Aus dem Institut für Geflügelkrankheiten
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

In Kooperation mit dem Friedrich Loeffler-Institut (FLI)
- Institut für molekulare Virologie und Zellbiologie,
Greifswald - Insel Riems

Epidemiological investigations and molecular characterization of avian influenza viruses in poultry in Egypt

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
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vorgelegt von
Ahmed Hatem Salaheldin Abdelhamid Mohamed
Tierarzt aus Alexandria, Ägypten

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Abbreviations

A/H5N1	Highly pathogenic AIV of H5N1 subtype
AI	Avian Influenza
AIV	Avian influenza virus
CEF	Chicken embryo fibroblast
CEK	Chicken embryo kidney
cRNAs	Complementary RNAs
DF-1	Chicken fibroblast
DIVA	Differentiation infected from vaccinated animals
ECE	Embryonated chicken eggs
EID_{50}	Median egg infectious dose
ELISA	Enzyme linked immunosorbent assay
FPV	Fowl Pox Virus
HA	Hemagglutinin
HI	Haemagglutination inhibition test
HP	Highly pathogenic
HVT	Herpes Virus of Turkey
IBV	Infectious bronchitis virus
iIFA	Indirect immunofluorescence assay
LBM	Live bird market(s)
LP	Low pathogenic
M	Matrix
M2e	M2 ectodomain
MDCK	Madin-Darby canine kidney
mRNA	Messenger RNA
NA	Neuraminidase
NDV	Newcastle Disease Virus
NEP	Nuclear export protein
NP	Nucleoprotein
NS	Non-structural
OIE	World Organization for Animal Health
PA	Polymerase acidic
PB	Polymerase basic
PCRs	Polymerase chain reactions
PCS	Proteolytic cleavage site
PPE	Personal protective equipment
QT-35	Quail fibroblast
RBD	Receptor binding domain
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT-PCR	Reverse-transcription polymerase chain reaction
RT-qPCR	Real-time RT-PCR
SA	Sialic acid
SAN	Specific antibody negative
SPF	Specific pathogen free
Tk16	A/turkey/Egypt/AR1507/2016(H5N1)

USA	United States of America
HVT	Turkey Herpes virus
Vero	African green monkey kidney
vRNP	Viral ribonucleoprotein
WHO	World Health Organization

Dedication

To my parents

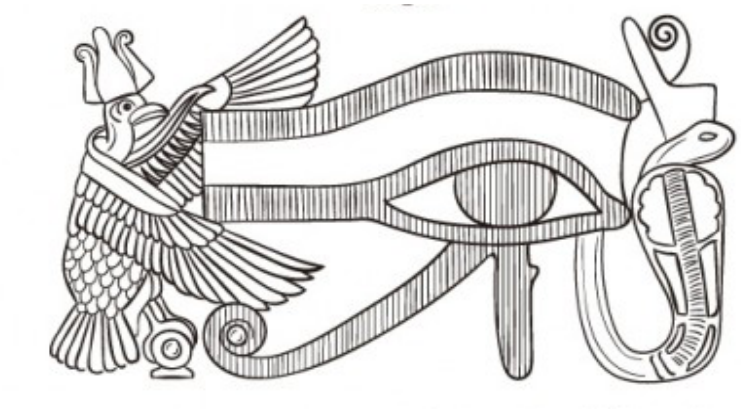
Dr. Hatem Salaheldin and Fatma Khalil

To my beloved wife and son

Marwa Hassan and Aly

This humble work is a sign of

love to you!



Chapter 1

General introduction

1. Introduction

Avian influenza viruses (AIV) belong to influenza A virus genus of the family *Orthomyxoviridae*. AIV contain segmented RNA genome composed of 8 gene segments, which encode at least 10 viral proteins. The surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) are divided into 16 HA (H1 to H16) and 9 NA (N1 to N9) distinct subtypes according to their antigenic properties. Each virus carries one HA and one NA with possible 144 HA-NA combinations. AIV infect a wide range of birds and mammals. Wild birds are the main reservoir of all AIV subtypes, where the infection is almost asymptomatic. In domestic birds, there are two pathotypes: low pathogenic (LP) AIV, induce mild clinical signs, if any, and highly pathogenic (HP) AIV, induce up to 100% mortality within few days. The HPAIV are naturally restricted to H5 and H7 subtypes and they evolve from LP precursors after mutations, particularly in the HA. Domestic turkeys and chickens are highly susceptible to the infection and therefore are considered "dead-end" hosts for the virus. Conversely, water fowls play a role as a reservoir for AIV because they are clinically more resistant than chickens and turkeys to AIV infection. Moreover, some AIV crossed the species barriers and infected humans with variable clinical outcomes ranging from mild or self-limiting to fatal (Figure 1).

Since 1996/1997, HP H5N1 Goose/Guangdong-like viruses caused significant losses in poultry industry worldwide. Since 2006 the virus was successfully eradicated in more than 55 countries and became endemic in poultry in few countries including Egypt. Nationwide vaccination strategy is the main prevention tool in poultry in Egypt, however, the virus is still circulating in vaccinated birds and continues to cause human infections and deaths. Little is known about the potential factors for the endemicity of the virus in Egypt in the last twelve years. Moreover, in 2017/2018, the panzootic H5N8 virus was spread from China to Asia, Russia, Europe, America and eventually to Africa. Wild birds were mostly the source of introduction for both H5N1 and H5N8 viruses into domestic birds worldwide.

In the present dissertation, genetic and antigenic characterizations of newly evolving H5N1 virus in vaccinated turkeys flock in Egypt were described (see chapter 2). Some potential biological and climatic factors foster the endemic situation of HPAIV H5N1 in Egypt were studied (see chapter 3). Moreover, sequence and phylogenetic analysis of the panzootic H5N8 viruses in commercial poultry flocks in Egypt in 2018 was carried out (see chapter 4). Findings in this study may help to better understand the epidemiology and evolution of

HPAIV H5Nx in Egypt and should be considered in the control and eradication of AIV in Egypt and prevent further spread to other countries in Middle East.

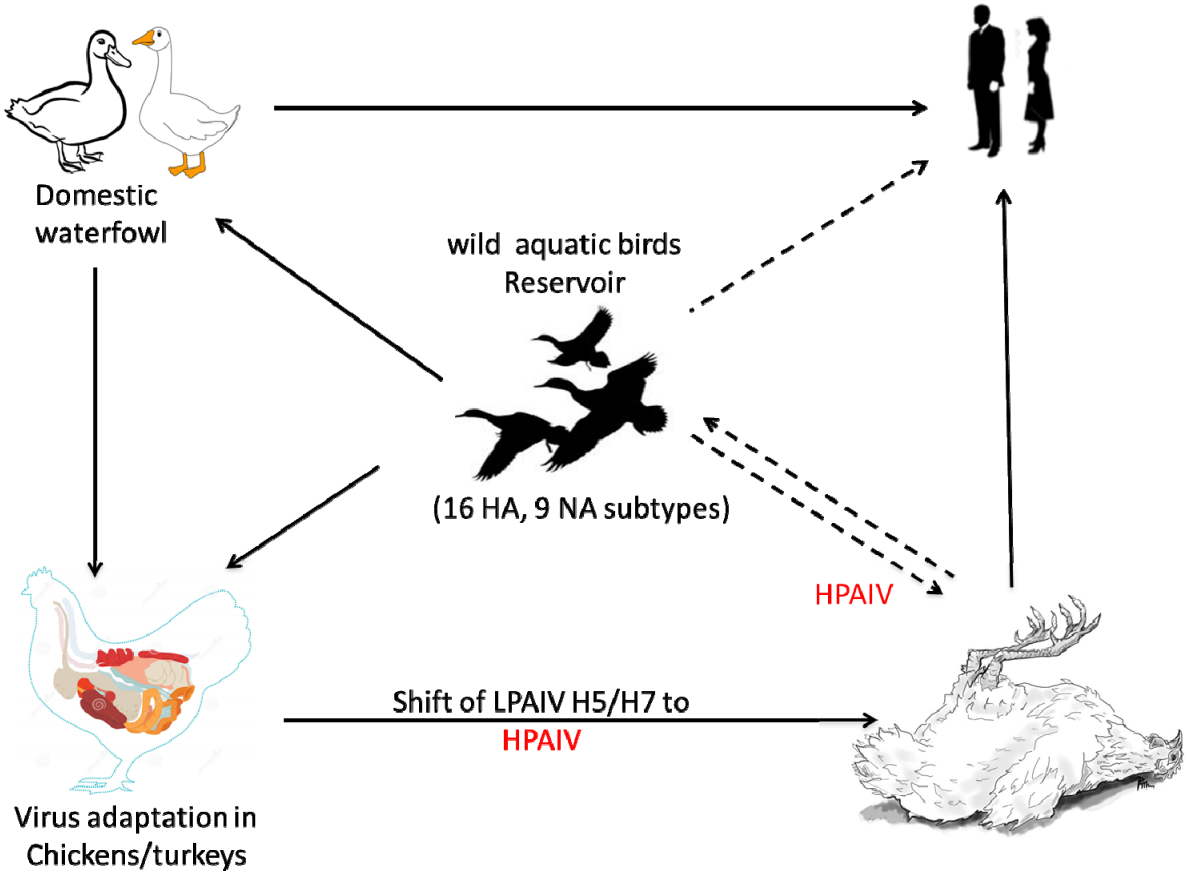


Figure 1: Avian influenza virus pathogenesis and epidemiology. Wild birds are the natural reservoir of influenza subtypes. LPAIV of subtypes H5 and H7 can mutate after transmission and adaptation to new poultry host shifting to HPAIV. The transmission of LPAIV and HPAIV from domestic birds to humans has been reported (1).

2. Review of literature

2.1 Avian influenza virus

2.1.2 Virus structure and taxonomy

Avian influenza viruses (AIV) are members of the genus influenza virus A in the family *Orthomyxoviridae* and possess a single-strand negative-sense RNA genome composed of eight gene segments that code for not less than ten viral proteins (2). The virus is a spherical or pleomorphic in shape and rarely filamentous with double bilayer membrane derived from the infected host cell. On the viral envelope, two glycoproteins haemagglutinin (HA) and neuraminidase (NA) in addition to Matrix 2 (M2) ion-channel protein are anchored (Figure 2) (3). The matrix protein M1 and nuclear export protein (NEP) are located just beneath the viral envelope. The polymerase basic (PB) subunits 1 and 2, polymerase acidic (PA) and nucleoprotein (NP) represent the minimal replicative unit of the virus forming the **r**ibo**n**ucleo**p**rotein complex (RNP). The core of the virus is made up of the viral RNA segments encapsidated by RNP. In addition, non-structural (NS1) protein is expressed in the host cell but is not packaged in the mature virus particles. Some AIVs possess PA-x and PB1-F2 proteins encoded by a frameshift or alternative frame of PA and PB1 gene segments, respectively (2).

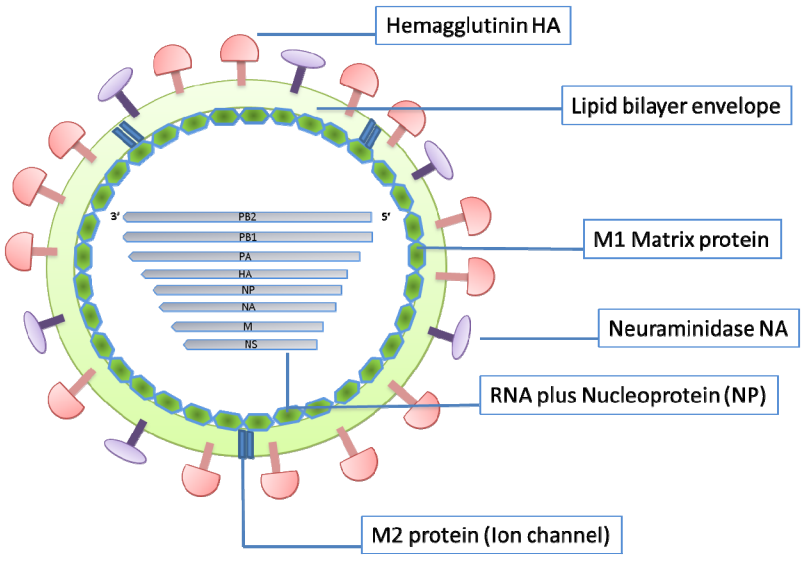


Figure 2: Diagrammatic illustration of an influenza virus and its genome. Virion of influenza contains three spikes: HA, NA and M2 proteins anchored in the host-derived lipid bilayer membrane. The genome of AIV consists of eight RNA gene segments (3).

Avian influenza viruses are classified according to the distinct antigenic properties of the HA and NA into 16 HA (H1-16) and 9 NA (N1–N9) subtypes. Both the HA and NA classified phylogenetically into two groups; group 1 encompassing (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16 & N1, N4, N5, N8) and group 2 encompassing (H3, H4, H7, H10, H14, H15 & N2, N3, N6, N7, N9) (Figure 3) (4).

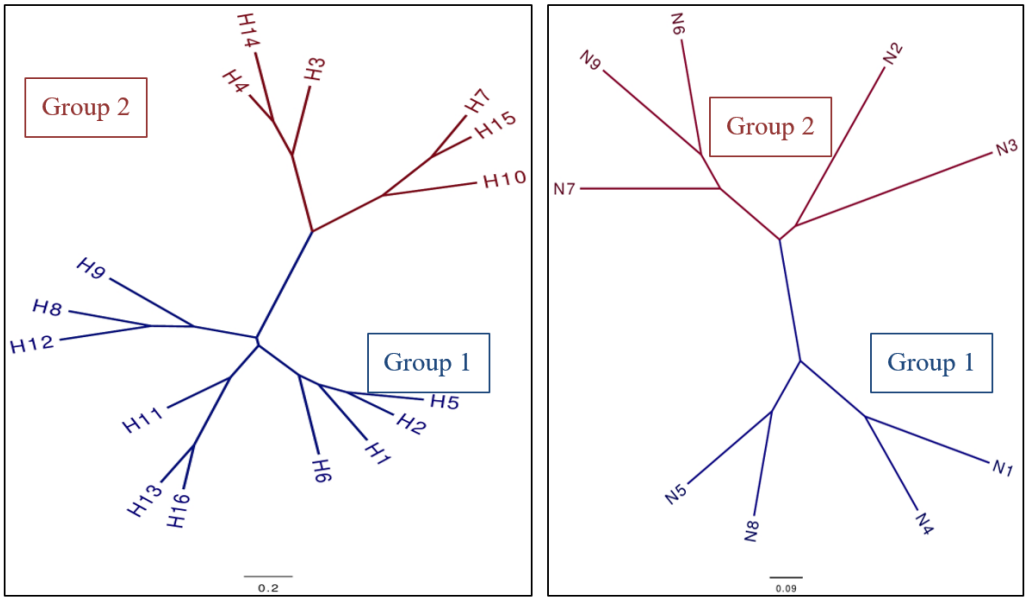


Figure 3: Phylogenic relatedness of influenza virus HA and NA subtypes. Maximum-likelihood trees were generated by comparing the nucleotide sequences of HA (left) and NA (right) genes using MEGA version 5.2 software (5) and allocated into groups and clades according to Palese and Shaw (4)

HA protein is the most abundant protein on the virus envelope. It mediates virus attachment to the sialic acid of the host cell, fusion with the host cell membrane, immunogenicity and pathogenicity (6). It is homotrimer; each single monomer is generated as a single polypeptide (HA0) then assembled all together. The HA is cleaved by host proteases to HA1 and HA2 subunits. HA1 first interacts with the receptors on the cell membrane triggering the endocytosis and then HA2 initiates cell membrane fusion. Tissue distribution of host proteases and the sequence of the HA proteolytic cleavage site (PCS) are crucial to determine the tropism and pathogenicity of AIV. Subtilisin-like enzymes are abundant in the host cells and recognize PCS motif consists of Arginine-X-X-Arginine↓ (R-X-X-R↓), whereas trypsin-like enzymes found in the respiratory and gastrointestinal tracts and recognize monobasic cleavage site motifs (R-X-R↓) (6). The PCS of HPAIV encompasses multibasic amino acids (e.g. arginine R or lysine K) and thus is cleaved by ubiquitous host subtilisin-like proteases.

The PCS of LPAIV possess monobasic amino acid motif and is activated by trypsin-like proteases causing local infections in the respiratory and digestive tracts (7, 8). The affinity of HA to bind to different sialic acid (SA) receptors is a main determinant for host specificity and interspecies transmission (9). The globular head of HA1 subunit contains 13 amino acids forming "pocket-like" receptor binding domain (RBD) (10). Viruses that infect humans bind to 2,6 SA linked carbohydrate side-chain, while AIV bind to 2,3 SA linked carbohydrate side-chain. Certain stepwise-mutations in the RBD increase the affinity of AIV to bind to human receptors which is a critical step in the emergence of viruses with pandemic potentials (11). Moreover, the HA1 harbors five immunogenic epitopes (designated A, B, C, D and E) which provoke the host immune system to produce anti-influenza antibodies. The accumulation of point mutations in these immunogenic epitopes allows the virus to escape the neutralizing antibodies and subsequently reduces the efficacy of a given vaccine (12, 13). Glycosylation of the HA and NA proteins is a posttranslational modification process by adding oligosaccharide to the asparagine side chain (14). Glycosylation sites (GS) play a key role in protein folding and transport (15, 16). The number and distribution of GS on the HA is a virus strategy to efficiently replicate in new hosts or under immune pressure. Glycosylation sites adjacent to the RBD could alter the receptor binding affinity of the virus to avian- or human-type receptors (17, 18). In the same manner, acquiring GS can help the virus to evade the immune system by masking the HA immunogenic sites. It can also sterically hinder the cleavability of the HA by host proteases and subsequently modulates virulence of the HPAIV (19-21)

The NA is an exosialidase capable of cleaving the sialic acid linkage between the HA protein and cell receptor enabling the release of the progeny virions from infected cells. Moreover, it removes the mucin in the upper respiratory tract facilitating the access of the virus to host cells (22). A functional balance between the HA and NA is crucial for virus replication, thus virus mutations in the HA are usually accompanied by alterations in the NA (2, 23). The NA protein is mushroom-like homotetramers consists of head, stem, transmembrane and N-terminal domains. The NA head domain contains catalytic and framework sites that are highly conserved among different influenza subtypes making it a target for influenza neuraminidase inhibitors (e.g. oseltamivir, zanamivir) (24) or neutralizing antibodies (25-27). Beside the active sites the head also harbors calcium binding site that stabilizes the enzyme structure at low PH (28). It was found that deletions within the stalk domain acts as an adaptation marker for wild-bird origin AIV on terrestrial birds (29). Lastly, the NA plays a less immunogenic role than the HA in protection against homologous influenza virus infection (30).

