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Avian influenza infections in poultry farms in Egypt, a continuous challenge: Current problems related to pathogenesis, epidemiology and diagnosis

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List of abbreviations	
AA	Amino acid
AI	Avian influenza
AIV	Avian influenza virus
CS	Cleavage site
ECDC	European Centre for Disease Prevention and Control
ECE	Embryonated chicken eggs
FAO	Food and agriculture organization of the United Nations
FLI	Friedrich-Loeffler-Institut
GISAID	Global initiative on sharing all influenza data
gs/GD	goose/Guangdong lineage
HA	Hemagglutinin
HACS	Hemagglutinin cleavage site
HP	Highly pathogenic
HPAI	Highly pathogenic avian influenza
HPAIV	Highly pathogenic avian influenza virus
HPAIVs	Highly pathogenic avian influenza viruses
IFN-b	Interferon-beta
IVPI	Intravenous pathogenicity index
LP	Low pathogenic
LPAI	Low pathogenic avian influenza
LPAIV	Low pathogenic avian influenza virus
M	Matrix protein
mRNA	Messenger ribonucleic acid
NA	Neuraminidase
NCBI	National center for biotechnology information
NEP	Nuclear export protein
NLQP	National laboratory for quality control on poultry production, Egypt
NP	Nucleoprotein
NS	Non-structural protein
NS1	Nonstructural protein-1
NS2	Nonstructural protein-2
OIE	The World Organisation for Animal Health (Office International des Epizooties)
PA	Polymerase acidic protein
PB1	Polymerase basic-1 protein
PB2	Polymerase basic-2 protein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RBCs	Red blood cells
RBS	Receptor binding site
RITA	Riems influenza A typing array
RNA	Ribonucleic acid
RNPs	Ribonucleoproteins
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Semi quantitative real time RT-PCR
SA	Sialic acid
SPF	Specific pathogen free
UN	United Nations
WHO	World Health Organization

Dedication

To my parents

Eid $ Azza

To my beloved wife and daughter

Yasmin $ Jala

This humble work is a sign of

Love to you!

CHAPTER 1:

1. Introduction

1. Introduction

Avian influenza virus (AIV) represents a continuous health threat for poultry and human beings. The first description of avian influenza (AI) as a poultry disease dates back to Italy in 1878 [140]. It was not before 1955 that the causative agent of the fowl plague was shown to be a type A influenza virus based on the presence of type A influenza virus type-specific ribonucleoprotein by electron microscopy in infected cells [82]. Until to date AIV are perpetuated in wild bird reservoir hosts and may infect and circulate in poultry [9].

Avian influenza continues to be responsible for devastating economic losses in poultry production worldwide [29]. Highly pathogenic (HP) variants naturally occurring for the subtypes H5 and H7 of AIV are able to induce severe fatal disease leading up to 100% mortality in gallinaceous poultry [4]. The majority of AIV expresses a phenotype of low pathogenicity (LP) which may cause mild respiratory signs, and decreased egg production in layers and breeders of gallinaceous poultry; however, also asymptomatic courses of infection have been reported [140]. Infections with LPAIV that are associated with co-infection of other respiratory viruses such as Infectious bronchitis virus (IBV), Newcastle disease virus (NDV) or bacterial infections such as *E. coli* and *Haemophilus paragallinarum* often are accompanied with more severe clinical signs [52].

The World Organisation for Animal Health (OIE) classified HPAI as a notifiable disease [105]. Notification of an outbreak has severe negative consequences on international poultry production trade. In addition, concern is growing with respect to zoonotic propensity and pandemic potential of certain HPAIV causing losses of human lives [161]. A particularly high toll of lethal human AIV infections due to poultry-human virus transmission was noticed in Egypt during the last decade [159].

