ger-12-16.1 and Ger-12-16.2, Ger-12-16-N5.1 and Ger-12-16-N5.2, Ger-12-17-N6, and Ger-01-20) have been identified by WGS to date. The work collected in this thesis presents studies on the outbreak dynamics of the major 2016/2017 HPAIV epidemic and the most recent HPAIV H5N8 outbreak in 2020. Further, the zoonotic propensity of the circulating H5N6 and H5N5 strains was evaluated and genetically analysed, proving to carry very low zoonotic potential. The most recent incursion of a reassorted clade 2.3.4.4b HPAI H5N8 virus (Ger-01-20) to Germany was genetically characterised by the previously described third-generation nanopore sequencing protocol to achieve rapid whole genome and reassortment evaluation, allowing the categorisation into clade 2.3.4.4b and reassortment dissection.

Likewise achieved by WGS, this thesis includes the detection of a novel HPAIV H5N2 strain in Egypt. Although the previously circulating HPAIV H5N1 and LPAIV H9N2 strains appeared genotypically stable, the introduction of clade 2.3.4.4b HPAI H5N8 viruses to Egypt in 2016 resulted in the emergence of multiple novel reassorted HPAIV. Due to the endemic situation in Egypt and zoonotic propensity of particular strains, active surveillance and rapid detection is of utmost importance to aid in prevention.

IAV will continue to evolve, mutate and reassort in the future. The role of easy and swift WGS for IAV surveillance, especially frequently reassorting strains like clade 2.3.4.4b, is indispensable. This thesis encompasses a novel IAV third-generation nanopore sequencing workflow to aid in rapid full genome evaluation, in-depth genetic analysis of previous clade 2.3.4.4b outbreaks in Germany allowing molecular epidemiology studies and insights into outbreak dynamics, and the novel detection of reassortment of circulating AIV strains in Egypt. The findings of this thesis can help with imminent outbreaks and the essential surveillance of IAV, potentially curbing future epidemics or even pandemics.

CHAPTER VII: ZUSAMMENFASSUNG

VII. ZUSAMMENFASSUNG

Die ständige Weiterentwicklung und das pandemische Potenzial von Influenza A Viren (IAV) machen sie zu einem der komplexesten und gefährlichsten viralen Erreger weltweit. Neben den Auswirkungen auf die Tiergesundheit und die Tierhaltung können einige Stämme ein zoonotisches Potenzial mit weitreichenden Folgen für die öffentliche Gesundheit haben. Je nach Herkunftswirt werden die IAV als aviäre influenza/Vogelgrippe Viren (AIV), Schweinegrippe Viren (swIAV) oder in anderer Weise entsprechend dem Wirt klassifiziert. Darüber hinaus werden AIV je nach Grad ihrer Pathogenität in hochpathogene AIV (HPAIV) oder niedrigpathogene AIV (LPAIV) eingeteilt. Charakteristisch für IAV ist die hohe Evolutionsrate und schnelle Anpassung an Wirts- oder Umweltveränderungen durch Punktmutationen, Segment-Reassortierung oder, in seltenen Fällen, Ribonukleinsäure (RNA)-Rekombination. Daraus ergibt sich das bestehende Risiko neu auftretender und potenziell zoonotischer IAV-Stämme, was eine schnelle und kontinuierliche Überwachung mit am besten vollständiger genomischer Erfassung und Auswertung der IAV unabdingbar macht.

In dieser Arbeit wird die Entwicklung und Validierung eines schnellen und mobilen Nanoporen-Sequenzierungsprotokolls für die Vollgenomsequenzierung der IAV vorgestellt. Im Vergleich zu großen, teuren und arbeitsintensiven Sequenziergeräten der zweiten Generation ermöglicht der vorgeschlagene Nanopore-Workflow durch den Einsatz des etwa 100 Gramm leichten MinION-Sequenzers (Oxford Nanopore Technologies, ONT) mit Multiplexing und schneller, Transposasen-basierter Sequenzierbibliothek-Vorbereitung eine große Zeit- und Kostenersparnis. Nur eine vollständige Genomcharakterisierung kann ein detailliertes Verständnis der Ausbruchsdynamik und der molekularen Epidemiologie ermöglichen und gleichzeitig zu einer schnellen Reaktion auf Ausbrüche und zur Etablierung geeigneter Präventionsstrategien beitragen.