The M2 protein is the third surface viral envelope protein. The transmembrane domain of M2 has ion channel activity causing acidification of the virus core, uncoating of the virus and release of the viral RNPs (vRNPs) into the cytoplasm (31). Moreover, the M2 protein has an important role in virus packaging and budding. Mutation in the M2 protein can also alter the viral phenotype (32). The role of M2 protein as an antiviral target has been fully studied. The M2-blockers (e.g. amantadine and rimantadine) fit into the proton ion-channel preventing the transport across the channel. Interestingly, mutations in positions L26, V27, A30, S31, and/or G34, making the channel smaller or larger so that M2-blockers cannot perfectly fit and thus resistant strains continue to replicate in the presence of M2-blockers (33-36). Moreover, M2 is less abundant than the HA and NA proteins particularly in inactivated viruses (i.e. vaccines). Thus, the detection of antibodies against the conserved M2-ectodomain (M2e) can be useful to differentiate between infected and vaccinated chickens (37). Other studies suggested that M2e protein confers partial protection against AIV infection through interfering with the virus budding thus reducing virus replication and increasing virus clearance (38-41).

M1 protein connects the viral envelope to the vRNPs and interacts with the HA and NA cytoplasmic tail which is important for budding of the progeny viruses (42, 43). Moreover, M1 protein has a significant role in virus morphology either spherical or filamentous (44-47). The NP encapsidates the viral genome to form vRNP complex responsible for virus replication, transcription, intercellular transport and packaging (48). NP encoded by segment 5 has been proven to interact with cellular proteins including actin, nuclear import and export machinery and a nuclear RNA helicase (49). Viral polymerases (PB2, PB1 and PA proteins), encoded by segments 1, 2 and 3, respectively are the longest influenza genome segments. The three polymerase proteins form the enzymatic unit required for viral RNA transcription and replication (50). Nuclear export protein (NEP) was previously known as non-structural protein 2 (NS2). NEP is encoded by the bicistronic segment 8 and plays an important role in virus assembly as well as export of the vRNP from the nucleus into the cytoplasm (51). NS1, encoded also by segment 7, is a non-structural protein that antagonizes the host interferon response, thus allowing virus replication (52). Furthermore, NS1 has been proven to play a role as a virulence marker, host range determinant and increased virus replication (53).

2.1.2 Virus replication

Avian influenza viruses infect birds via nasal, ocular and/or oral routes. Therefore, the primary target of influenza virus is the epithelial cells of the respiratory and gastrointestinal

tracts. The HA1 binds to the SA receptors on the cell surface, as mentioned earlier, mediating the endocytosis (i.e. uptake of the virus into an endocytic vesicle). Proteolytic activation of the HA is done by host proteases either extracellular (for both LPAIV and HPAIV) or intracellular (for HPAIV only) and so the HA2 N-terminal act as fusion peptide "sticky ends". Uncoating of influenza virus genome is pH-dependent. The acidification of the vesicle occurs through the M2 ion-channel protein by pumping protons into the vesicle. Lowering of the pH results in irreversible conformational changes to merge the HA2 fusion peptide with the endosomal membrane and ends with the release of the vRNPs into the cytoplasm. The vRNPs are transported into the nucleus, where influenza virus transcription occurs. The first round of transcription results in 5′-capped and 3′-polyadenylated mRNAs. The viral polymerase cleaves mRNAs of the host cell forming a short, capped RNA fragment, which is then used as a primer. Therefore, each segment of viral mRNA contains about 12 bases homologous to that of the host RNA derived sequence at the 5′ end. In influenza viruses, the mRNAs acquire a poly(A) tail from repetitive transcription of a short poly(U) stretch near the 5′ end of the viral RNA (vRNA) template. After this primary round, the vRNA serve as templates that are transcribed into exact positive-sense RNA copies of the template (cRNA). Meanwhile, mRNA exported to the cellular ribosomes for translation of viral proteins. The peptides that are produced are being transported to the endoplasmic reticulum for glycosylation (HA and NA only) and folding into trimers (HA) and tetramers (NA). Afterwards, the HA and NA proteins are transported to the Golgi apparatus for trimming and further processing. All viral proteins, except the NS1, are transported to the apical plasma membrane "Budding site". Packaging and assembly of the progeny viruses takes place at the budding site where all eight segments must be included, one copy of each segment to produce progeny virions. Budding is then initiated via HA lipid raft association and interaction between the M1 with NEP and vRNPs. After successful budding on the host plasma membrane, the NA cleaves the bond between the HA of progeny virions and SA allowing viral release from the cell surface. For more detailed information please refer to these reviews (4, 54, 55).

2.1.2 Virus evolution

The evolution of influenza virus in nature is maintained by three main mechanisms: antigenic shift, antigenic drift and recombination (56). Antigenic shift, also known as reassortment, occurs after swapping of gene segments of two different influenza A viruses upon the simultaneous infection of the same host cell (Figure 4) (54, 57). The emergence of 256 different genotypes is possible (i.e. 2^8 plus the 2 parent viruses). The progeny viruses may

exhibit different phenotypes compared to the parent ancestors. Some of these viruses may be more transmissible, exhibit higher virulent and/or gain higher replication efficiency in a certain host than their predecessors (55). It is also possible that the reassortment produces poorly replicating viruses and mostly will be eliminated from the quasispecies or results in the extinction of the virus. Reassortment of the HA and NA may result in complete shift in the antigenicity of the virus. Although this process is common in the wild bird reservoir, however antigenic shift of avian and human influenza viruses poses a pandemic threat, hence, humans are naïve to the virus with the new constellation. A recent example for reassortment is the pandemic H1N1 virus in 2009 (58). This pandemic virus was a result of combination of gene segments between European swine influenza H1N1, the North American swine flu H1N2 and a human H3N2 virus. Another recent example is the new H5N8 belonging to clade 2.3.4.4 that was evolved in China then spread globally to Asia, Europe and Americas resulting in dramatic economic losses (59). The virus was a result of reassortment between H5 clade 2.3.4.4, other H5N1 subtypes and local LP strains (60).

Antigenic drift is the accumulation of stepwise mutations (e.g. exchange, insertions or deletion) in virus genome, which may result in gradual alteration of the protein structure of the virus. The influenza virus polymerase lacks the proofreading activity and thus is prone to introduce new mutations (61, 62). Antigenic drift of the HA is responsible for the immune-escape of a virus from the vaccine-induced antibodies causing serious infection in vaccinated animals. The immune pressure exerted by the host system (i.e. due to infection or vaccination) can accelerate the mutation rate. Resembling seasonal influenza epidemics in vaccinated humans, the emergence of antigenic drift viruses in domestic poultry has been frequently reported after massive vaccination (63-68). Moreover, point mutations (mostly exchange or deletion) in the receptor-binding domain may result in higher propensities or shift in the affinity from avian to human-type receptors or increase airborne transmission in chickens or mammals (69, 70).

Recombination between different influenza gene segments (homologous recombination) or host ribosomal RNA (non-homologous recombination) has been also described. Although, less frequently reported than antigenic shift and drift, recombination of the HA with NP (71) or M (72) genes or chicken ribosomal RNA (73) resulted in the evolution of HPAIV from LPAIV in terrestrial poultry.

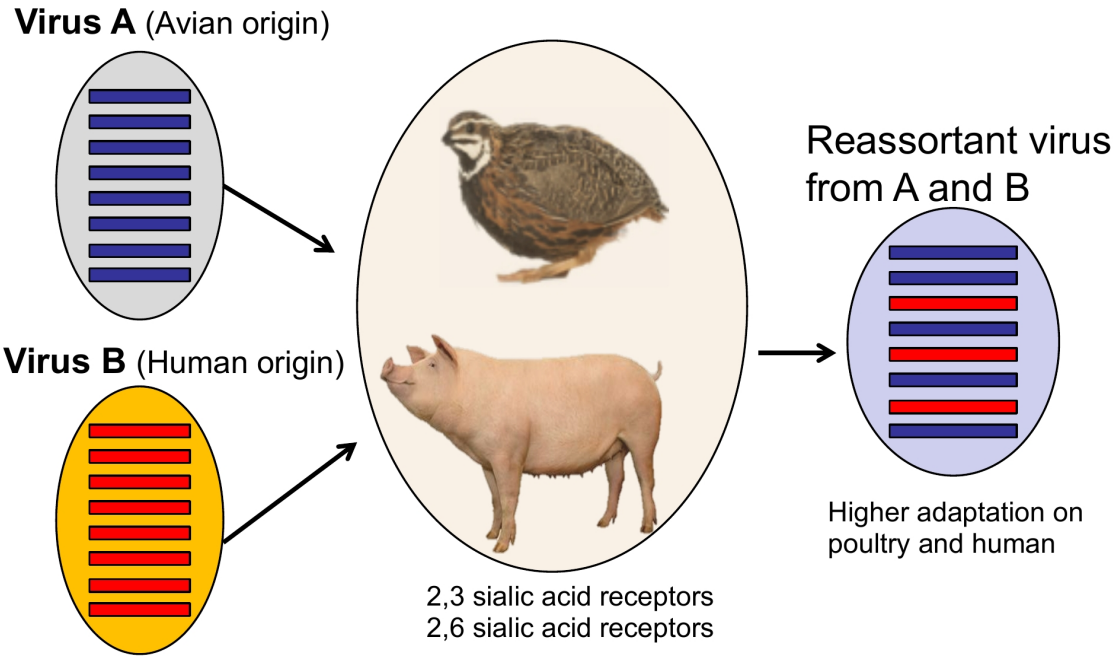


Figure 4: Reassortment of two influenza viruses. Shown are two different influenza A viruses co-infect the same cell. Theoretically, this could result in 2^8 different viral genotypes (i.e. 254 new genotypes in addition to the 2 parental viruses) (54).

H5N1 and H5N8. In 1996/1997, an HPAI H5N1 virus was generated through reassortment of the HA and NA segments of H5N1 with internal segments from H6N1 or H9N2 viruses in live bird markets (LBM) in Hong Kong. The virus killed 6 out of 18 infected humans and was successfully eradicated after depopulation of birds and closure of LBM (74). Nevertheless, the precursor viruses were circulating in the wild bird reservoir. After its reemergence in China in 2003, the virus rapidly spread to over 60 countries in Asia, Europe and Africa. The continuous circulation of H5N1 virus in birds has led to the evolution of 10 different phylogenetic clades (designated clade 0, 1, 2, 3, 4, 5, 6, 7, 8 and 9) and tens of first-, second-, third-, fourth-order clades (e.g. clade 2.2, 2.2.1, 2.2.1.2) according to the diversity of the HA gene (75). Most of the H5N1 clades died out, while viruses in clades 1, 2 and 7 are still circulating (75). To date, only few countries including Egypt, China, Indonesia, Viet Nam, and Bangladesh declared endemic status with H5N1 in poultry. To July 15, 2018 a total of 454 died out of 860 human infections worldwide (76). Several reassortment events with different LPAIV in wild birds in Asia have resulted in the generation of clade 2.2.3.4 H5N1, H5N2, H5N3, H5N5, H5N6 and H5N8 viruses (59, 77, 78). Some of these viruses (e.g. H5N8 and H5N6) became panzootic and transmitted to several Asian, European and African countries and for the first time to North America (59, 77-81).

2.1.2 Virus diagnosis

The preliminary diagnosis depends on clinical signs and gross pathology. The severity of the clinical signs is greatly influenced by the pathogenicity of the virus either highly pathogenic or low pathogenic as well as the species infected, age, immune status, concurrent viral or bacterial infection and the environment (82).

In case of LPAIV the **clinical signs** range from unapparent to mild or severe respiratory disease with mortalities ranged from 5 to 30 percent in broiler chickens and 3 percent in layer hens. The egg production in layer flocks may be dropped dramatically from 5 to 30 percent of the normal egg production yield and can take up to 24 weeks to return to normal production yield. Many **gross lesions** can be seen; the tracheal mucosa may be edematous with exudate either serous or caseous, inflammation in the air sac with fibrinous or caseous exudate. Catarrhal to fibrinous enteritis may be seen in the caeca and/or the intestine. Exudates can be seen in oviduct of laying hens (83, 84).

In peracute cases of HPAIV sudden deaths occur without showing any clinical signs or after onset of depression. In acute cases, the deaths mostly happen after 24 to 38 hours of the first signs of the disease. **Clinical signs** include severe respiratory distress with watery eyes and sinusitis, cyanosis of the comb and wattle, petechial haemorrhage on shanks, head/facial edema, ruffled feathers, diarrhea, nervous signs and complete cessation of egg production in layer flocks. **Gross pathology:** haemorrhagic, necrotic, congestive and transudative changes in almost all internal organs with characteristic dark red to blue areas of ischemic necrosis in the comb and wattles may be seen. Generally, these clinical signs and gross lesions are not pathognomic for AIV infection. Therefore, confirmatory diagnosis via virus isolation and/or RNA detection is required (82, 85).

Virus isolation is carried out by the inoculation of tracheal and/or cloacal swabs into 9-11 day-old specific pathogen free (SPF) embryonated chicken eggs (ECE) via chorioallantoic sac route (86). HPAIV usually kill the embryos within 2-3 days post inoculation, while the LPAIV may or may not kill the embryos and in some cases several egg passages are required (86). Allantoic fluid of inoculated eggs is usually collected and haemagglutination test is performed using 1% chicken erythrocytes to confirm the presence of haemagglutinating agent (87). Moreover, the samples could be inoculated on primary cell culture like chicken embryo kidney (CEK) and chicken embryo fibroblast (CEF) cells as well as different cell

lines including, but not limited to, Madin-Darby canine kidney (MDCK), African green monkey kidney (Vero) or chicken fibroblast (DF-1) cells (88, 89).

Molecular diagnosis is very useful for the detection of AIV-RNA. Molecular techniques are very sensitive, rapidly preformed, accurate, high throughput with reasonable cost (90). Therefore, they are important to control outbreaks through rapid diagnosis. Conventional reverse transcription-polymerase chain reaction (RT-PCR), real-time RT-PCR (RT-qPCR) and sequencing are the most common tools. The main challenges for RT-PCR is the RNA extraction as high yield and good quality of RNA are required for successful detection. The difficulty of processing tissue samples as well as the cloacal swabs and the presence of RNA inhibitors may influence the RNA extraction (91-93). However, the development of automated extraction devices enhanced the extraction process with less carryover contamination (94). Many validated assays for RT-PCR and RT-qPCR have been developed for accurate detection and subtyping of AIV. However, mismatches in the primer and probe specific sites may induce false negative results and therefore continuous update of oligonucleotides is important. Moreover, genome sequencing and phylogenetic analysis is very important for studying the AIV epidemiology, pathogenicity, evolution and update of vaccines (92, 95, 96).

Serological tests are commonly used to detect anti-AIV antibodies or antigens (97). Serological tests are used to determine AIV HA or NA subtypes (98-100) as well as evaluation of vaccine efficacy (66, 101-104). Enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition test (HI) and indirect immunofluorescence assay (iIFA) are commonly used serological techniques (97).

2.1.2 Control strategies for avian influenza outbreaks

Avian influenza outbreaks cause severe economic losses worldwide and some AIV pose a serious public health threat (105, 106). Wild birds are the main source for the primary introduction of AIV into terrestrial birds. Thereafter, the secondary spread of AIV in poultry is by direct and indirect contact with infected birds or fomites. Backyard birds and LBM can act as a reservoir for AIV. Airborne transmission for long distances among farms has not been proven yet (107). The implementation of high standard **biosecurity** measures, restriction of movement of infected birds or contaminated materials, cleaning and disinfection of equipment, vehicles and infectious organic matter are helpful to control the spread of or prevent AIV outbreaks (108, 109). **Stamping out** policy of infected flocks and those in

nearby farms is an effective tool to control and limit the AIV spread. However, in developing countries the costs of culling and compensation are not affordable (110, 111). Therefore, **vaccination** has been implemented as an ancillary or alternative control strategy in some countries including Egypt, China, Indonesia and Vietnam. Vaccination could be a powerful strategy, if combined with other control measures. Two major vaccines are being used: inactivated whole-virus and recombinant live vaccines (112). Inactivated whole-virus vaccines produce mainly humeral immunity and could be either homologues (same HA and NA subtype of the field virus) or heterologous (same HA but different NA subtype). The latter is of great importance for establishing the DIVA system (differentiating infected from vaccinated animals) to support the sero-surveillance and eradication programs (113, 114). Vaccination of poultry against HPAI as well as LPAI was systematically applied to mitigate the socioeconomic impact of HPAIV H5N2 in Mexico in 1994, H9N2 in several countries in Asia and Middle East since mid-1990s, H7N1 in Italy in 1999 and H7N3 in Pakistan. Vaccination accompanied with high biosecurity and movement restriction was helpful to eradicate some of these outbreaks (e.g. HPAIV H7N1 and H7N3 in Italy and Pakistan as well as , HPAIV H5N2 in Mexico), while AIV infections in poultry in other countries remained endemic (e.g. H9N2 in Asia and the Middle East, H5N1 in Egypt and Asia) (115, 116). Many commercial recombinant vaccines have been developed and used in some countries. Fowl pox virus (FPV), Newcastle disease virus (NDV) or Turkey herpesvirus (HVT) have been used as a vector expressing the AIV HA gene giving dual protection against the vector virus and the AIV (116). Effective vaccines decrease the virus shedding, increase the resistant to field challenge, reduce horizontal transmission, maintain normal egg production and mask clinical signs (110, 117). However, the intensive use of the vaccine can lead to the evolution of immune-escape mutants reducing the vaccine efficiency (118). Moreover, maternal immunity transferred from vaccinated breeders to their offspring can interfere with vaccination of chicks at early days of life (119-122).

2.1.2 Factors contribute to the evolution of influenza viruses in poultry

The life span of poultry is short compared to other animals and humans. Moreover, before 1990s HPAIV in terrestrial poultry was successfully eradicated within few months by culling of infected birds. Due to the rapid growth of poultry industry particularly in the developing countries and frequent introduction of AIV into commercial poultry, long-term circulation of AIV in (vaccinated) domestic birds has been reported (115, 123, 124). Unlike human influenza viruses, there is a paucity of information on the factors contribute to the persistence

and reemergence of AIV in birds. Resembling human influenza viruses, recent studies have shown that the emergence of antiviral resistance is accelerated after the applications of M2-blockers for AIV treatment or prophylaxis (125-127). Likewise, vaccination accelerates the evolution rate of AIV in birds and therefore the periodical update of the vaccine (annual or biennially) is recommended (114, 116). Climatic factors (e.g. temperature, relative humidity) influence the prevalence and transmission of influenza viruses in humans (128, 129), but little is known about the influence of climates on endemicity of AIV in poultry. Importantly, sustainable exposure to infected birds may increase the interspecies transmission and adaptation of AIV to mammals including humans (130).

2.2 Current situation of AIV in Egypt

2.2.1 Structure of poultry industry in Egypt

In Egypt, domestic birds represent the main source of animal protein for human consumption. Poultry industry was estimated in 2004/2005 to contribute around 8.8 %of the value of the agricultural production and 24.6 % of the value of the country's animal production (131). Labor force was estimated about 2.5 million workers either permanent workers (about 1.5 million workers) or temporary workers (about 1 million workers) representing approximately 6% of the Egyptian labor force (132). The poultry production was enough for the market demand as well as exporting to other Arab and African countries. Around 850 million birds are kept in the commercial sector and 250 million birds are reared in the backyard sector. The latter sector has low biosecurity level standards where birds are reared in small cages or barns and have access to the ponds and streets with close contact to humans especially women and children (133, 134).