In Egypt, in 2006, the first detection of AI with trading consequences was due to an incursion by HPAIV H5N1 of Chinese origin. The virus was a descendent of the goose/Guangdong (gs/GD) lineage, classified as clade 2.2.1 [11]. Also, LPAIV of subtype H9N2 was detected in quail in Egypt for the first time in 2010 and widely spread to poultry farms thereafter [38]. Both viruses became entrenched in Egyptian poultry production until to date [130]. Moreover, in 2016, several reassortant lineages of gs/GD HPAI H5N8 viruses of

clade 2.3.4.4b were detected in Egypt in parallel with a worldwide epizootic in 2016 [8]. Recently, in 2019, novel reassortant HPAIV H5N2 was detected in a commercial apparently healthy duck flock in Dakahlia governorate in Egypt, which resulted from reassortment between the circulating HPAIV H5N8 and LPAIV H9N2 [49]. Reassortment between AIVs of different subtypes and origin is not an unexpected outcome following co-circulation of and co-infection with multiple AIV strains endemically circulating in Egypt [51]. Such emerging reassortant viruses may have unpredictable phenotypic features including altered zoonotic propensity [159].

The O.I.E., the Food and Agriculture Organization (FAO) of the United Nations (UN), and other international organizations, provide legally binding advice on notifiable AI (i.e. all infections in poultry with subtype H5 and H7 viruses) and its control strategies depending on the evaluation of the different local situations [21]. National legislation for control of AI is in place in most countries. Stamping out of infected farms is a harsh, but often successful method for AI eradication, however, it can be financially demanding given the extent of outbreaks [85]. Vaccination is an attractive and powerful tool to support eradication programs in conjunction with other control methods [20] especially in endemically infected countries like Egypt [108].

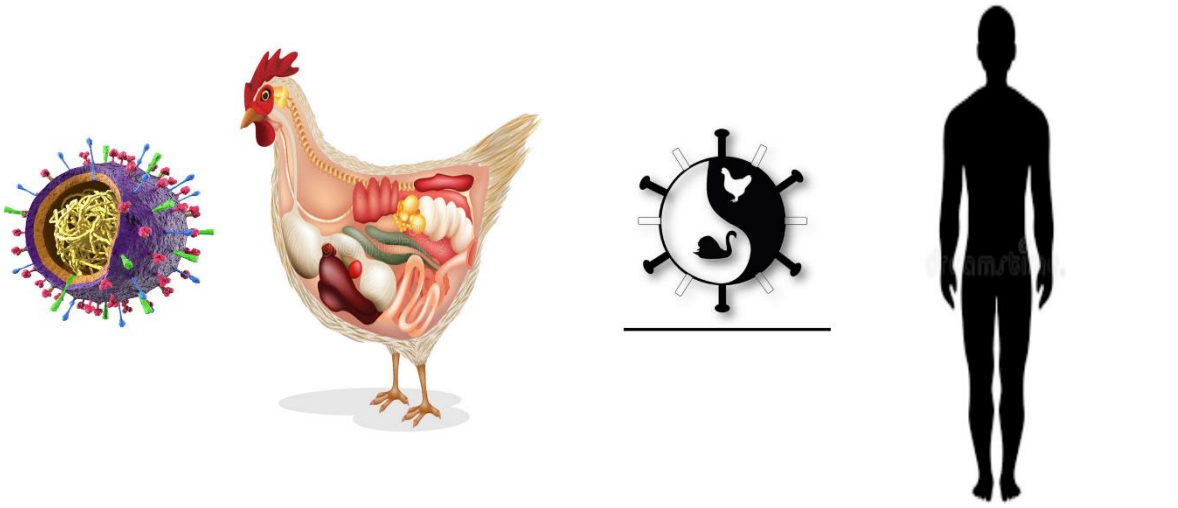
A global long-term and well-planned strategy in combatting AIV infections in poultry is urgently required to minimize the current global threat of zoonotic HPAI in domestic poultry. In order to progress into this direction, the way needs to be paved with several "stepping stones".

With the purpose of providing "stepping stones" towards better control of AI in general and specifically in Egypt, this study aims to specify, analyse, and seek solutions for, problems related to the pathogenesis, epidemiology and diagnosis of currently circulating AIV subtypes:

1. Provide an update of the epidemiological situation of AIV subtypes circulating in poultry in Egypt between 2017 and 2019.
2. Improve diagnostic tools for AIV detection and characterization.
3. Provide molecular characterization and epidemiologic data of detected AIV.