Seit dem Aufkommen der Goose/Guangdong (gs/GD) Linie im Jahr 1996 haben insbesondere HPAI H5 Viren der gs/GD Klade 2.3.4.4 eine hohe Reassortierungstendenz einhergehend mit einer hohen Sterblichkeit in der Wildvogelpopulation und der Geflügelindustrie gezeigt. Hier ist die Anwendung von Vollgenomsequenzierung von größter Bedeutung, um neue Reassortierungen rechtzeitig zu identifizieren. Nach der ersten Einschleppung von HPAI H5 Viren der Klade 2.3.4.4b nach Deutschland im Jahr 2016 wurden durch Vollgenomsequenzierung bisher drei Subtypen (H5N8, H5N5 und H5N6) mit insgesamt sieben Reassortanten (Ger-11-16, Ger-12-16.1 und Ger-12-16.2, Ger-12-16-N5.1 und Ger-12-16-N5.2, Ger-12-17-N6 und Ger-01-20) identifiziert. Diese Promotionsarbeit stellt Studien zur Ausbruchsdynamik der großen HPAIV Epidemie 2016/2017 vor. Darüber hinaus wurde das zoonotische Potenzial der zirkulierenden H5N6- und H5N5-Stämme bewertet und genetisch analysiert, wobei sich herausstellte, dass sie ein sehr geringes zoonotisches Potenzial besitzen. Die jüngste Einschleppung

eines neu reasortierten HPAI H5N8 Virus der Klade 2.3.4.4b (Ger-01-20) nach Deutschland wurde genetisch durch das zuvor beschriebene Nanoporen-Sequenzierungsprotokoll charakterisiert, um eine rasche Bewertung des Gesamtgenoms und der Reassortierung zu erreichen.

Auch ein neuartiger HPAIV H5N2-Stamm aus Ägypten konnte mit Hilfe dieser in der Arbeit beschriebenen Technik vollständig genetisch charakterisiert werden. Obwohl die zuvor zirkulierenden HPAIV H5N1- und LPAIV H9N2-Stämme genotypisch stabil zu sein schienen, führte die Einführung von HPAI H5N8 Viren der Klade 2.3.4.4b nach Ägypten im Jahr 2016 zur Entstehung mehrerer neuartiger reassortierter HPAIV. Aufgrund der endemischen Situation in Ägypten und der zoonotischen Neigung bestimmter Stämme ist eine aktive Überwachung und schnelle Erkennung für die Prävention von größter Bedeutung.

IAV werden sich auch in Zukunft weiterentwickeln, mutieren und reassortieren. Die Rolle der Vollgenomsequenzierung bei der Überwachung von IAV, insbesondere bei der häufigen Reassortierung von Stämmen aus der Klade 2.3.4.4b, ist unverzichtbar. Diese Arbeit umfasst einen neuen IAV-Arbeitsablauf zur Nanoporen-Sequenzierung der dritten Generation, der eine schnelle und vollständige Genomauswertung sowie eine eingehende genetische Analyse früherer und laufender Ausbrüche, z.B. der Klade 2.3.4.4b in Deutschland, erlaubt. Damit werden in der Folge dann auch retrospektive oder sehr zeitnahe molekular-epidemiologische Studien zur Ausbruchsdynamik möglich. Die gleiche Methodik hat schließlich auch zum Nachweis einer neuen HPAIV-H5-Reassortante in Ägypten geführt.

Insgesamt könnten die Ergebnisse dieser Arbeit bei bevorstehenden Ausbrüchen und der unerlässlichen Überwachung der IAV helfen, wodurch künftige Epidemien oder sogar Pandemien möglicherweise besser erkannt und kontrolliert werden können.

CHAPTER VIII: REFERENCES

VIII. REFERENCES

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CHAPTER IX: SUPPLEMENT

IX. SUPPLEMENTARY MATERIAL

1. List of Abbreviations

AIV	Avian influenza viruses
ASIC	Application-specific Integrated Circuit
bp	Base pairs
cDNA	Complementary DNA
ddNTP	Dideoxynucleosidtriphosphates
DNA	Deoxyribonucleic acid
EBOV	Ebola virus
ENA	European Nucleotide Archive
GB	Gigabytes
GPU	Graphics processing unit
gs/GD	Goose/Guangdong lineage
HA	Hemagglutinin
HACS	HA endoproteolytic cleavage site
HIV	Human immunodeficiency viruses
HPAIV	Highly pathogenic avian influenza viruses
IAV	Influenza A viruses
LPAIV	Low pathogenic avian influenza viruses
MAP	MinION Access Program
MP	Matrix protein
NA	Neuraminidase
NEP	Nucleic export protein
NGS	Next-generation sequencing
nm	Nanometres
NP	Nucleoprotein
NS	Non-structural protein
ONT	Oxford Nanopore Technologies
PA	Polymerase acidic protein
PacBio	Pacific Biosciences
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction

RAM	Random-access memory
RdRP	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT-PCR	Reverse transcription polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SGS	Second-generation sequencing
SMRT	Single molecule real-time
SSD	Solid-state drive
SwIAV	Swine influenza A viruses
TB	Terabyte
TGS	Third-generation sequencing
vRNA	Viral RNA
cRNA	Complementary RNA
WGS	Whole-genome sequencing

2. List of Figures

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CHAPTER X: ACKNOWLEDGEMENTS

X. ACKNOWLEDGEMENTS

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