The Food and Agriculture Organization (FAO) of the United Nations divided the commercial enterprise in Egypt into 3 categories according to the biosecurity measures. The first category includes integrated vertical farms applying high level of biosecurity regulations and their production is marketed commercially through slaughterhouses. The second category has poultry farms with moderate to high-level biosecurity standards and their production is mostly marketed also through slaughterhouses. The third category includes poultry farms with low biosecurity measures and their production is marketed through LBM (133, 135).

Most of the poultry production in Egypt is marketed through LBM. Retail shops are opened the whole week selling live birds (135). Different poultry species (chicken, ducks, geese and turkeys) from different farms or villages are gathered in the same market or within the same retail shops without information about the source or producers (Figure 5). Therefore, tracing back the infection is mostly impossible (131, 133).

Figure 5: A poultry merchant feeds a pigeon by mouth in a popular market in Cairo, Egypt, Nov. 19 2014 (136)

2.2.2 Epidemiology of AIV in Egypt

2.2.2.1 AIV subtypes in poultry in Egypt

Egypt is an important passage for migratory birds between Europe, Africa and Asia. Two-migration pathways crosslink Egypt: the Black Sea–Mediterranean and West Asian-East African (Figure 6) (137). Therefore, multiple introductions of new viruses especially during the season of migration of wild birds are common in Egypt (138-142).

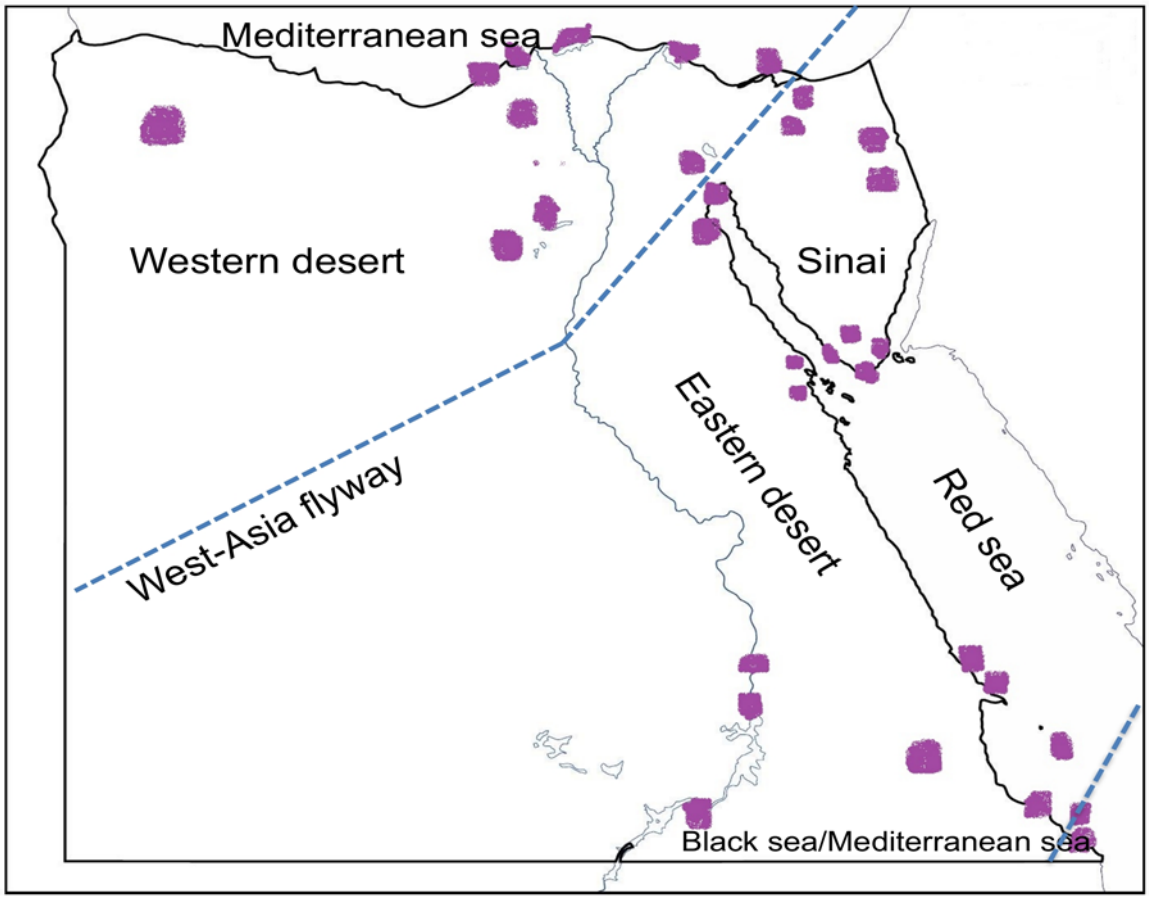


Figure 6: Flyways of migratory birds and location of the major wetlands in Egypt. Purple spots areas refer to the location of the major wetlands of migratory birds. Dotted lines refer to the migratory birds' flyways (143).

2.2.2.1.1 Fowl plague (H7N1)

The first record for AIV in poultry in Egypt was in 1912 (144) as a part of the global outbreak of H7 fowl plague (145-147). The virus was isolated from chickens, turkeys, waterfowl, parrots and pheasants. Vaccination was used to control the disease using inactivated whole-virus vaccine from the field strain. The vaccination strategy was effective and it succeeded to protect chickens and turkeys against fowl plague infection (148, 149). Between 1948 and 1950, escape mutant variants were reported. Later on, the virus was classified as HPAIV H7N1 (A/fowl/Egypt/45) with a polybasic amino acid at the cleavage site of the HA protein. Interestingly, in 1960s the virus was no longer reported and suddenly disappeared (150-152).

2.2.2.1.2 H5N1

HPAIV H5N1 was firstly reported in wild birds at the end of 2005, in domestic poultry in mid-February 2006 and in humans in March 2006 (99). Since then, the virus has become

endemic and still causes a significant threat to the poultry industry and public health. HPAIV H5N1 in Egypt has been reported in a variety of bird species, pigs, donkeys and humans. Many species including domestic ducks are assumed to be a potential reservoir of the virus (143). To reduce the economic losses and human risk major interventions were taken by the authorities including active surveillance, culling of infected birds and restriction of movement (133). Mass vaccination of poultry was implemented using over 15 types of inactivated H5N1 and H5N2 AIV vaccines from many vaccine manufacturers (Table 1) with low vaccination coverage (1% in the villages and 50-60% in the commercial sector) (153).

Table 1: Common vaccine strains used in Egypt

Vaccine strain	Sequence similarity %	Manufacturer
A/chicken/Italy/22A/1998(H5N9)	90.7	FATRO, Italy
A/chicken/Mexico/232/1994(H5N2)	84	Ceva,Mexico
A/turkey/England /N28/1973(H5N2)	91.4	Yebio, Qingdao, China
A/turkey/Minnesota/3689–1551/1981(H5N2)	89.8	Lohmann, Unites states
A/duck/Potsdam/1402/1986(H5N2)	91.6	Merck,United States
A/turkey/Wisconsin/1968(H5N9)	88.3	Ceva Biomune, Mexico
RG A/goose/Guangdong/1996(H5N1)(Re-1)	93.8	Zhaoqing DaHuaNong Biology Medicine, China
RG A/chicken/VN/C58/2004(H5N3)	95.1	Zoeits, USA
RG A/chicken/Egypt/Q1995D/2010(H5N1)	93.8	ME-VAC, Egypt
RG A/chicken/Egypt/M2583D/2010(H5N1)	99.6	ME-VAC, Egypt
RG A/chicken/Egypt/Q1995D/2010(H5N1)	93.8	ME-VAC, Egypt
RG A/duck/Egypt/M2583D/2010(H5N1)	99.6	Veterinary Serum and Vaccine Institute, Egypt
RG A/chicken/Egypt/18-H/2009(H5N1)	94.9	Harbin Veterinary Research Institute, China
RG A/duck/Guangdong/S1322/2006(H5N1)	-	Yebio, Qingdao, China
RG A/duck/Anhui/1/2006(H5N1)(Re-5)	94.9	Merial, United States

*RG= reverse-engineered

Therefore, circulation of the virus in vaccinated poultry has been frequently reported. Moreover, the immune pressure induced by the vaccines resulted in antigenic drift of the virus and subsequently reduced the efficacy of the vaccines to protect against HPAIV H5N1 infections (154-157). Moreover, Egypt has reported the highest number for human infections with H5N1 worldwide (Table 2) (76, 158). A total of 120 fatalities out of 359 infected were

confirmed. Infected persons reported exposure to backyard poultry (70%), bred domestic birds (26%), slaughtered poultry (14%), or were exposed to dead birds (4%) (159). It is worth mentioning that reported cases of H5N1 in Egypt differed significantly by sex (higher among female patients), age (increased with age), and time to hospitalization (decreased with faster hospitalization) (159-161).

Several genetic analyses indicated that the antigenic-drift viruses, emerged in early 2007, clustered in a distinct clade designated 2.2.1.1, meanwhile viruses in humans and non-vaccinated backyard birds since 2008 clustered in 2.2.1.2 clade (154, 162). Viruses in both clades possessed distinguished genetic markers in all gene segments assuming disparate evolutionary traits in virus adaptation. Handful mutations in the HA of clade 2.2.1.2 increased the affinity of the virus to human-type sialic acid receptors and retained its specificity to avian-type receptors (123, 163-165).

Table 2: Cumulative number of confirmed human cases for avian influenza A(H5N1) in Egypt reported to WHO from 2006 to May 2018 (76).

Year	Cases	Deaths	Fatality rate
2006	18	10	55.6
2007	25	9	36.0
2008	8	4	50.0
2009	39	4	10.3
2010	29	13	44.8
2011	39	15	38.5
2012	11	5	45.5
2013	4	3	75.0
2014	37	14	37.8
2015	136	39	28.7
2016	10	3	30.0
2017	3	1	33.3
2018	0	0	0.0
Total	359	120	33.4

2.2.2.1.3 H9N2

In December 2010, H9N2 of G1-lineage was isolated from commercial poultry in Egypt (166, 167). Since then, the virus widely spread in farmed chickens, quails, ducks, turkeys and pigeons causing respiratory distress and/or decrease in egg production (166, 168). Inactivated vaccines are used either from local or non-local isolates. Nevertheless, the prevalence of

H9N2 outbreaks in Egypt has been frequently reported in breeder, layer and broiler flocks. The emergence of antigenic-drift variants has been also described (162). In 2015, sero-survey among poultry workers revealed 7.5% positivity (159). Despite the co-circulation and co-infections of H5N1 and H9N2 viruses in poultry in Egypt since 2010, no evidence of reassortment has been reported (169, 170).

2.2.2.1.4 H5N8

During two independent surveillance, the panzootic HPAIV H5N8 of clade 2.3.4.4 was detected in swab samples collected from diseased and dead migratory birds in Egypt in November and December 2016 (141, 142). The virus spread to backyard birds and several commercial farms. The current vaccines used for the control of H5N1 are less effective to protect poultry against HPAIV H5N8 in Egypt (142, 171). Moreover, the virus has higher affinity to bind to avian 2,3 sialic acid receptor than human receptor 2,6 sialic acid and it was fully inhibited by antivirals targeting the M2 and NA proteins assuming low zoonotic potentials (142).

3. Outline of the publications in the current dissertation

3.1 Publication 1:

Ahmed H. Salaheldin, Jutta Veits, Hatem S. Abd El-Hamid, Timm C. Harder, Davud Devrishov, Thomas C. Mettenleiter, Hafez M. Hafez and Elsayed M. Abdelwhab 2017. **Isolation and genetic characterization of a novel 2.2.1.2a H5N1 virus from a vaccinated meat-turkeys flock in Egypt.** Virology Journal 14(1): 48.(172)

Field reports on vaccinal breaks in turkeys are scarce in contrast to chickens. In this chapter we described an H5N1 outbreak in a commercial turkeys flock caused by a novel HPAIV H5N1 designated 2.2.1.2a virus. The flock was vaccinated with two different inactivated H5 vaccines at 8 and 34 days of age. Few weeks after the last vaccination, turkeys exhibited clinical signs and high mortality rate reaching ~29% within 10 days. An H5N1 virus was isolated and confirmed by RT-qPCR and sequencing of all gene segments. Antigenic characterization revealed significant antigenic drift against vaccine strains. These viruses possessed common mutations of 2.2.1.1 and 2.2.1.2 that enabled evasion from humoral immune response and high affinity to human-like receptors. Phylogenetic analysis showed

clustering of the virus in clade 2.2.1.2a, which mostly spread, to poultry in Israel, Gaza and Egypt.

3.2 Publication 2:

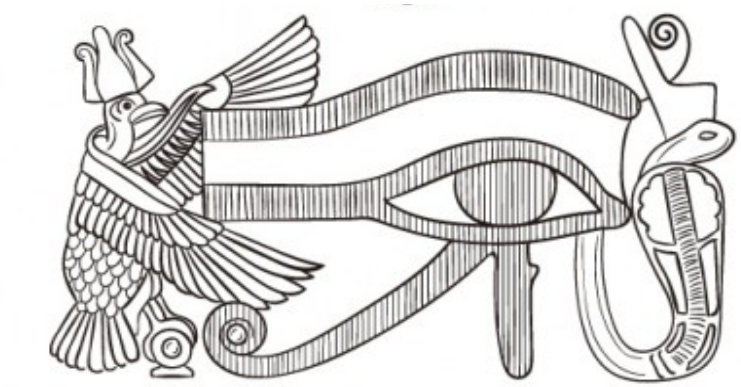
Ahmed H. Salaheldin, Elisa Kasbohm, Heba El-Naggar, Reiner Ulrich, David Scheibner, Marcel Gischke, Mohamed K. Hassan, Abdel-Satar A. Arafa, Wafaa M. Hassan, Hatem S. Abd El-Hamid, Hafez M. Hafez, Jutta Veits, Thomas C. Mettenleiter and Elsayed M. Abdelwhab 2018. **Potential biological and climatic factors that influence the incidence and persistence of highly pathogenic H5N1 avian influenza virus in Egypt.** Frontiers in Microbiology 9: 528. (173)

In the second publication, we describe three potential factors which probably affect the evolution of A/H5N1 since 2006 in poultry in Egypt: (1) the climatic factors from October to March 2006-2016 where the incidence of the outbreaks rises, (2) the variation in biological fitness of 2.2.1.1 and 2.2.1.2 viruses in different cells and niche, and (3) pathogenicity in domestic Pekin and Muscovy ducks. Statistical analyses using negative binomial regression models showed that ambient temperature in winter season affected the spread of A/H5N1 in different geographic regions in Egypt. Viruses from clade 2.2.1.2 and recent 2.2.1.1 were more stable at 4°C and 56°C than other viruses in this study. Moreover, viruses from clade 2.2.1.2 replicated at higher level than other viruses on human lung cells. Further, four groups of Pekin ducks (groups 1 and 2) and Muscovy ducks (groups 3 and 4) were inoculated with 2.2.1.1 (groups 1 and 3) or 2.2.1.2 (groups 2 and 4). One-day post inoculation (dpi), 5 birds were added in each group to assess duck-to-duck transmissibility of the viruses. Swabs were collected at days 2, 4, 7, 11 and 14 post-inoculation and tested by RT-qPCR for viral RNA. Results indicated that Pekin ducks were more resistant than Muscovy ducks for the infection. A total of 0/7, 1/7, 6/7 and 6/7 inoculated birds died in groups 1 to 4, respectively. None of contact Pekin ducks died, whereas 5/5 and 4/5 contact Muscovy ducks died in groups 3 and 4, respectively. Pekin ducks were all seropositive and they shed the virus for up to 14 dpi. Together, Pekin ducks are one of the major sources for silent transmission of the AIV in poultry in Egypt and pose a serious public health threat.

3.3 Publication 3:

Ahmed H. Salaheldin, Hatem S. Abd El-Hamid, Ahmed R. Elbestawy, Jutta Veits, Hafez M. Hafez, Thomas C. Mettenleiter and Elsayed M. 2018. **Multiple introductions of influenza A(H5N8) virus into poultry, Egypt, 2017.** Emerging Infectious Diseases 24(5) (174)

The HPAIV H5N8 in Egypt was firstly isolated during November/December 2016 from three wild birds. However, in this publication we isolated the H5N8 virus from four commercial flocks (i.e. 3 chicken and 1 duck flocks). During 2017, 48 poultry flocks from Nile delta suffered from severe mortalities. Tracheal and cloacal swabs were tested using RT-qPCR and virus isolation for AIV. Four flocks were positive for H5N8. Full genome sequencing suggested 4 different introductions of H5N8 virus into poultry in Egypt, independent of viruses isolated from captive wild birds.



Chapter 2

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Veits, J.
Abd El-Hamid, H. S.
Harder, T. C.
Devrishov, D.
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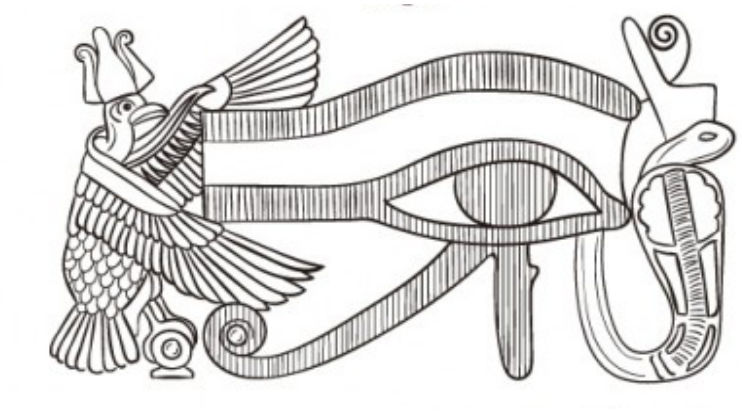

Chapter 3

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Potential Biological and Climatic Factors That Influence the Incidence and Persistence of Highly Pathogenic H5N1 Avian Influenza Virus in Egypt

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Highly pathogenic H5N1 avian influenza virus (A/H5N1) of clade 2.2.1 is endemic in poultry in Egypt where the highest number of human infections worldwide was reported. During the last 12 years the Egyptian A/H5N1 evolved into several genotypes. In 2007-2014 vaccinated poultry suffered from antigenic drift variants of clade 2.2.1.1 and in 2014/2015 an unprecedented upsurge of A/H5N1 clade 2.2.1.2 occurred in poultry and humans. Factors contributing to the endemicity or re-emergence of A/H5N1 in poultry in Egypt remain unclear. Here, three potential factors were studied: climatic factors (temperature, relative humidity, and wind speed), biological fitness *in vitro,* and pathogenicity in domestic Pekin and Muscovy ducks. Statistical analyses using negative binomial regression models indicated that ambient temperature in winter months influenced the spread of A/H5N1 in different geographic areas analyzed in this study. *In vitro*, at 4 and 56°C 2.2.1.1 and recent 2.2.1.2 viruses were more stable than other viruses used in this study. Further, Pekin ducks were more resistant than Muscovy ducks and the viruses were excreted for up to 2 weeks post-infection assuming a strong role as a reservoir. Taken together, ambient temperature in winter months potentially contributes to increasing outbreaks in some regions in Egypt. Heat stability of clade 2.2.1.1 and recent 2.2.1.2 viruses probably favors their persistence at elevated temperatures. Importantly, asymptomatically infected Pekin ducks may play an important role in the spread of avian and human-like A/H5N1 in Egypt. Therefore, control measures including targeted surveillance and culling of silently infected Pekin ducks should be considered.