4. Assess the presence of co-infections with different AIV subtypes and/or other respiratory viruses such as Infectious bronchitis virus (IBV) and Newcastle diseases virus (NDV).
5. Evaluate the impact of AIV subtypes circulating on poultry and human health in Egypt.

2. Review of relevant scientific literature



2.1. Natural history of avian influenza

2.1.1. First detection of avian influenza

Fowl plague is the historic designation of highly pathogenic avian influenza (HPAI) when firstly identified and differentiated from other diseases by Perroncito in 1878 in Italy [9]. Avian influenza (AI) defined as a disease is caused by infection with avian influenza type A viruses (AIV). These viruses occur naturally among wild aquatic birds worldwide and can infect domestic poultry, other birds and animal species. Avian influenza viruses do not normally infect humans or other mammalian species [155].

According to virus pathogenicity, AIVs can be classified into two pathotypes; highly pathogenic avian influenza viruses (HPAIVs) causing up to 100% mortality in susceptible poultry flocks and low pathogenic avian influenza viruses (LPAIVs) which cause no or mild disease in infected poultry. Co-infection with other micro-organisms and/or adverse environmental conditions (e.g. high ambient temperatures) may exacerbate LPAIV infections leading to much more serious disease [9]. The world animal health organization, OIE, has defined HPAI as an "infection of poultry caused by any influenza A virus that has an intravenous pathogenicity index in 6-week-old SPF chickens greater than 1.2, or any infection with influenza A viruses of subtype H5 or H7 for which nucleotide sequencing has demonstrated the presence of multiple basic amino acids at the cleavage site of the hemagglutinin" [105].

2.1.2. Emergence of HPAIV of the goose/Guangdong lineage

During a 40-year period from 1959 to 1998 there are only 17 HPAI episodes reported worldwide in domestic poultry [9]. However, since 1997, multiple outbreaks were reported resulting in eight episodes involving 12 countries during 1997 to March 2004. These were located in southeast Asia and caused by emerging HPAIVs of subtype H5N1, first detected in a geese flock in the Chinese province of Guangdong in 1996. Since then, viruses of this so-called goose/Guangdong lineage (gs/GD) have undergone rapid evolution into multiple genotypes [156]. Also, the gs/GD hemagglutinin (HA) H5 protein, the major immunogenic protein, underwent massive evolutionary changes; in order to follow the dynamic

phylogenetic emergence of different HA H5 lineages a complex cladistics nomenclature system had to be established [135]. About 6414 gs/GD H5N1 outbreaks officially were reported from 2003 to 2008, affecting both domestic poultry and wild birds in 61 countries. Due to disease control measures or as a consequence of infection, more than 122 million head of poultry had to be discarded during 2013-2018 [107]. According to an OIE report [107], since January 2003 until 31 August 2018 there were 7122 HPAI outbreaks reported in domestic birds in 68 countries and territories with 12 different gs/GD HPAIV H5Nx subtypes.

Recently, novel gs/GD HPAI re-assortant viruses have emerged and generated the new subtype H5N8 of clade 2.3.4.4. These viruses were first detected in an outbreak in poultry in China in 2013 [170], followed by outbreaks in South Korea in January 2014 [75]. During these outbreaks, two distinct groups of H5N8 viruses were identified; group A (Buan-like) and group B (Gochang-like). Clade 2.3.4.4a and b H5N8 viruses appear to have been re-introduced from poultry into wild bird populations, likely in Asia, and then spread globally through wild bird migration during 2014 –2015 (2.3.4.4a) and 2016–2017 (2.3.4.4b) [62, 73]. Migratory aquatic birds are suspected to be responsible for the long distance, transcontinental spread of these viruses from southeast Asia China. HPAIV H5N8 clade 2.3.4.4 was reported in Europe and East Asia since late autumn 2014, and spread to North America where it was detected in wild birds, captive falcons and poultry in 2014 and 2015 [63]. The intercontinental spread co-incided with the autumn bird migration out of Russia; the H5N8 virus was identified in a long-distance migratory bird in Russia in September 2014 and subsequently in Japan and Europe, and on the west coast of North America [150]. Meanwhile, clade 2.3.4.4 H5 viruses have evolved into at least four genetic groups (nominally designated A, B, C, and D).