Keywords: H5N1, highly pathogenic avian influenza virus, poultry, meteorological factors, epidemiology, ducks, clade 2.2.1, Egypt

INTRODUCTION

Highly pathogenic avian influenza virus (HPAIV) H5N1 (A/H5N1) caused enormous economic losses in poultry in many countries worldwide and genetically diversified into 10 clades and several subclades since 1996/1997 (Smith et al., 2015). Clade 2 viruses spread from China to Europe and Africa since 2003, and eventually became endemic in poultry in Egypt and several Asian countries. Since 2006, Egyptian A/H5N1 of clade 2.2.1 have diversified into several genetic groups. Most of these phylogroups disappeared but two major clades circulated for several years (Abdelwhab et al., 2016). Clade 2.2.1.1 represented antigenic-drift variants, which were primarily isolated from vaccinated commercial poultry leading to three human infections so far according to the official reports to the World Health Organization. These viruses appeared in early 2007 and predominated in 2008-2010 challenging the efficacy of the highly diverse H5 vaccines in Egypt. In 2011-2014, the prevalence of 2.2.1.1 viruses dramatically decreased and they are most likely extinct by now (Abdelwhab et al., 2016; El-Shesheny et al., 2017; Rohaim et al., 2017). The second clade are 2.2.1.2 viruses which circulated in non-vaccinated backyard birds and were introduced into small-scale farmed poultry since early 2008. The vast majority of infected humans were infected by this genotype (Younan et al., 2013). In 2014/2015, an unprecedented upsurge of 2.2.1.2 was reported in poultry and humans marking Egypt as the country with the highest number of human infections with A/H5N1 worldwide (Arafa et al., 2015; WHO, 2017). These viruses spread to neighboring countries posing a serious threat in the Middle East (Naguib et al., 2016a; Salaheldin et al., 2017). Driving forces for the emergence, extinction or spread of A/H5N1 clades in Egypt are not well-studied except for the massive application of vaccines and antivirals in poultry (Abdel-Moneim et al., 2011; Cattoli et al., 2011; Abdelwhab et al., 2016; El-Shesheny et al., 2016; Naguib et al., 2016b).

The spread of influenza viruses may be influenced by several factors related to environment, virus, and host. Previous research has shown that meteorological factors, biological fitness, and/or domestic ducks play significant roles in shaping the spread of influenza viruses (Li et al., 2004, 2015). Apart from the seasonal incidence of human influenza viruses, there is a paucity of information about the impact of climatic factors on the spread and course of influenza viruses' infection in domestic birds. Studies on wild bird populations showed that the regional prevalence of avian influenza viruses (AIV) may follow a seasonal pattern and can be influenced by climatic conditions (Gilbert et al., 2008a; Herrick et al., 2013; Ferenczi et al., 2016).

Abbreviations: A/H5N1, Highly pathogenic avian influenza virus H5N1; A549, Adenocarcinomic human alveolar basal epithelial cells; AIC, Akaike information criterion; AIV, Avian influenza viruses; BSA, Bovine serum albumin; CBS, Citrate-buffered saline; CEK, Chicken embryo kidney cells; dpi, days post-inoculation; ECE, Embryonated chicken eggs; FLI, Friedrich-Loeffler-Institut; HA test, hemagglutination test; HA, hemagglutinin; HPAIV, Highly pathogenic avian influenza virus; MDCKII, Madin-Darby canine kidney cells II; NA, Neuraminidase; NLQP: National Laboratory for Veterinary Quality Control on Poultry Production; PFU, Plaque-forming unit; SA, Sialic acid; SPF, Specific pathogen free; TRBCs, Turkey erythrocytes.

However, little is known about the correlation and the impact of climatic factors on introduction and persistence of HPAIV in domestic poultry. Biological fitness (e.g., stability in harsh niches, rapid replication, or spread) may be advantageous for virus perpetuation outside the host or increase adaptation to birds and human (Terregino et al., 2009). The role of domestic ducks for generation and persistence of A/H5N1 in Asian countries is well-studied. In contrast to the high mortality in chickens, domestic ducks were considered a "Trojan horse" because they are usually infected without exhibiting clinical signs or mortality (Chen et al., 2004; Hulse-Post et al., 2005; Gilbert et al., 2006; Songserm et al., 2006) enabling silent spread of the virus to other hosts (e.g., chickens, humans) (Kim et al., 2009; Lebarbenchon et al., 2010). In Egypt, about 40 million ducks raised in backyard and commercial farms where Pekin and Muscovy ducks are the most prevalent breeds (Hassan et al., 2013). A/H5N1 was isolated from asymptomatic domestic ducks in hot summer seasons in some localities in Egypt (Hassan et al., 2013). Also, viruses isolated from different organs of ducks were more genetically diverse than those isolated from chickens (Watanabe et al., 2011a), suggesting a role for ducks in perpetuating the endemicity of A/H5N1 in Egypt.

Here, we investigated three potential factors which could affect the evolution of A/H5N1 in poultry in Egypt: the climatic factors from October to March where the incidence of outbreaks rises, the variation in biological fitness of 2.2.1.1 and 2.2.1.2 viruses in different cells, and the role of Pekin and Muscovy ducks as a reservoir for two representative viruses from both major clades isolated from poultry in Egypt.

MATERIALS AND METHODS

Climatic and Epidemiological Data Collection and Processing

The daily data on temperature (minimum, maximum, and average), relative humidity, and wind speed were retrieved from Weather Underground Website (Dugas et al., 2013). Data were collected for 10 seasons from 2006 to 2015. Each season lasted from the 1st of October to 31st of March of the following year, when the incidence of outbreaks peaked according to previous surveillance (Arafa A. et al., 2012; Arafa A. S. et al., 2012; El-Zoghby et al., 2013; Arafa et al., 2015; Kayali et al., 2016). For example, the "2006-2007" season represented the data from 01.10.2006 to 31.03.2007. The means and standard deviations for each month were calculated and used for analysis. Four governorates namely Alexandria, Cairo, Minya, and Luxor (ordered from north to south) were selected based on their geographic location, climate zones, and variable number of A/H5N1 outbreaks (**Figure 1**). While Alexandria is situated close to the Mediterranean coast and therefore characterized by a maritime climate with higher precipitation and moderate temperatures, following the river Nile further south the climate changes into a hot desert climate with little to no precipitation and high temperatures. The total number of A/H5N1 outbreaks per month was summarized for each season based on the official reports of national surveillance conducted by the Egyptian

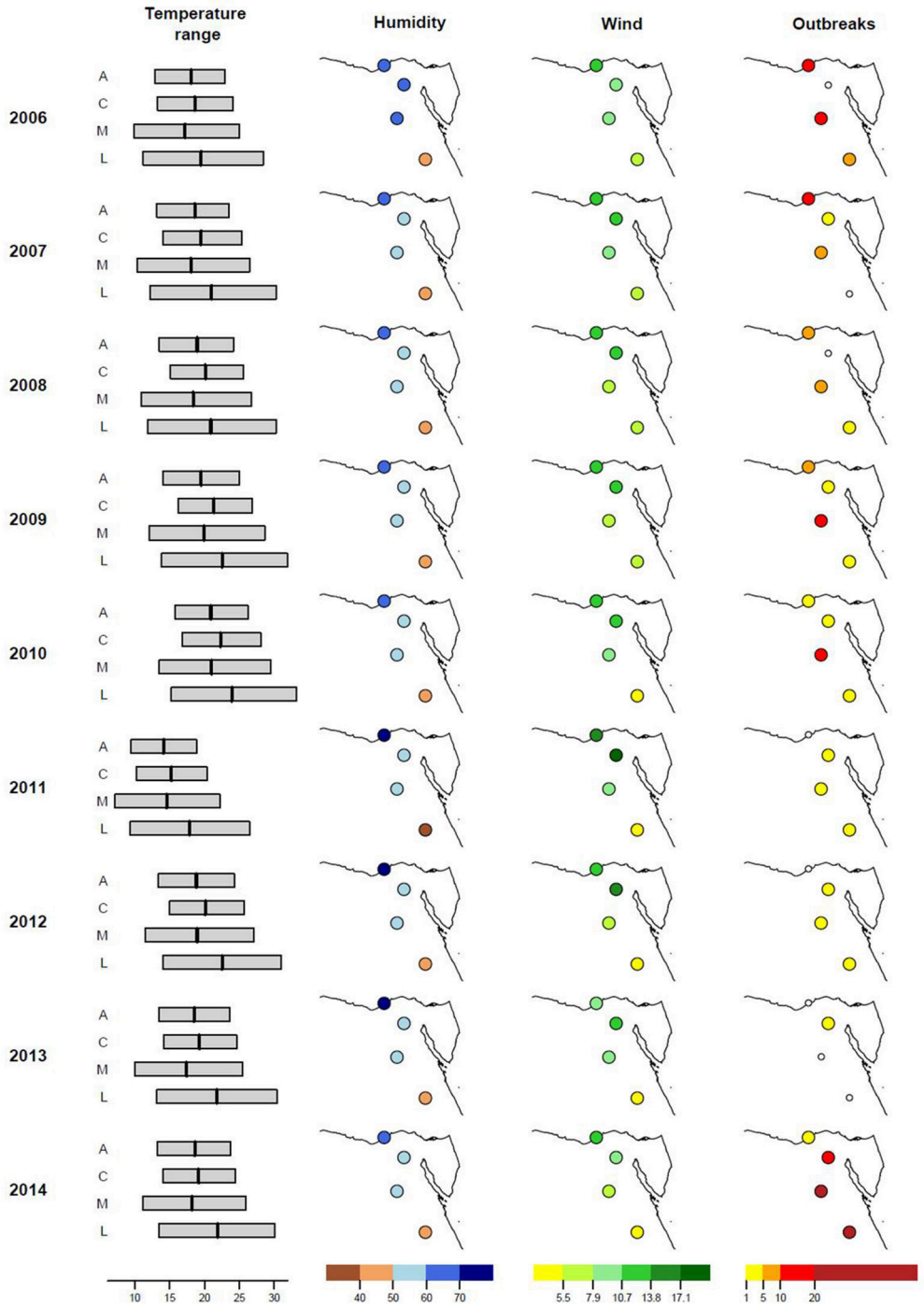

FIGURE 1 | Climatic factors and number of H5N1 outbreaks in four selected governorates in Egypt. Illustrated are the ranges from minimum to maximum temperature (°C, average temperature marked as vertical line), relative humidity (%), wind speed (m/s), and number of avian influenza outbreaks in domestic poultry for four selected provinces in Egypt (from north to south: A, Alexandria; C, Cairo; M, Minya; L, Luxor) from 2006 to 2015 (October 1st to March 31st of the next year).

National Laboratory for Veterinary Quality Control on Poultry Production (NLQP) and reported to the World Organization for Animal Health (OIE) and to Food and Agriculture Organization of the United Nations (FAO). Five unreported cases, which were detected in a retrospective surveillance from vaccinated birds, were also included in the analysis. The effect of selected climate parameters on the number of outbreaks in each region and all-over Egypt was analyzed as described below.

The correlation between each of the climate factors and the reported number of outbreaks was calculated as Spearman's rank correlation coefficient for the whole of Egypt and also for each of the governorates. Furthermore, negative binomial regression models were used to assess the combined contribution of several climate factors on the observed number of outbreaks per governorate. We investigated a "full" model, which includes regional effects and all climate factors as explanatory variables. Since climate factors are interrelated, significant effects of these variables might be obscured in the full model. Thus, a "reduced" model with fewer variables was derived from the full model by stepwise backward elimination minimizing the Akaike information criterion (AIC). AIC was chosen for model selection for the reason that this criterion evaluates the goodness of fit (based on the likelihood) and simultaneously takes the number of explanatory variables into account. Additionally, we investigated a model based solely on the available climate variables without correcting for regional differences and an additional model which includes only regional effects and hence assumes that the observed numbers of outbreaks follow a random (climate independent) pattern.

Viruses and Cells

To study the biological fitness of different A/H5N1 clades in Egypt (2.2.1, 2.2.1.1, and 2.2.1.2), six viruses were obtained from the repository of the Friedrich-Loeffler-Institut (FLI), Germany as summarized in **Table 1**. Two viruses belonged to the clade 2.2.1, A/chicken/Egypt/083-NLQP/2008(H5N1) (designated 2.2.1-A), A/chicken/Egypt/0815-NLQP/2008(H5N1) (designated 2.2.1-B), one virus belonged to the early clade 2.2.1.2 (A/duck/Egypt/0897-NLQP/2008(H5N1) (designated 2.2.1.2-A), one virus of clade 2.2.1.2 originate from the recent upsurge in 2014/2015 A/turkey/Egypt/AR238-SD177NLQP/2014(H5N1) (designated 2.2.1.2-C) and a putative predecessor virus from 2013 A/chicken/Egypt/NLQP7FL-AR747/2013(H5N1) (designated 2.2.1.2-B). The last virus belonged to clade 2.2.1.1 A/chicken/Egypt/0879-NLQP/2008(H5N1) (designated 2.2.1.1) which was extensively studied as an A/H5N1 immune-escape variant in vaccinated chickens (Abdelwhab et al., 2011; Grund et al., 2011). It is antigenically distinct from clade 2.2.1.2 viruses. Furthermore, in addition to 2.2.1.1 virus, A/turkey/Egypt/R1507/2016 (designated 2.2.1.2-D) isolated in 2016 from a vaccinated turkey flock (Salaheldin et al., 2017) was used for infection of Pekin and Muscovy ducks. The 2.2.1.2-D virus was not available when the project started. Not all *in-vitro* characterization experiments were done for this virus and therefore these data (except for receptor binding) are not provided. The 2.2.1.2-D was used for the infection of ducks because it was the most recent Egyptian isolate from clade 2.2.1.2. Lastly, A/PR/8/1934(H1N1) (designated PR8), a human virus, and A/quail/California/D113023808/2012(H4N2) were used as controls in receptor binding assays. The H4N2 virus was kindly provided by Beate Crossley, UC Davis. Viruses were inoculated into the allantoic cavity of specific pathogen free (SPF) embryonated chicken eggs (ECE) for 3–5 days. Chorioallantoic fluid (AF) was tested by hemagglutination test using 1% chicken erythrocytes (OIE, 2015). Bacteria-free AF was pooled and virus was titrated by plaque assay as described below.

Madin-Darby canine kidney cells II (MDCKII) and adenocarcinomic human alveolar basal epithelial cells (A549) were obtained from the FLI. Chicken embryo kidney cells (CEK) were prepared from kidneys of 18-day-old SPF ECE (Lohmann Animal Health, Germany) according to Standard procedures.

Plaque Assay

Titration of viruses was performed in MDCKII by ten-fold serial dilutions. The cells were infected for 1 h, then washed twice with PBS and overlaid with 3 ml plaque test medium, minimal essential medium (MEM) containing 37% fetal calf serum (Sigma, Germany) and 1.8% Bacto-agar (BD, USA) at a 1:1 ratio. Trypsin was added not to the cells because HPAIV can grow in the presence of serum without trypsin. Plates were incubated at 37°C, 5% CO_2 for 3 days and then fixed with formalin containing crystal violet. The number of plaques was counted and the final titers were calculated and expressed as plaque forming unit per ml (PFU/ml). For measuring the size of plaques produced by different viruses in MDCKII, Nikon Instruments NIS elements basic research software was used.

TABLE 1 | Viruses isolated or used in this study.

H5N1 Viruses	Abbreviation	HA accession number*	Clade
VIRUSES USED IN BIOLOGICAL CHARACTERIZATION			
A/chicken/Egypt/083-NLQP/2008	2.2.1-A	CY044032	2.2.1
A/chicken/Egypt/0815-NLQP/2008	2.2.1-B	GQ184221	2.2.1
A/chicken/Egypt/0879-NLQP/2008	2.2.1.1	GQ184238	2.2.1.1
A/duck/Egypt/0897-NLQP/2008	2.2.1.2-A	JF746738	2.2.1.2**
A/chicken/Egypt/NLQP7FL-AR747/2013	2.2.1.2-B	EPI557170	2.2.1.2
A/turkey/Egypt/AR238-SD177NLQP/2014	2.2.1.2-C	EPI573268	2.2.1.2
VIRUSES USED IN DUCK EXPERIMENT			
A/chicken/Egypt/0879-NLQP/2008	2.2.1.1	GQ184238	2.2.1.1
A/turkey/Egypt/AR1507/2016	2.2.1.2-D	EPI827065	2.2.1.2

GISAID/GenBank accession numbers,Early 2.2.1.2= 2.2.1/C clade.*

Replication Kinetics

CEK, MDCKII and A549 cells were infected with 1PFU per 1000 cells in 2–4 independent assays. After 1 h, cells were washed with citrate-buffered saline (CBS) pH 3.0 to inactivate extracellular virions. Then, the cells were washed twice with isotonic PBS, and infection medium, MEM with bovine serum albumin (BSA), was added and incubated for 1, 8, 24, 48, and 72 h post-infection (hpi) at 37°C and 5% CO_2. Harvested cells and supernatants were stored at −80°C until use. The results were expressed as average and standard deviation of PFU/ml of all replicates.

Thermo- and pH Stability

The titre of indicated viruses used in biological characterization (**Table 1**) were adjusted to 10^5-10^6 PFU/ml and aliquots were incubated in duplicates at 4°C for 1, 2, 3, and 4 months. Also, duplicates were incubated at 56°C for 0, 1, 2, 3, and 4 h or were incubated with an equal volume with PBS pH 4, 5, 6, 7, or 7.4 at room temperature (20 to 22°C) for up-to 7 days followed by neutralization with sodium hydroxide. Aliquots were removed and stored at −80°C until use. The decrease in HA titre and infectivity was investigated using HA test and plaque assay, respectively. The HA test was conducted in duplicate for each replicate. The experiments were repeated twice and the average and standard deviation of each experiment were given.

Receptor Binding Assay

Affinity to avian α2,3 and human-like α2,6- sialic acid (SA) receptors was assessed using modified turkey erythrocytes (TRBCs) (Herfst et al., 2012). Briefly, SA was removed from TRBCs by incubation with Vibrio cholerae neuraminidase (Sigma-Aldrich, Germany) in the presence of calcium chloride (Herfst et al., 2012). After washing with PBS, desialylated TRBCs were suspended in PBS containing 1% BSA. Complete loss of hemagglutination of the TRBCs was confirmed by incubation with control viruses (i.e., PR8 and H4N2). Resialylation was done using α2,6-(N)-sialyltransferase (Takara, Germany) or α2,3-(N)-sialyltransferase (Sigma-Aldrich, Germany) in final concentrations of 1.5 mM Cytidine 5′-monophosphate (CMP)-sialic acid (Sigma-Aldrich, Germany). Modified TRBCs were suspended in PBS containing 1% bovine serum albumin to a final concentration of 0.5%. Resialylation was confirmed by hemagglutination of viruses using human PR8 with high affinity to α2,6-SA and H4N2 with high affinity to avian α2,3-SA receptors. HA test was done using the modified TRBCs, desialylated RBCs and original turkey RBCs (OIE, 2015). The assay was run in duplicates and repeated twice.

Experimental Infection of Ducks

The animal experiment in this study was conducted in the biosafety level 3 animal facilities of the FLI following the German Regulations for Animal Welfare after approval by the authorized ethics committee of the State Office of Agriculture, Food Safety, and Fishery in Mecklenburg—Western Pomerania. The experiment was approved by the commissioner for animal welfare at the FLI representing the Institutional Animal Care and Use Committee.

Sixty 4 to 5-week-old Pekin ($n = 30$) and Muscovy ($n = 30$) ducks were purchased from a commercial, influenza-free breeder flock. Birds were housed for 4 days before virus inoculation. Water and feed were supplied *ad-libitum*. Blood samples as well as oropharyngeal and cloacal swabs were collected pre-infection. At day of inoculation (day 0), 10 birds per group were inoculated via the oculo-nasal route with 0.2 ml inoculum containing 10^5 PFU/bird of each virus. At day 1 post-inoculation (dpi), 5 sentinel birds were added to each group. Ducks were observed daily for 14 days post-inoculation. Pathogenicity index (PI) based on clinical scoring was done as following: 0 for healthy birds, 1 for birds with one clinical sign (depression, nervous signs, respiratory signs, diarrhea, or facial oedema), 2 for birds that showed more than one clinical sign, and 3 for dead birds. Moribund birds which could not eat or drink were euthanized and scored 3 at the next day. The PI was calculated from the daily mean score of all birds during a 14-day observation period. All survived birds at the end of the experiment were anesthetized by inhalation of isoflurane (CP-Pharma, Germany), and then slaughtered and the blood was collected from the jugular veins.

To determine the level of viral excretion swabs were collected in MEM containing BSA and antibiotics from surviving ducks at 2, 4, 7, 11, and 14 dpi. Also, lung and spleen samples were collected at 3 dpi from three birds killed for histopathology as described below. Viral RNA was extracted from swabs using NucleoSpin 8/96 PCR Clean-up Core Kit (Macherey & Nagel GmbH) and from organs using NucleoMag kit according to the manufacturer instructions in automatic extraction. Real-time reverse-transcription polymerase chain reaction (RT-qPCR) targeting the matrix gene was used (Hoffmann et al., 2016). Standard curves for virus quantification were generated in each RT-qPCR plate using RNA extracted from 10-fold serial dilutions of 2.2.1.1 virus. Ct values of samples were plotted against the standard curves and the results were presented as PFU/ml.

To investigate the distribution of influenza antigens in different tissues, 3 birds per group were euthanized at 3 dpi by isoflurane inhalation and blood withdrawal. Samples were collected from trachea, lung, heart, spleen, liver, pancreas, duodenum, jejunum, cecal tonsils, bursa of Fabricius, thymus, and brains. All samples were fixed in formalin and embedded in paraffin-wax, then subjected to histopathologic and immunohistochemistry (IHC) examination. Primary anti-influenza NP-antibodies and secondary biotinylated goat anti-rabbit IgG1 (Vector) antibodies (1:200) were used to detect H5N1 antigens in different tissues (Klopfleisch et al., 2006). The intensity of signals of influenza nucleoprotein was semi-quantified by scoring on a 0 to 4 scale for tissues: 0 = negative; 1 = single cells, 2 = scattered foci, 3 = numerous foci, 4 = coalescing foci or diffuse; and on a scale of 0 to 3 for endothelium: 0 = negative; 1 = single blood vessel, 2 = multiple blood vessels, 3 = diffuse, as described previously (Klopfleisch et al., 2006).

Serum samples collected at day 0 and at the end of the experiment from surviving birds were inactivated at 56°C for 2 h and tested by a commercial enzyme linked immunosorbent assay (ELISA) targeting the NP of AIV as recommended by the manufacturer (ID Screen® Influenza A Antibody Competition

Multi-species, IDvet). The results of ELISA were confirmed using hemagglutination inhibition (HI) test against 8 HAU of the challenge viruses (OIE, 2015).

Statistic Analysis

For the duck experiment, differences in viral excretion at 2 and 4 dpi, respectively, were evaluated using Kruskal-Wallis tests followed by Wilcoxon tests using the Benjamini-Hochberg procedure for multiple testing correction. Clinical scoring was compared across groups based on the mean clinical score per bird during a 14 days observation period in the same manner. *In vitro* replication kinetics were analyzed by a repeated measures ANOVA with Benjamini-Hochberg-corrected *post-hoc* tests. All computations in this study were performed in R version 3.3.1 from the R-project website (http://www.r-project.org) with packages nlme, car, multcomp and MASS (Venables and Ripley, 2002; Hothorn et al., 2008; R Core-Team, 2015; Pinheiro et al., 2017).

RESULTS

Ambient Temperature Influenced the Prevalence of A/H5N1 Outbreaks in Examined Regions in Egypt

Data on temperature, humidity, and wind speed from 2006 to 2015 were analyzed for overall Egypt and for four selected provinces (**Figure 1**). In 2006, 185 outbreaks of avian influenza in domestic poultry were reported in Egypt. In the following 2 years, the number of outbreaks decreased to 69 cases in 2008 but increased to over 200 cases in 2009 and 2010. After a decline for the following 3 years, the number of outbreaks peaked at 445 cases in 2014/2015, where Cairo, Minya, and Luxor had the highest number of outbreaks. On the contrary, Alexandria showed a steady decrease in outbreaks from 2006 onwards.

Potential correlations of single climate factors with outbreaks of A/H5N1 in Egypt were analyzed by Spearman's correlation coefficients (ρ). As shown in **Table 2**, only weak to moderate correlations were observed for overall Egypt and also for each of the governorates. For instances, the decrease in the number of outbreaks in Alexandria correlates with an increase of humidity ($\rho = -0.61$) and the increase of outbreaks in Minya correlated with an increase of the minimum temperature per winter season ($\rho = 0.45$). However, none of the correlations were statistically significant ($P < 0.05$). Accordingly, the observed variation in A/H5N1 outbreaks could not be attributed to a single climate factor.

Nevertheless, the variation in A/H5N1 outbreaks might have been influenced by a combination of several climate factors. Therefore, five negative binomial regression models, which differed in the choice of explanatory variables (with/without climate factors and with/without regional effects, one model resulted from the variable selection procedure), were constructed for the governorate-level data. All proposed negative binomial regression models showed similar deviances and fit reasonably well to the data according to goodness of fit tests (χ^2 tests on the deviances) (**Table 3**). However, likelihood ratio tests comparing the full model with all other proposed models indicated that incorporation of both regional and climate effects significantly improved model performance. Yet some climate variables may be omitted from the full model as the difference in log-likelihood between our reduced model (resulting from the variable selection procedure) and the full model was not statistically significant. As expected, our reduced model showed the best fit regarding the AIC. It comprised regional effects, maximum temperature and average temperature as explanatory variables. The regression coefficients indicated a significantly increased baseline risk for Minya and Luxor, but not for Cairo, in comparison to Alexandria (**Table 3**; Supplementary Figure S1). Furthermore, according to this model an increase in maximum temperature (while average temperature remains unchanged) corresponded to a decrease in the number of outbreaks ($\beta = -2.38$). Conversely, an increase in average temperature (while maximum temperature remains unchanged) corresponded to an increase in the number of outbreaks ($\beta = 2.79$). Both effects were statistically significant (**Table 3**). Thus, an increase in average temperature appears to promote A/H5N1 outbreaks whereas very hot days in winter seasons presumably counteract this trend.

TABLE 2 | Correlation of climatic factors with A/H5N1 outbreaks in poultry in all Egypt and the four selected governorates.

	Temp. (min)	Temp. (max)	Temp. (average)	Humidity (%)	Wind speed
Entire Egypt	0.38	0.03	0.35	−0.18	0.29
Alexandria	−0.03	−0.03	0.17	−0.61	0.39
Cairo	0.28	0.23	0.20	0.02	−0.02
Minya	0.45	0.29	0.37	0.17	−0.14
Luxor	0.21	−0.16	0.19	0.17	−0.10

Spearman's correlation coefficient.
None of the reported correlations is statistically significant ($P < 0.05$).

This finding could be validated using the nation-level data for all Egypt. Based on this data, a negative binomial regression model using only average temperature and maximum temperature per winter season as explanatory variables indicated that an increase in average temperature increased the risk of an outbreak ($\beta = 1.79$) while an increase in maximum temperature decreased the risk of an outbreak ($\beta = -1.50$). Again, both effects were statistically significant (Supplementary Table S1, Supplementary Figure S2). Hence, even though this model represents a strong simplification of the actual outbreak scenario, it reveals a potential connection between ambient temperature and A/H5N1 outbreaks and confirms the findings of the best fitting-model for the governorate-level data.

Taken together, ambient temperature is a potential driving climate factor for A/H5N1 outbreaks in Egypt. Our results imply that average temperature of the respective winter season affected the number of outbreaks. Since all models simplify the complexity of the epidemiology of A/H5N1 in Egypt, none of them fully explains the observed variation in A/H5N1 outbreaks (Supplementary Figure S1, Supplementary Figure S2).

TABLE 3 | Negative binomial regression models for explaining the observed number of A/H5N1 outbreaks in winter season (d.f., degrees of freedom).

		Model parameters			Model performance				
		β	SE	*P*-value (Wald test)	Deviance	*P*-value (χ^2 test, goodness of fit)	AIC	2 x log-likelihood	*P*-value (Likelihood ratio test to full model)
Full model	Cairo	−1.45	1.25	0.24	39.78	0.05	206.16	−186.16	–
	Minya	6.38	3.00	0.03	(27 d.f.)				
	Luxor	4.68	4.10	0.25					
	Temp (min)	0.46	1.40	0.75					
	Temp (max)	−2.02	1.08	0.06					
	Temp (average)	1.92	2.30	0.40					
	Humidity	−0.08	0.08	0.33					
	Wind	−0.09	0.17	0.59					
Reduced model	Cairo	−0.32	0.60	0.59	39.82	0.11	201.00	−187.00	0.84
	Minya	7.53	2.27	<0.01	(30 d.f.)				
	Luxor	6.64	2.53	<0.01					
	Temp (max)	−2.38	0.79	<0.01					
	Temp (average)	2.79	0.88	<0.01					
Climate factors only	Temp (min)	0.39	0.98	0.69	39.41	0.12	211.14	−197.14	0.01
	Temp (max)	0.12	0.74	0.87	(30 d.f.)				
	Temp (average)	−0.51	1.69	0.76					
	Humidity	0.09	0.04	0.03					
	Wind	−0.29	0.16	0.06					
Regional effects only	Cairo	−0.65	0.65	0.31	39.74	0.16	208.28	−198.28	0.03
	Minya	0.86	0.63	0.17	(32 d.f.)				
	Luxor	−0.25	0.64	0.70					
Null model					39.92	0.26	208.26	−204.26	0.02
					(35 d.f.)				

Underlined values indicate significant difference ($P < 0.05$).

Viruses in Clade 2.2.1.1 and Recent 2.2.1.2 Exhibit Increased Stability at Low and High Temperature

At 4°C, all viruses survived for at least 4 months. The 2.2.1.1 virus and 2.2.1.2-B were more stable and showed the highest titres compared to the other viruses used in this study (**Figure 2A**). After 4 months, 2.2.1.1 virus and 2.2.1.2-B had 10- to 100-fold higher titres than the other viruses (**Figure 2A**). Likewise, at 56°C all viruses were relatively stable for 2 h. The recent 2.2.1.2 and 2.2.1.1 viruses were more stable than other viruses after 3 h. The 2.2.1.1 virus and 2.2.1.2-B were not totally inactivated even after 4 h (**Figure 2B**). Moreover, all viruses were stable at different pH for 7 days. 2.2.1.2-B showed a ∼10-fold decreased titre at lower pH (**Figure 2C**).

Antigenic Drift 2.2.1.1 Virus Replicated at Lower Levels in Human Lung Cells Compared to the Other Viruses

All Egyptian viruses reacted at similar levels using unmodified TRBCs (HA titre ∼256) as well as against α2,3-SA carrying RBCs (HA titre 64 to 128). The viruses varied in binding to α2,6-SA RBCs although at 2- to 16-fold lower efficiency than to avian α2,3-SA receptors. The 2.2.1.1 and 2.2.1-B reacted 2- to 8-fold less than other viruses. 2.2.1.2-D bound to α2,6-SA receptors 4-fold higher than 2.2.1.1 (**Figure 2D**). PR8 bound at similar levels to unmodified TRBCs and α2,6-SA carrying RBCs and did not bind to the avian α2,3-SA receptors. Conversely, H4N2 did not bind to α2,6-SA-TRBCs (data not shown). All viruses reached the maximum titer at 24 hpi in CEK (**Figure 3A**) and MDCKII (**Figure 3B**). Interestingly, in A549, 2.2.1.1 replicated at significantly lower titre than 2.2.1.2-C at 8, 24, 48, and 72 hpi and lower than 2.2.1-A at 24 hpi (**Figure 3C**). While 2.2.1-A produced the largest plaques, 2.2.1-B and 2.2.1.2-B produced the smallest plaques. Plaque size induced by the 2.2.1.1 virus was significantly larger than the recent 2.2.1.2 viruses (2.2.1.2-B and 2.2.1.2-C) (**Figure 3D**).

Pekin Ducks Are More Resistant Than Muscovy Ducks Toward Infection With Different Egyptian H5N1 Viruses

Pekin ducks (groups 1 and 2) were more resistant than Muscovy ducks (groups 3 and 4) after inoculation with the Egyptian H5N1 viruses of clade 2.2.1.1 and 2.2.1.2 (**Figure 4**). A total of 1/7, 0/7, 6/7, and 6/7 inoculated birds died in groups 1 to 4,

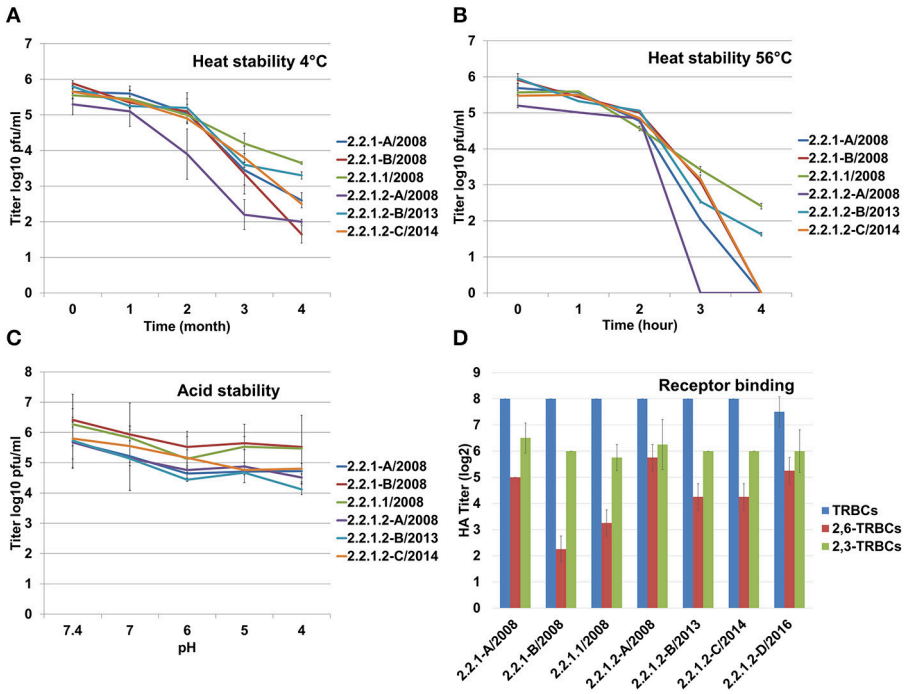

FIGURE 2 | Stability of the Egyptian H5N1 viruses at different temperatures and pHs. Heat stability at 4°C **(A)** or 56°C **(B)**, and acid stability **(C)**. **(D)** Receptor binding affinity was tested against turkey RBCs (TRBCs) and modified TRBCs carrying avian α2,3 and human-like α2,6- sialic acid. All experiments were conducted in duplicates, heat stability at 56°C and acid stability was assessed in two independent experiments. Shown are the average and standard deviations of all experiments. Titration of viruses was carried out in MDCKII cells.

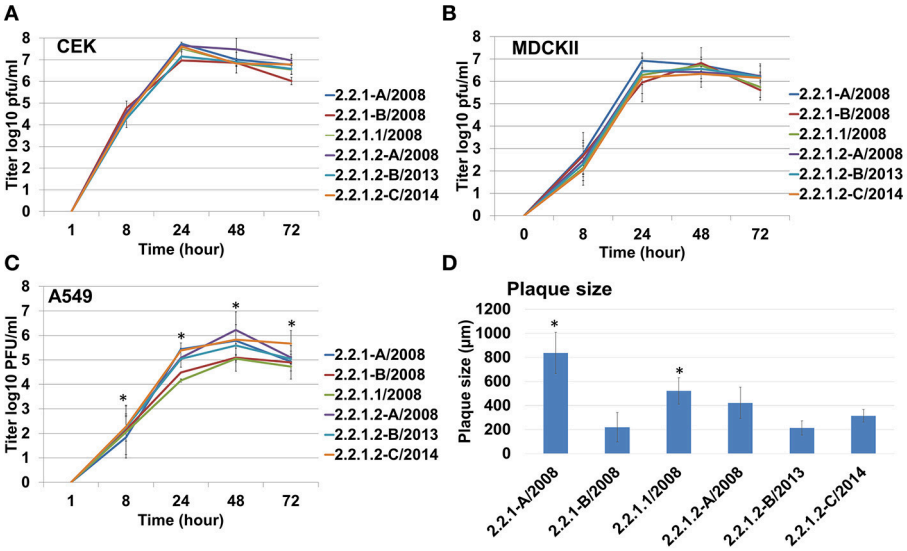

FIGURE 3 | Replication and cell-to-cell spread of different Egyptian-origin H5N1 viruses. Replication of different viruses in chicken embryo kidney (CEK) cells **(A)**, Madin-Darby canine kidney type II (MDCKII) cells **(B)**, human adenocarcinoma lung cells (A549) **(C)**. All experiments were repeated 2–4 times. Cell-to-cell spread was analyzed by measuring plaque size induced by different viruses in MDCKII cells **(D)**. *$P < 0.05$.

respectively. None of contact Pekin ducks died, whereas 5/5 and 4/5 contact Muscovy ducks died in groups 3 and 4, respectively (**Figure 4**). In group 1, all inoculated Pekin ducks except bird number 1 and 5 were clinically healthy up to the end of the experiment. Bird number 1 showed depression beginning at 4 dpi, whereas bird number 5 showed severe nervous signs and

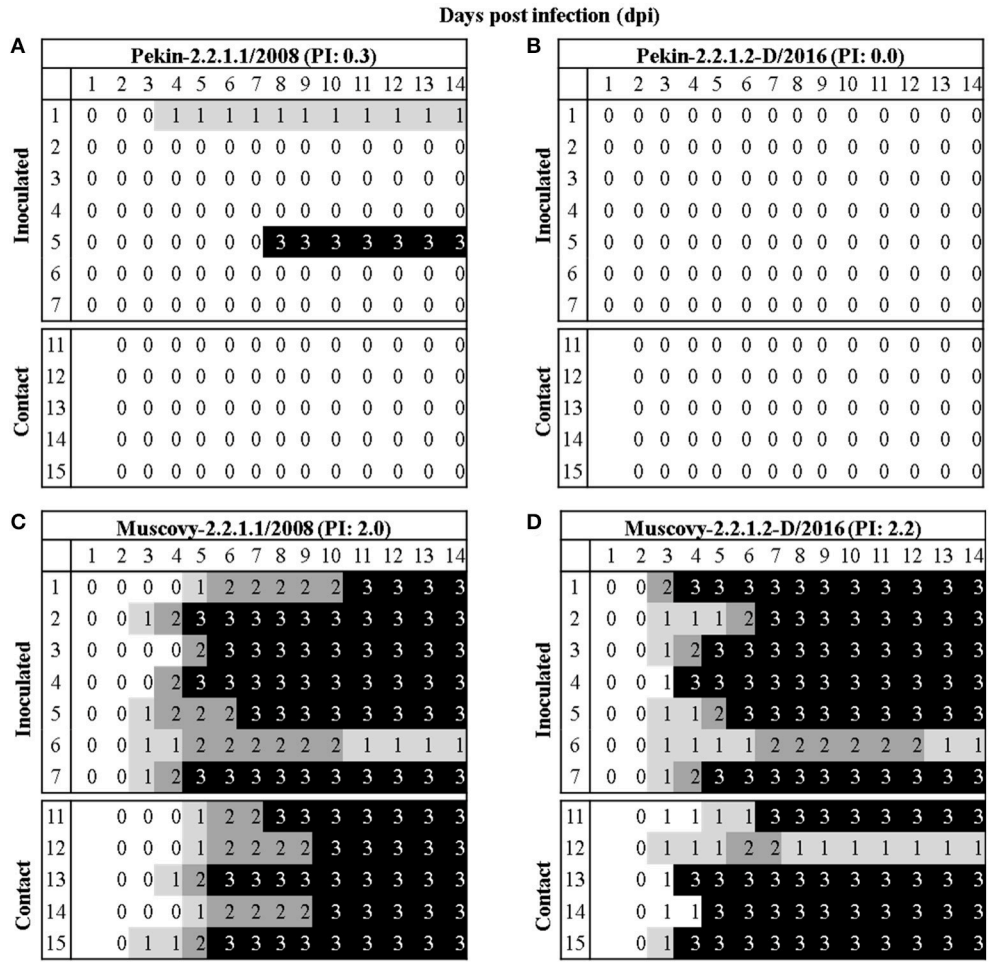

FIGURE 4 | Morbidity and mortality in Pekin and Muscovy ducks after inoculation with two different A/H5N1 of clades 2.2.1.1 and 2.2.1.2. Clinical scoring after oculonasal infection of 4–5 weeks old Pekin **(A,B)** or Muscovy **(C,D)** ducks with H5N1 viruses belonging to clades 2.2.1.1 **(A,C)** or 2.2.1.2 **(B,D)**. At 1 day post inoculation, five naïve ducks were added to assess virus transmissibility. All birds were observed for up to 14 days. Ducks without clinical symptoms were scored "0". The score "1" was applied to ducks showed one of the following clinical signs: depression, ruffled feathers, diarrhea, discharges, torticollis, opisthotonus, or rolling. These ducks were categorized as ill. Severely ill ducks showed two or more clinical signs were scored "2," whereas dead ducks were scored "3." Birds which could not eat or drink were euthanized and scored 3 at the next day of observation. The pathogenicity index (PI) for each group was expressed as the mean sum of the daily arithmetic mean values divided by 14; the number of observation days.

therefore was killed at 7 dpi and scored dead at day 8 post-inoculation (**Figure 4A**). In group 2, all inoculated Pekin ducks remained healthy. Likewise, all contact ducks in both groups were apparently healthy (**Figure 4B**). On the contrary to Pekin ducks, the majority of Muscovy ducks inoculated with 2.2.1.1 (group 3) or 2.2.1.2 (group 4) died by day 11 post-inoculation. Clinical signs started at 3 dpi with no significant difference in groups 3 and 4 ($p = 0.29$). In group 3, 6 out of 7 Muscovy birds died with a mean death time of 6.5 days and a PI of 2. All contact birds in this group died by day 10 post-inoculation after showing mild to severe clinical signs. Clinical signs started with depression and progressed quickly to become moderate to severe (**Figure 4C**). In group 4, 6 out of 7 inoculated birds died by day 7 post-inoculation with a mean death time of 5.2 days and a PI of 2.2. The onset of death in group 4 started 1 day earlier than in group 3. Four out of 5 contact birds died in group 4 by day 7. The onset of death started at 4 dpi, 2 days earlier than in group 3 (**Figure 4D**). One inoculated bird each from groups 3 and 4, and 1 contact bird from group 4 survived. However, they remained sick during the duration of the experiment showing torticollis but were able to obtain food and water.

At 2 dpi, the majority of ducks in group 1 and all ducks in groups 2, 3, and 4 excreted viruses confirmed by oral and cloacal swabs (**Figure 5**, Supplementary Table S2). Muscovy ducks excreted significantly higher amounts of the viruses than Pekin ducks. Also, the level of virus excretion in ducks inoculated with the 2.2.1.2 virus at 2 dpi was significantly higher than those infected by 2.2.1.1 virus. At day 4, the majority of Pekin ducks excreted viruses although at lower levels than Muscovy ducks. All Muscovy ducks excreted viruses orally and/or cloacally. Muscovy

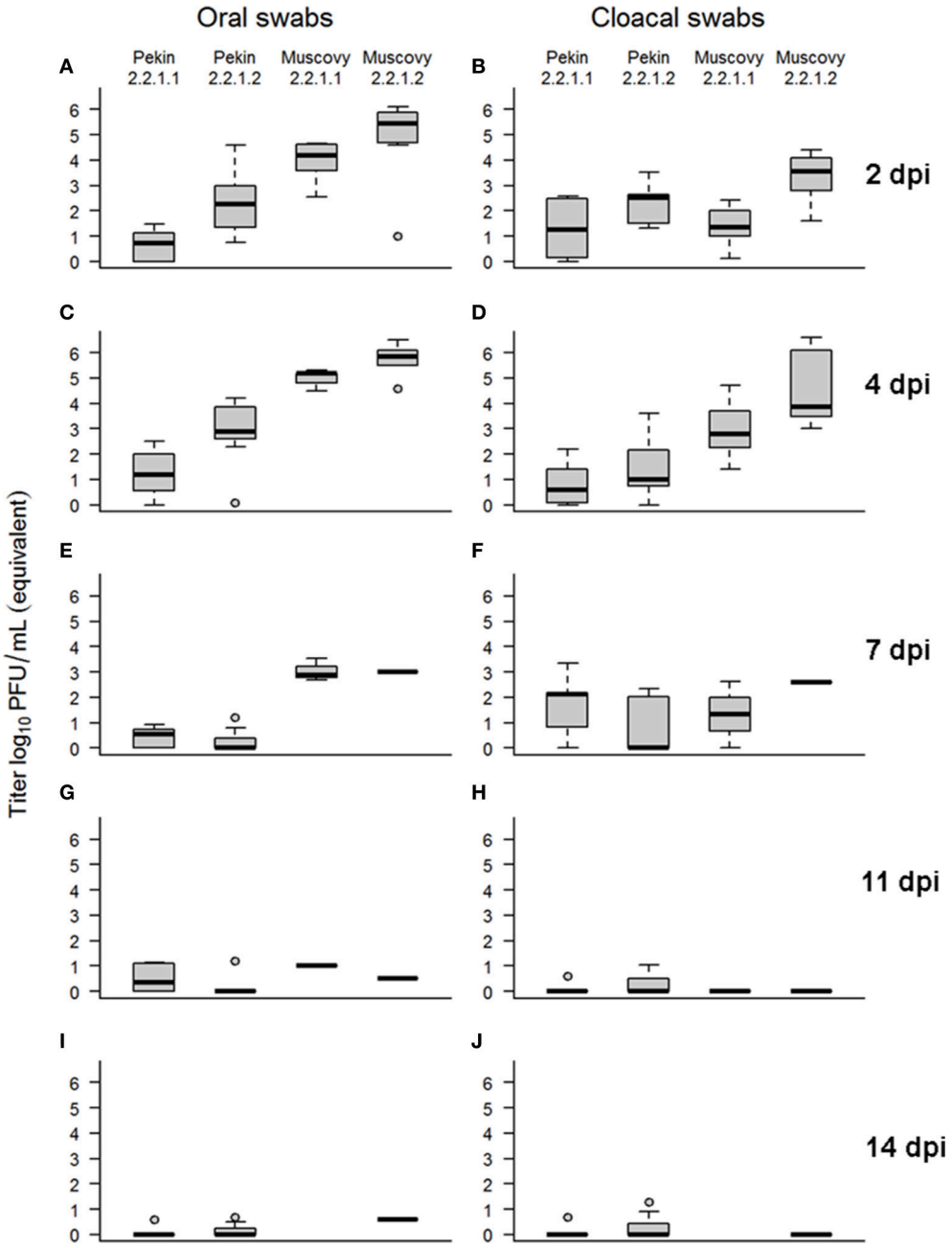

FIGURE 5 | Virus excretion in swab samples collected from Pekin and Muscovy ducks at different time points after infection with Egyptian 2.2.1.1 or 2.2.1.2 viruses. Swab samples were collected from surviving birds at 2 **(A,B)**, 4 **(C,D)**, 7 **(E,F)**, 11 **(G,H)**, and 14 **(I,J)** days post inoculation (dpi) and examined by real-time reverse transcription polymerase chain reaction. Standard curves were generated using serial dilutions of 2.2.1.1 virus. Amount of virus excretion was determined by plotting CT against the log of 2.2.1.1 dilution expressed in equivalent log10 PFU/ml.

ducks infected with 2.2.1.2 had a higher amount of virus in cloacal swabs than those infected with 2.2.1.1 ($p = 0.0078$). At 11 and 14 dpi some surviving ducks secreted viruses orally and/or cloacally, although at low levels (Supplementary Table S2, **Figure 5**).

Distribution of influenza antigen in different organs was analyzed by IHC (**Figure 6**; Supplementary Table S3), and in lung and spleen samples using RT-qPCR (Supplementary Figure S3). Breed has a major effect on distribution of A/H5N1 in different organs. No NP-antigen was detected in Pekin ducks at 3 dpi using IHC, however, using RT-qPCR, 2.2.1.1 and 2.2.1.2 viruses were detected in the lungs, but not spleen, of Pekin ducks (Supplementary Figure S3). In contrast, in primary inoculated Muscovy ducks NP antigen was detected in neuroglial

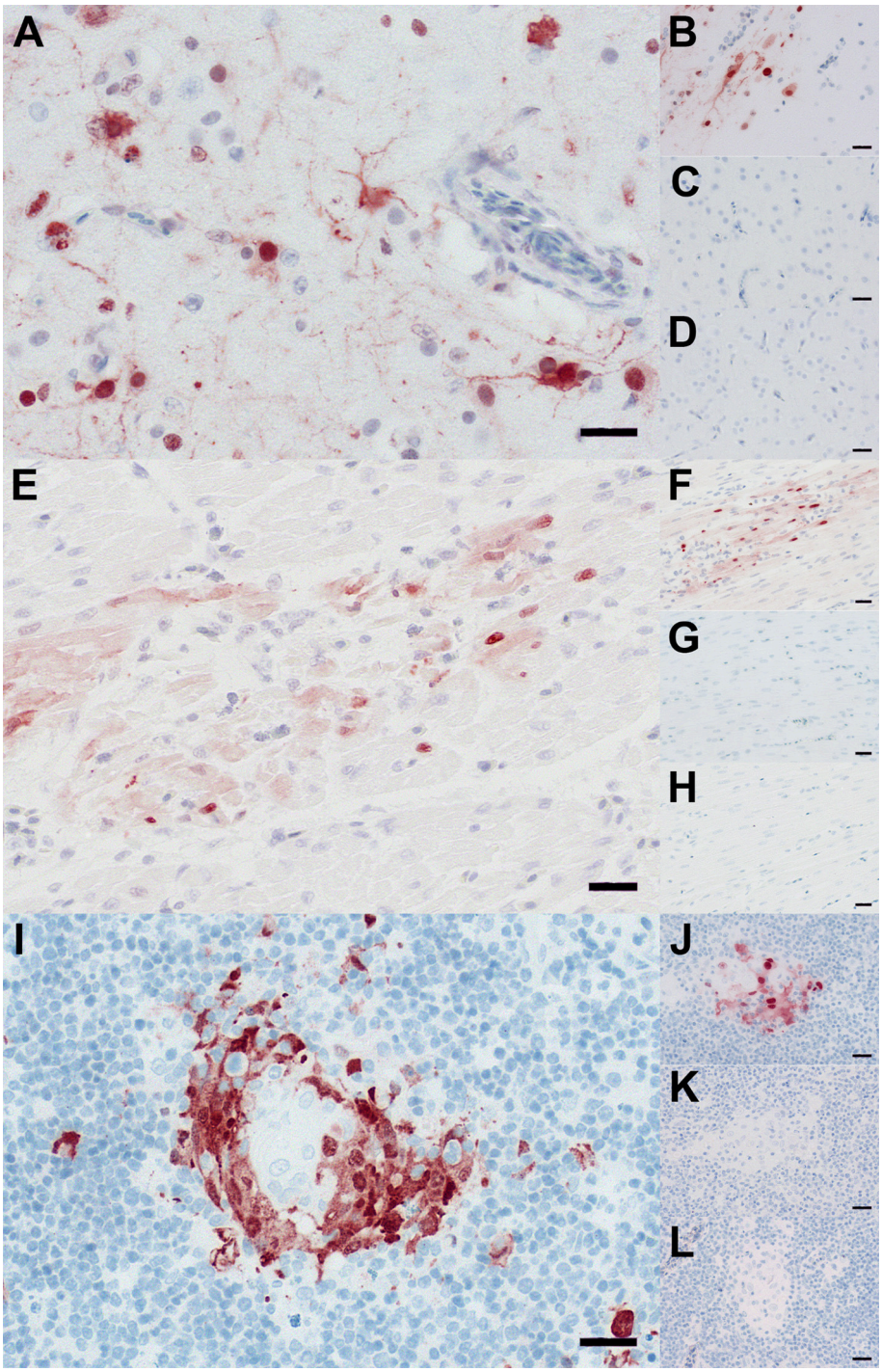

FIGURE 6 | Distribution of A/H5N1 in tissues of Pekin and Muscovy ducks. Immunohistochemistry revealed influenza A-virus nucleoprotein-antigen in neuroglial cells within the brain **(A,B)**, cardiomyocytes within the heart **(E,F)**, and thymocytes within the thymus **(I,J)** of Muscovy ducks **(A,B,E,F,I,J)**. In contrast, influenza A-virus nucleoprotein-antigen was not detected within brain **(C,D)**, heart **(G,H)**, and thymus **(K,L)** of Peking ducks **(C,D,G,H,K,L)**. Immunohistochemistry; polyclonal rabbit anti- influenza A FPV/Rostock/34-virus-nucleoprotein antiserum; avidin-biotin-peroxidase-complex method; 3-amino-9-ethyl-carbazol chromogen (red-brown); hematoxylin counterstain (blue); Bars = 20 μm. More immunostaining slides are available upon request.

cells, cardiomyocytes, and thymocytes (**Figure 6**) as well as in a contact bird (data not shown). Variation in the distribution of 2.2.1.1 and 2.2.1.2 A/H5N1 antigen in Muscovy ducks was also observed. In Group 3, the antigen was not detected in the tracheal epithelium, liver, and kidneys; conversely all three ducks in group 4 had remarkable infiltration in these organs.

In the gastrointestinal tract (duodenum, jejunum, cecal tonsils, proventriculus, and gizzard), the virus was detected only in birds of group 4, particularly in the neurons in peripheral ganglia. In Group 3, infected areas found in the lung and brain were less frequent compared to birds in group 4. In the lung of animals from group 4, virus antigen was detected in the upper and lower respiratory tract, while birds in group 3 showed viral antigen only in the bronchial epithelium. Viral antigen was not detected in endothelial cells in any organ or the circulatory system in Pekin or Muscovy ducks. Using RT-qPCR, viral RNA was also detected in the lungs and spleen of Muscovy ducks in groups 3 and 4 (Supplementary Figure S3).

Serum samples collected before infection were negative for AIV NP antibodies using ELISA. At the end of the experiment, sera from group 1 ($n = 11$), group 2 ($n = 12$), group 3 ($n = 1$), and group 4 ($n = 2$) were examined by NP-ELISA and HI test against homologous and heterologous antigens. Using ELISA, all sera post-infection were positive. Using HI test against 2.2.1.2-D antigen, all samples in groups 2 and 4 were positive (HI titer $\geq$8) with a mean titer of between 3.8 and 5 $\log_2$, in inoculated birds and 3.2 and 5 $\log_2$ in contact birds, respectively. Surviving birds in Group 1 and Group 3 were tested negative (HI titer $<$ 8). Using 2.2.1.1 antigen, the sera of inoculated birds in groups 1 and 3 reacted at similar levels with a mean titer 4.7 and 6 $\log_2$, respectively, and no cross reaction with the 2.2.1.2-D antigen was obtained.

DISCUSSION

Since the introduction of 2.2.1 clade of A/H5N1 into poultry in Egypt in 2006, the virus established an endemic status and spilled over to humans making Egypt the country with the longest endemic status outside Asia and with the highest number of human infections. Understanding the factors driving the evolution and persistence of the Egyptian A/H5N1 may enable prediction and control of future outbreaks.

In this study, climatic factors during moderate to relatively cold months (October to March) where the number of outbreaks (i) is high and (ii) allows statistical analysis were collected. In summer, the prevalence of outbreaks is very low (Arafa A. et al., 2012; Arafa A. S. et al., 2012; El-Zoghby et al., 2013) and therefore statistical analysis will be misleading. Statistical analysis of selected climatic factors in this study indicated that ambient temperature influenced the prevalence of A/H5N1 outbreaks in Egypt from 2006 to 2015. Also, the thermostability of viruses from clade 2.2.1.1 and recent clade 2.2.1.2 at 56°C degrees is remarkable. Thus, we assume that the ability of some A/H5N1 viruses to survive at elevated temperatures (i.e., in summer season when temperature is over 40°C) is an important factor for the persistence and spread of H5N1 in Egypt. In a previous study, clade 2.2.1.2 virus was isolated from domestic ducks in mid-summer in Egypt (Hassan et al., 2013). Two major waves of the A/H5N1 in Egypt were reported due to the emergence of clade 2.2.1.1 virus in vaccinated poultry in 2008-2010 and clade 2.2.1.2 virus from October 2014 to March 2015. Viruses isolated from the two waves (2.2.1.1 and 2.2.1.2-B/2.2.1.2-C, respectively) exhibited increased thermal stability than the other viruses, which may be advantageous for virus persistence in harsh environment and spread when the average temperature is moderate (i.e., in winter months). Our results are partially in accordance with the analysis conducted by Murray and Morse (2011) who found that the incidence of A/H5N1 infections in humans in Egypt in 2006-2008 was strongly associated with moderate temperature and humidity. The current study does not explain the disappearance of clade 2.2.1.1 which was probably due to the extensive vaccination using local field-strains, competition with co-circulating H9N2 viruses and/or other unknown reasons (Abdelwhab et al., 2016; Hassan et al., 2016; Naguib et al., 2017). Moreover, climatic factors alone did not explain the high prevalence of HPAIV in domestic poultry in 2014/2015. Williams et al. (2011) suggested that anthropogenic factors (human population density, movement, etc.) were important for the spread of A/H5N1 in the Middle East and Northeastern Africa (Williams and Peterson, 2009). Likewise, increased incidence of A/H5N1 outbreaks in the commercial farms in Egypt was most strongly correlated with road network distances (Young et al., 2017).

All viruses replicated well and at similar levels in avian cell culture and had binding affinity to both avian and human-like receptors, meanwhile cell-to-cell spread was mostly virus-specific. An interesting observation is that the virus in clade 2.2.1.1, which is highly adapted to (vaccinated) chickens, replicated at lower levels in human lung cells than human-like viruses in clade 2.2.1.2. Mutations in clade 2.2.1.2 viruses increased replication in human cells while maintaining the ability for replication in avian cells (Watanabe et al., 2011b). Although this topic should be further investigated using a broader panel of clade 2.2.1.1 viruses, it has been postulated that the adaptation of A/H5N1 to terrestrial poultry may prevent the evolution of human-adapted viruses (Long et al., 2015). This may partially explain the lower prevalence of clade 2.2.1.1 viruses in humans compared to viruses in clade 2.2.1.2.

Ducks exhibit mild or no clinical signs after infection with A/H5N1 and, therefore, play an important role in the genesis and silent transmission of highly pathogenic viruses to susceptible gallinaceous poultry and probably to humans (Fan et al., 2014; Lee et al., 2014). However, the pathogenicity of H5N1 viruses in ducks may vary according to the duck species, age of ducks, virus strain and inoculation route (Pantin-Jackwood and Swayne, 2009; Szeredi et al., 2010; Cagle et al., 2011; Pantin-Jackwood et al., 2012, 2013; Yuan et al., 2014). In the recent 2014/2015 upsurge in poultry and humans in Egypt, ducks were speculated to be a major source for infection (Arafa et al., 2015). In this study, Muscovy ducks proved to be more sensitive than Pekin ducks which is in accordance with previous studies (Guionie et al., 2010; Cagle et al., 2011). Nearly all inoculated and in-contact Muscovy ducks died after infection with human-like clade 2.2.1.2 virus. Conversely, none of the Pekin ducks showed clinical signs or mortality, while producing antibodies and

excreting a considerable amount of virus from the respiratory and digestive tract for up to 14 dpi. Viral RNA was detected in the lungs at 3 dpi. The virus was also transmitted to sentinel Pekin ducks without affecting health as shown by virus excretion in swabs and seroconversion. Previously, two Egyptian viruses from 2007 and 2008 from clade 2.2.1 and early clade 2.2.1.1 killed all 2-week-old Muscovy ducks, while 2008-virus killed all 2-week-old Pekin ducks and 2007-virus killed only 10 to 30% depending on the route of inoculation (Pantin-Jackwood et al., 2013). Furthermore, all Pekin ducks infected with Turkish A/H5N1 of clade 2.2 at 8-weeks-old died, while 12-week-old Pekin ducks survived the challenge without significant impact on the amount of virus excreted in both groups (Londt et al., 2008, 2010). Pekin and Muscovy ducks challenged intranasally with a clade 2.3.4 A/H5N1 died, after developing neurological signs, within 3.6 and 3.1 days, respectively (Cagle et al., 2011). Variable pathogenicity in domestic ducks may be due to variation in the immune response between the two breeds (Cagle et al., 2011). It is worth mentioning that virus excretion from "silently" infected ducks in this study is in accordance with intermittent excretion of Chinese A/H5N1 from clinically healthy ducks for up to 17 dpi (Hulse-Post et al., 2005). Together, the silent infection of Pekin ducks particularly with human-like clade 2.2.1.2 virus poses public health hazards and intervention strategies (e.g., targeted surveillance in Pekin ducks, segregation of Pekin ducks from backyards, etc.) should be considered.

Limitations of the Study

Some limitations for the current study should be considered: statistical analysis was conducted for a limited number of regions only, using national surveillance data (i.e., reported outbreaks) and a limited number of factors analyzed herein. Our model reveals an association between temperature and spread of A/H5N1. However, other factors can not be excluded. Moreover, we collected data for the outbreaks from official reports for national surveillance conducted by the ministry of agriculture in cooperation with the FAO. However, these numbers likely do not represent all cases observed in the field. Underreporting of outbreaks in Egypt is not uncommon due to lack of compensation for culling of infected flocks, false information on protection of poultry by vaccination, and masking infection (silent infection) due to partial protection induced by the vaccines (Vergne et al., 2012). Also, although governorates in this study are well known for their high-density poultry population, we did not find accurate estimates for annual poultry density in these four regions which may affect the number of outbreaks. Importantly, climatic weather for governorates in the Nile delta were not fully available. Lastly, we analyzed only a limited number of factors, while other potential important parameters such as elevation (Loth et al., 2011), chicken density (Pfeiffer et al., 2007), species of birds and density of human population (Gilbert et al., 2008b), and movement of birds, particularly for marketing or hatcheries to rearing areas should be included in future models. Likewise, the impact of seasonal variation on movement or migration of feral or wild birds should be also considered. Biological fitness was assessed using representative viruses from each clade; however, more viruses particularly from clade 2.2.1.1 should be analyzed in the future. Clinical outcome in inoculated ducks with A/H5N1 may be affected by viral doses and ages of ducks (Londt et al., 2010; Pantin-Jackwood et al., 2012) which should be considered in the future. In summary, our study sets a baseline on the importance of several parameters in shaping the epidemiological situation of A/H5N1 infection in poultry and humans in Egypt.

AUTHOR CONTRIBUTIONS

EA, TM, JV, and HH conceived and designed the study. AS, DS, and EA conducted the animal experiment. EK, HE, and EA collected data and conducted the climatic factors analysis. MH, AA, WH, and HA provided the official data on the outbreaks in Egypt and/or viruses used in this study. RU conducted the histopathological analysis. EK and EA conducted the statistical analysis. AS, DS, MG, and EA conducted the *in-vitro* characterization. EA wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00528/full#supplementary-material

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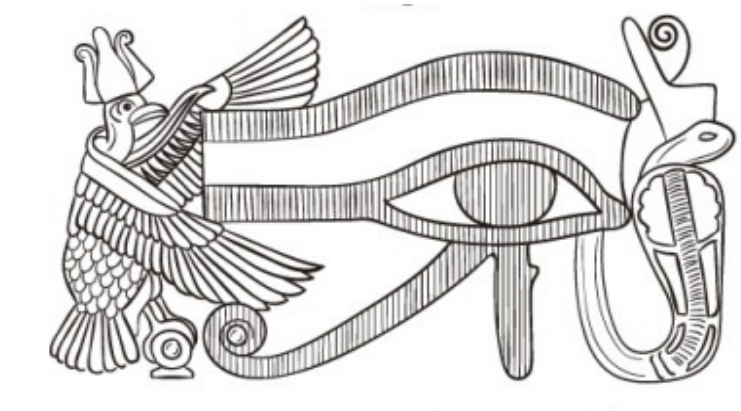

Chapter 4

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Multiple introductions of influenza A(H5N8) virus into poultry, Egypt, 2017

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Multiple Introductions of Influenza A(H5N8) Virus into Poultry, Egypt, 2017

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After high mortality rates among commercial poultry were reported in Egypt in 2017, we genetically characterized 4 distinct influenza A(H5N8) viruses isolated from poultry. Full-genome analysis indicated separate introductions of H5N8 clade 2.3.4.4 reassortants from Europe and Asia into Egypt, which poses a serious threat for poultry and humans.

In Egypt, highly pathogenic avian influenza A(H5N1) clade 2.2.1 virus was introduced to poultry via migratory birds in late 2005 (*1*) and is now endemic among poultry in Egypt (*2*). Also in Egypt, the number of H5N1 infections in humans is the highest in the world, and low pathogenicity influenza A(H9N2) virus is widespread among poultry and has infected humans (*2*). Despite extensive vaccination, H5N1 and H9N2 viruses are co-circulating and frequently reported (*2*). In 2014, highly pathogenic avian influenza A(H5N8) virus clade 2.3.4.4 was isolated, mostly from wild birds, in several Eurasian countries and was transmitted to North America. However, in 2016 and 2017, an unprecedented epidemic was reported in Asia, Africa, and Europe (*3*). In Egypt, during November 30–December 8, 2016, a total of 3 H5N8 viruses were isolated from common coot (*Fulica atra*) (*4*) and green-winged teal (*Anas carolinensis*) (*5*). To provide data on the spread of the virus in poultry, we genetically characterized 4 distinct H5N8 viruses isolated from commercial poultry in Egypt in 2017.

During February–May 2017, a high mortality rate was observed for 48 poultry flocks in the Nile Delta, Egypt. Up to 20 tracheal and cloacal swab samples were collected from each flock for initial diagnosis by reverse transcription PCR and virus isolation at the Faculty of Veterinary Medicine, Damanhour University (Damanhour, Egypt). Results were positive for H5N8 virus in samples for 4 flocks not vaccinated for H5 in 3 governorates (Figure). Sudden deaths also occurred in 3 broiler chicken flocks (Ck12, Ck15, Ck21) and 1 duck flock (Dk18); mortality rates were 29%–52% (online Technical Appendix 1 Table 1, https://wwwnc.cdc.gov/EID/article/24/5/17-1935-Techapp1.pdf). No epidemiologic links between farms were observed.

Positive samples were spotted onto FTA cards (*6*) and submitted to Friedrich-Loeffler-Institut (Insel Riems-Greifswald, Germany), where H5N8 virus was confirmed

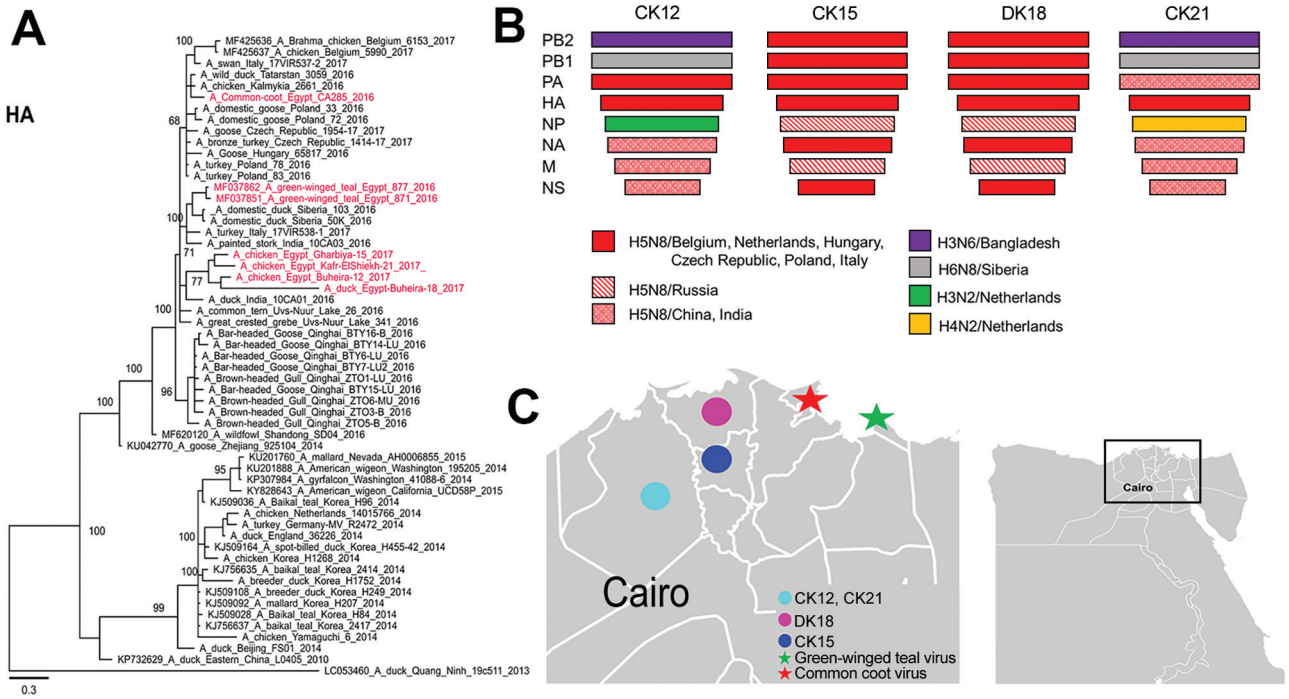

Figure. Characterization of highly pathogenic avian influenza A(H5N8) viruses of clade 2.3.4.4 from Egypt, 2017. A) Phylogenetic relatedness of the HA gene and schematic representation of potential precursors of different H5N8 viruses. The maximum-likelihood midpoint rooted tree was constructed by using MrBayes (http://mrbayes.sourceforge.net/). Gray indicates viruses from this study. Scale bar indicates nucleotide substitutions per site. B) Putative ancestors of the different gene segments of H5N8 viruses from Egypt characterized in this study compared with reference viruses. C) Governorates in Egypt where H5N8 viruses had been reported in domestic birds (circles) and where viruses in birds had been previously reported (stars). Inset shows study location in Egypt. Ck, chicken farm; Dk, duck farm; HA, hemagglutinin; M, matrix; NA, neuraminidase; NP, nucleocapsid protein; NS, nonstructural; PA, polymerase acidic; PB, polymerase basic.

by reverse transcription PCR and full-genome sequences (*7*) from 4 viruses (GISAID [https://www.gisaid.org/] accession nos. EPI1104268–EPI1104299) (online Technical Appendix 2, https://wwwnc.cdc.gov/EID/article/24/5/17-1935-Techapp2.pdf). We retrieved sequences with high similarity and all H5N8 virus sequences from GISAID and GenBank and aligned them by Multiple Alignment using Fast Fourier Transform (https://mafft.cbrc.jp/alignment/server/index.html). The most highly related viruses are summarized in online Technical Appendix 1 Table 2. We calculated sequence identity matrices in Geneious (https://www.geneious.com/) (online Technical Appendix 1 Figure 1) and studied phylogenetic relatedness to H5N8 virus isolated in Eurasia and in Egypt by using IQtree (http://www.iqtree.org/). Representative viruses were selected for generation of maximum-likelihood midpoint rooted trees by MrBayes (http://mrbayes.sourceforge.net/) using a best-fit model (GTR+G) (*8*) and were further edited by using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape (https://inkscape.org/en/).

The hemagglutinin (HA) and neuraminidase (NA) genes of the 4 viruses shared 95.8%–99.2% nt and 93.1%–99.4% aa identity and shared 96.5%–99.2% nt and 94.2%–99.7% aa identity with viruses from wild birds in Egypt (*4*,*5*). Other segments showed 92.6%–99.6% nt and 96%–99.7% aa identity, where the polymerase acidic (PA) genes and proteins of viruses from Dk18 showed the lowest similarity to those of other viruses (online Technical Appendix 1 Figure 1).

All viruses possess the polybasic HA cleavage site PLREKRRKR/G and contain mammal-adaptation and virulence markers (*9*) in polymerase basic (PB) 2 (T63I, L89V, G309D, T339K, Q368R, H447Q, R477G), PB1 (A3V, L13P, K328N, S375N, H436Y, M677T), PA (A515T), HA (T156A, A263T; H5 numbering), matrix (M) 1 (N30D, T215A), and nonstructural (NS) 1 (P42S, T127N, V149A) proteins. Therefore, protection of humans and risk assessment of bird-to-human transmission is crucial. The NS1 protein from viruses from Ck15 and Ck18 is 217 aa long because of truncation in the C-terminus, whereas NS1 of the other H5N8 viruses from Egypt are 230 aa long. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis indicated that these 4 viruses differ from viruses isolated from birds in live bird markets in Egypt in 2016 (*4*,*5*). Gene segments were closely related to viruses isolated from wild birds, poultry, and zoo birds in Europe (including Belgium, Czech Republic, the Netherlands, Poland, Hungary), Russia, and Asia (including Bangladesh, China, India) (Figure; online Technical Appendix 1 Figures 2, 3).

HA of the 4 H5N8 viruses in this study clustered in 1 distinct branch (Figure), and NA clustered in 2 phylogroups (online Technical Appendix 1 Figure 2). The PB2, nucleoprotein, M, and NS genes of viruses from Ck12 and Ck21 (from chickens in the same governorate, February and May 2017) clustered together, and the same genes from viruses from Dk18 and Ck15 (from ducks and chickens in 2 governorates) clustered in 2 distinct phylogenetic groups. However, viruses from Ck12 and Ck15 have similar but not identical PA gene segments (online Technical Appendix 1 Figure 3).

These data suggest 4 different introductions of H5N8 virus into poultry in Egypt, independent of viruses isolated from captive birds (*4*,*5*). Multiple separate introductions of H5N8 virus into Europe also occurred (*10*). Further studies are needed to identify the source(s) of introduction. The separate introductions of different reassortants of H5N8 clade 2.3.4.4 virus from Europe and Asia into Egypt indicate a serious threat for poultry and human health.

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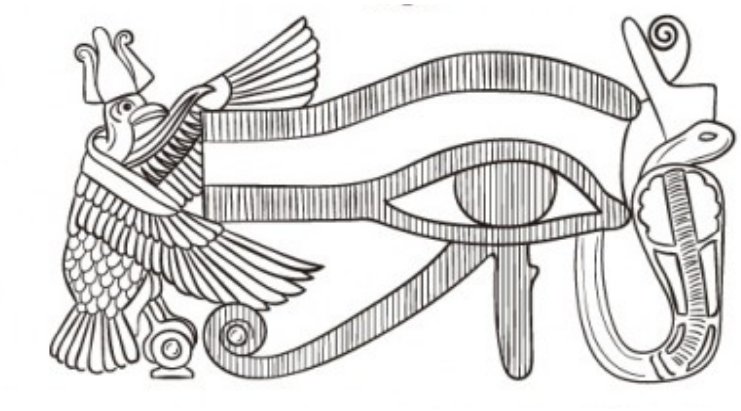

Chapter 5

General Discussion

General discussion

The rapid and intercontinental spread of avian influenza viruses (AIV) causing tremendous socioeconomic losses in poultry and humans and is the best example for "One world, One health" concept. The geographic location of Egypt connecting the Asian, African and the Mediterranean countries and a long two migration flyways poses a continuous threat for the introduction and "spillover" of transboundary animal diseases. Therefore, the endemic situation of AIV in poultry and continuous human infections in Egypt is of high concern to the international organizations for animal and human health (e.g. OIE, WHO, FAO). The co-circulation of H5N1 subtype with divergent H5N8 and H9N2 viruses in Egypt poses a serious threat for both poultry and human health. There is a need for strong public health vigilance and monitoring on the evolution of AIV in Egypt and to better understand the epidemiology and factors contribute to the long-term persistence in poultry.

After the re-emergence of the HPAIV H5N1 in 2003 in China, Egypt started intensive surveillance in poultry and wild birds to early detect and control the virus through the immediate culling of birds, restriction of movement of poultry and eggs to and from the infected farms and improve the biosecurity measures. In February 2006, the HPAIV H5N1 of clade 2.2.1 was isolated in limited numbers of backyards and LBM in 3 out of 27 governorates. Within few weeks HPAIV H5N1 was reported nationwide and vaccination was used as an ancillary control measure to the main test-and-slaughter-campaign (133, 167, 175). Vaccination was successful to decrease the number of outbreaks in 2006 (176). However, in 2007, the number of outbreaks in the commercial sector dramatically increased due to the emergence of antigenic-drift variants in the novel clade 2.2.1.1. Viruses in the backyard sector and humans evolved at lower rate than in commercial farmed chickens (177). In 2008, human-like H5N1 viruses from backyard birds and humans in Egypt clustered in 2.2.1.2 clade (156, 178-181). In 2010-2011, the prevalence of 2.2.1.1 viruses decreased significantly and no longer isolated since 2014 (182). The use of updated-vaccines seeded by local strains, the cross-protection caused by the H9N2 in 2010, the illegal application of amantadine, and/or the higher rate of mutations and fitness cost of this clade in addition to other unknown factors were responsible for the extinction of viruses from clade 2.2.1.1 since 2014(125, 154, 170, 183, 184). However, in 2014-2015, an unprecedented upsurge of A/H5N1 clade 2.2.1.2 occurred in poultry and subsequently in humans marking Egypt an influenza epicentre outside Asia (159, 185, 186).

Several recombinant and inactivated vaccines are used to prevent H5N1 outbreaks in poultry in Egypt. Seed viruses in these vaccines are highly variable representing viruses from different clades belonging to north American and Eurasian lineages as well as local field viruses isolated in 1968 to 2010 (table 1).

In the first publication of the present work, a novel HPAIV H5N1 belonging to clade 2.2.1.2a was isolated from an outbreak in a vaccinated meat-turkey flock in Egypt (TK2016). In general, turkeys are highly susceptible to AIV than other domestic poultry species (187-189). Therefore, vaccination of turkeys with H5 vaccines was implemented in the field to protect turkeys from lethal infection by HPAIV in many countries as well as in Egypt (190). However, recommendations for vaccination of turkeys are mostly given based on experiments in chickens. In contrast to chickens, single vaccination of turkeys at day seven was not successful to protect the birds against the Egyptian HPAIV H5N1 (191). Little is known about the protection of H5-vaccinated turkeys under field conditions and performance before, during and after the outbreak. The current turkey flocks were vaccinated twice with inactivated H5N2 and H5N1 vaccines at day 8 and day 34 of age, respectively, 38 day post the last vaccination birds showed respiratory and up to 29 % mortality within 10 days was recoded. Birds showed reduction in body weight and food-conversion ratio and some birds survived to the end of the fattening period (i.e. to 27 weeks of age).

Many factors probably contributed to this “vaccinal break” including vaccine mismatch to the circulating strain, suboptimal protection of turkeys after two vaccinations or immunosuppression. Antigenic characterization of isolated virus (TK2016) showed weak cross reactivity against serum sample generated against clade 2.2.1.1 and also against HPAIV H5N8 (clade 2.3.4.4), which is a descendent clade from the vaccine strain. Moreover, the full genome sequencing showed a considerable number of mutations in all gene segments compared to the vaccinal strains. Many of these mutations were found in the HA and NA immunogenic epitopes, which may be linked to the lethal infection in vaccinated turkeys. Moreover, there is a need for accredited vaccination programs specifically for turkeys and particularly in endemic situations, where maternal antibodies can interfere with early vaccination of birds (119-122). Importantly, TK2016 strain possess genetic signatures of both antigenic drift variants and human-like viruses. Phylogenetic analysis indicated that similar viral strains were reported in neighboring countries too. Therefore, the spread of zoonotic HPAIV H5N1 in the commercial poultry sector in Egypt and spillover to poultry in other countries pose a serious health threat for both poultry and humans in the Middle East. The

control measures should be taken on national and international levels. In endemic situations of AIV in poultry, as the situation in Egypt, relying on the vaccine, as a sole control measure is very common, which is inadequate for successful eradication of the disease.

In the second publication of this dissertation, we aimed at understanding the potential factors contribute to the persistence of HPAIV H5N1 in Egypt since 2006. A part from the insufficient protection of vaccinated birds in the commercial sector and extensive genetic analysis of HPAIV H5N1 in Egypt; there are gaps of knowledge on the impact of climatic factors, biological properties and the biological reservoir of the virus to foster the endemicity of AIV in poultry. Field observations described an "epidemiological twist" of HPAIV H5N1 in Egypt after 2008, where outbreaks were not only restricted to winter months (November to January) but also in hot summer seasons (June to September) (192, 193). In addition, the number of outbreaks differs from winter season to another (156, 158, 178, 192), which suggested that climatic factors play a role in the persistence or emergence of HPAIV H5N1 in Egypt. Our statistical analysis for the correlation of number of outbreaks in Egypt from 2006 to 2015 with the ambient temperature, relative humidity and wind speed was indeed useful. We showed that the moderate ambient temperature in winter months correlated with the higher number of HPAIV H5N1 outbreaks in different regions in Egypt. This can be helpful to intensify control and biosecurity measures particularly in these months.

A second notion was the possible fitness of some HPAIV H5N1 to resist high temperature in summer and stay "viable" in/on inanimate objects for the next winter, where the conditions favor widespread. It is known that avian influenza viruses remain infectious in faecal materials, water or the environment for longer periods according to the temperature in addition to other factors (e.g. pH and salinity) (194, 195). We compared the inactivation of different clades of the Egyptian HPAIV H5N1 at high temperature. Strikingly, viruses isolated during the upsurge in 2008-2010 and 2014 have higher thermal stability than other viruses. The correlation of ambient temperature with the number of outbreaks in poultry together with the ability of some HPAIV H5N1 to remain infectivity in high temperature lead to the assumption that the endemic HPAIV H5N1 has progressively adapted to environmental conditions (e.g. temperature) in Egypt.

A third factor, which is a missing link in the epidemiology of HPAIV H5N1 in Egypt compared to other Asian countries (196, 197), is domestic ducks. It is widely accepted dogma that wild waterfowl are responsible for long-distance transmission of AIV; hence, the infection is silent but the virus is excreted in a considerable amount. Domestic breeds of

ducks showed variable resistance to HPAIV according to the breed and age of ducks as well as the involved strain , dose and route of infection. Muscovy and Pekin ducks are more susceptible than Mallards to some HPAIV (124, 198-204). Surveys in poultry in Egypt revealed higher prevalence of HPAIV in clinically healthy ducks particularly in household and LBM sectors (186, 205). However, susceptibility of ducks to different Egyptian HPAIV H5N1 from 2.2.1.1 and 2.2.1.2 remained unknown. In the present dissertation (publication 2), Muscovy and Pekin ducks were infected with two virusal strains representing antigenic-drift 2.2.1.1 and human-like 2.2.1.2 clades. Muscovy ducks died within few days post infection after showing severe neurological signs, while Pekin ducks were apparently healthy, survived, and excreted the virus from the respiratory and digestive tracts for up to 14 days post infection. The clinical resistance of Pekin ducks to HPAIV H5N1 can be a major factor for the persistence of the virus in poultry in Egypt. Moreover, the close genetic relationship of HPAIV H5N1 from ducks and humans in Egypt warrants targeted surveillance in (Pekin) ducks and separation from backyard birds and humans nationwide.

In the third publication of this dissertation, the isolation of HPAIV H5N8 clade 2.3.4.4 from commercial chickens and ducks in different governorates in the Nile Delta and Northern of Cairo, was reported. The H5N8 virus was firstly reported in China in 2010 and has spread rapidly via migratory wild aquatic birds. Since 2014, HPAIV H5N8 of clade 2.3.4.4 devastated the poultry industry in several countries in Asia, Europe and America and recently in Africa (59, 81). Many bird species, including wild and domestic waterfowl, domestic poultry, and zoo birds are susceptible for the infection and seem to be responsible for transmission and spread of the virus. Compared to previous HPAIV H5N1, the H5N8 in 2016 showed higher virulence in wild ducks (206-208). In Egypt, two previous reports described the isolation of three distinct HPAIV H5N8 2.3.4.4b viruses from wild birds (141, 142). In the present investigation, we isolated four viruses from commercial chickens and ducks farms vaccinated against H5 and showing high mortality. The full genome sequences of the four viruses in this study were distinct from those isolated from wild birds (141, 142) and revealed close relationship to viruses isolated from wild and commercial birds in Europe, Russian and Asia. All four viruses carried different gene constellations suggesting four independent introductions of H5N8 into commercial poultry in Egypt. These findings revealed the wide diversity of the panzootic HPAIV H5N8 in wild and domestic birds and illustrated the importance of Egypt as a bridge for the transmission of AIV from Eurasia into Africa. Furthermore, the isolation of these viruses in H5-vaccinated birds and the poor cross-reactivity to the current Egyptian H5N1 2.2.1.2 virus (publication 1) merit the need for

effective vaccines to protect poultry against both H5N1 and H5N8 in Egypt. Moreover, many genetic signatures linked to the adaptation of AIV in mammals were found in the Egyptian HPAIV H5N8, which pose a zoonotic threat.

In conclusion, vaccination of turkeys was not sufficient to prevent infection, morbidity and mortality due to the infection with novel 2.2.1.2a HPAIV H5N1 under field conditions in Egypt. Thermostability, an adaptation trait of the endemic HPAIV H5N1 to harsh environmental conditions (e.g. ambient temperature), and climatic factors are additional factors that foster the persistence of the virus in Egyptian poultry. Pekin ducks are clinically resistant to the Egyptian HPAIV H5N1 and excrete the virus for long period. Thus, targeted surveillance in Pekin ducks, separation of ducks from chickens and humans in backyard birds or temporary prohibition of keeping Pekin ducks should be considered to decrease the risk of H5N1 transmission to poultry and humans in Egypt. The panzootic H5N8 clade 2.3.4.4 was isolated from commercial poultry in early 2017 after the isolation of H5N8 viruses from wild birds in November 2016. That means that the introduction of other AIV subtypes from Eurasia in the future should be expected. The co-circulation of two antigenically distinct H5Nx viruses merit the need for continuous update of the vaccines. Nevertheless, the enforcement of biosecurity measures remains the gold standard to prevent the infection of poultry with AIV.

4. Summary

Avian influenza H5N1 clade 2.2.1 and H5N8 clade 2.3.4.4 viruses were introduced into Egypt by wild birds in February 2006 and November 2017, respectively.

The H5N1 virus has evolved into several genetic and antigenic distinct clades/subclades: 2.2.1.1 clade dominated viruses in the commercial sector of poultry, while 2.2.1.2 was prevalent in humans and backyards. Likewise, similar diversity was seen among the newly emerging H5N8 viruses. These viruses caused significant losses in the poultry industry and posed a public health threat. In this dissertation, some aspects of the epidemiology of H5N1 and H5N8 viruses in poultry in Egypt are described.

In chapter two, a novel H5N1 was isolated from commercial turkey flock vaccinated with inactivated H5 vaccines. The virus was genetically distinct from the parent 2.2.1.2 viruses and therefore was allocated in a new phylogroup designated 2.2.1.2A along with viruses from poultry in Israel and Gaza. Viruses in this group carried mutations in the receptor binding domain resembling those 2.2.1.2 viruses isolated from humans in Egypt. In addition to other mutations in the immunogenic epitopes correlated with antigenic drift of 2.2.1.1 viruses in vaccinated birds. The spread of such viruses in poultry in Egypt and neighboring countries emphasized the need to revise the current control measures (i.e. massive vaccination) to mitigate the socioeconomic losses in both poultry and humans.

In chapter three, the effect of climatic and biological factors on the endemicity of H5N1 in Egypt were investigated. The ambient temperature and increased thermostability of the involved strains are probably two important factors for persistence and/or spread of the virus in poultry in Egypt. In addition, while Muscovy ducks were severely affected and exhibited high mortality after infection with 2.2.1.1 and 2.2.1.2a H5N1 viruses, Pekin ducks were apparently healthy and excreted viruses for up to 14 days post inoculation suggesting an important role as a reservoir.

In chapter four, the first record for isolation of the panzootic H5N8 viruses of clade 2.3.4.4b from four commercial poultry farms in early 2018 was described. Strikingly, the four viruses possessed different gene constellations giving evidence for multiple and independent introductions of H5N8 viruses into Egypt. Interestingly, viruses isolated from commercial poultry were different from the H5N8 viruses isolated from captive birds in Egypt in late

2017. The Egyptian H5N8 viruses were closely related to divergent avian influenza viruses isolated from wild birds and commercial poultry in Europe, Asia and Russia.

Findings described in this dissertation are important to better understand the epidemiology of the endemic and emerging avian influenza viruses in Egypt, spillovers to poultry in neighboring countries and multiple introductions from Europe and Asia into Africa. Continuous monitoring and surveillance is needed. Effective biosecurity measures should be applied to prevent the infection of poultry with AIV and /or to reduce the spread of infection.

5. Zusammenfassung

Epidemiologische Untersuchungen und molekulare Charakterisierung von aviären Influenzaviren bei Geflügel in Ägypten.

Aviäre Influenzaviren der Subtypen H5N1 (*Clade* 2.2.1) und H5N8 (*Clade* 2.3.4.4b) wurden im Februar 2006 bzw. November 2017 von Wildvögeln nach Ägypten eingetragen.
Das H5N1-Virus hat sich im Geflügel zu diversen genetischen *Clades* / *Subclades* weiterentwickelt: Während Vertreter der *Clade* 2.2.1.1, bei geimpftem Geflügel im kommerziellen Sektor dominierten, waren bei Menschen und privaten Kleinhaltungen Viren der *Clade* 2.2.1.2 vorherrschend. Eine vergleichbare Diversität wurde auch unter den neu eingetragenen H5N8-Viren beobachtet. Diese Viren verursachten erhebliche wirtschaftliche Verluste in der Geflügelindustrie und stellten darüber hinaus eine Gefahr für die öffentliche Gesundheit dar. In dieser Dissertation werden einige Aspekte der Epidemiologie von H5N1 und H5N8-Viren bei Geflügel in Ägypten näher beleuchtet.

In Kapitel 2 wird die Isolierung eines neuen H5N1-Virus aus einer kommerziellen Putenherde beschrieben, die zuvor mit inaktivierten H5-Impfstoffen geimpft war. Das isolierte Virus war genetisch mit den *Clade* 2.2.1.2- Viren nicht identisch und wurde in eine neue Phylogruppe, 2.2.1.2A, zusammen mit Viren, die bei Geflügel in Israel und Gaza nachgewiesen worden waren, eingeteilt. Viren in dieser Phylogruppe wiesen Mutationen in der rezeptorbindenden Domäne auf, die denjenigen der *Clade* 2.2.1.2-Viren ähnelten, die von Menschen in Ägypten isoliert worden waren. Darüber hinaus wurden weitere Mutationen in den immunogenen Epitopen festgestellt, welche mit der antigenen-*Drift* von *Clade* 2.2.1.1-Viren bei geimpften Puten korrelierten. Die Verbreitung derartiger Viren beim Geflügel in Ägypten und in den Nachbarländern unterstreicht die Notwendigkeit, die derzeitigen Bekämpfungsstrategien (d. h. massive Impfungen) zu überdenken, um die sozioökonomischen Verluste bei Geflügel zu verringern und die Übertragung auf den Menschen zu verhindern.

In Kapitel 3 wurde der Einfluss verschiedener klimatischer und biologischer Faktoren, die zur Endemie von H5N1 in Ägypten beigetragen haben könnten, untersucht. Die hohe Umgebungstemperatur einhergehend mit einer erhöhten Thermostabilität der untersuchten H5N1 Isolate könnten als zwei wichtige Faktoren für die Persistenz und / oder Ausbreitung des Virus bei Geflügel in Ägypten verantwortlich gemacht werden. Infektion von Moschusenten mit *Clade* 2.2.1.1- und 2.2.1.2a-Viren führten zu einer hohen Mortalität.

Hingehen traten bei Pekingenten bis zu 14 Tage nach der Infektion weder klinische Erscheinung noch Mortalität auf, jedoch schieden die Tiere das Virus aus, was auf eine wichtige Rolle als Reservoirwirt hindeutet.

In Kapitel 4 wurde zum ersten Mal die Isolierung der panzootischen H5N8-Viren der *Clade* 2.3.4.4b aus vier kommerziellen Geflügelbeständen zu Beginn des Jahres 2018 beschrieben. Bemerkenswerterweise besaßen die vier Viren unterschiedliche Gen-Konstellationen, was auf mehrere, unabhängige Eintragsgeschehen der H5N8-Viren in Ägypten hinweist. Die ägyptischen H5N8-Viren waren eng mit diversen aviären Influenzaviren, nachgewiesen bei Wildvögeln und kommerziellem Geflügel in Europa, Asien und Russland, verwandt.

Die in dieser Dissertation beschriebenen Erkenntnisse tragen zum Verständnis der Epidemiologie sowohl der endemisch- als auch der neu auftretenden aviären Influenzaviren in Ägypten bei, einschließlich deren Weiterverbreitung in den Nachbarländern sowie der Mehrfacheintragungen der Viren aus Europa und Asien nach Afrika. Eine kontinuierliche Überwachung ist erforderlich. Wirksame Biosicherheitsmaßnahmen müssen ergriffen werden, um die Infektion von Geflügel mit AIV zu verhindern bzw. die Verbreitung zu reduzieren.

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7. List of publications

1. **Salaheldin AH,** Veits J, Abd El-Hamid HS, Harder TC, Devrishov D, Mettenleiter TC, Hafez MH, Abdelwhab EM. Isolation and genetic characterization of a novel 2.2.1.2a H5N1 virus from a vaccinated meat-turkeys flock in Egypt. Virology Journal. 2017; 14(1): 48.

2. **Salaheldin AH,** Kasbohm E, El-Naggar H, Veits J, Ulrich R, Scheibner D, Gischke M, Hassan MK, Arafa A, Harder TC, Abd El-Hamid HS, Hafez MH, Mettenleiter TC and Abdelwhab EM. Potential Biological and Climatic factors that influence the incidence and persistence of highly pathogenic H5N1 avian influenza virus in Egypt. Forinters in Microbiology. 2018; 9: 528.

3. **Salaheldin AH,** Abd El-Hamid HS, Elbestawy AR, Veits J, Hafez MH, Mettenleiter TC and Abdelwhab EM. Multiple Introductions of Influenza A(H5N8) Virus into Poultry, Egypt, 2017. Emerging Infectious Diseases. 2018; 24. 10.3201/eid2405.171935.

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9. Declaration of authorship / Selbstständigkeitserklärung

Selbständigkeitserklärung

I hereby confirm that the present work was solely composed by my own. I certify that I have used only the specified sources and aids.

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin 02.04.2019 Ahmed Mohamed

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