From January 2013 to August 2018, all regions of the world except South America and Australia were affected by gs/GD HPAIV outbreaks in domestic birds (Fig. 1). The most affected regions were Asia, Africa and Europe [107].

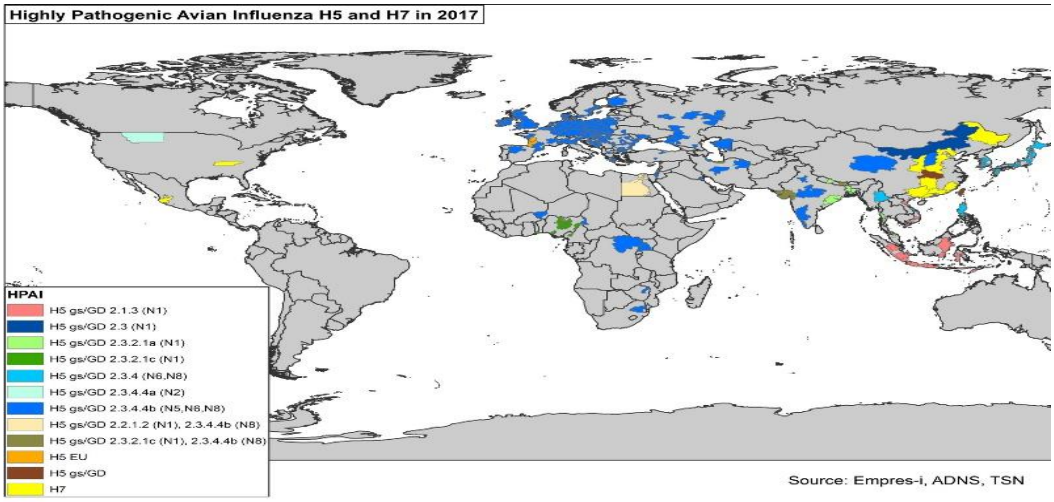


Figure 1. Snapshot of the geographical distribution in 2017 of gs/GD-derived HPAIV of subtype H5 and of different H7 subtypes around the world. Egypt is highlighted with the presence of two clades of HPAIV H5, 2.2.1 and 2.3.4.4. (adapted with permission from figure 1 of the EFSA report ,2017, [34], title modified).

2.1.3. Emergence of LPAIV H9N2

AIV of subtype H9N2 was first reported in 1966 in the United States [58]. Subsequently, this subtype was detected in various countries and poultry species like chickens, turkeys, pheasants and ostriches but also in many wild bird species. Occurrence of epizootic-like outbreaks in poultry were reported from various countries and regions including Germany (1995-1998), Italy (1994-1996), Ireland (1997), South Africa (1995), USA (1995-1996), Korea (1996), China (1990s) and Middle East since 1998 [9, 16, 17, 19, 39, 80, 97, 98, 104]. Based on phylogenetic analysis of the HA gene of H9N2 viruses, two major geographically restricted genetic lineages are distinguishable: North American and Eurasian H9 [16]. Later, several further subgroups emerged within the Eurasian lineage: The G1-like sublineage (A/quail/Hong Kong/G1/1997) was established in the Middle East [38, 54] and on the Indian subcontinent in the 1990s [128]. Other sublineages such as BJ94 (A/chicken/Beijing/1/94), later also called Y280 or G9 lineage, circulated mainly in countries of the Far East [128] (Fig. 2) similar to Y439 also sometimes known as the Korean lineage [114]. The G1 lineage has established enzootic status in poultry populations in Asia and in the Middle East including Egypt and was classified into four distinct, co-circulating

phylogenetic groups provisionally named A, B, C, and D [40]. Also, certain countries (China, Russia) reported co-presence of more than one lineage (Fig. 2) [114].

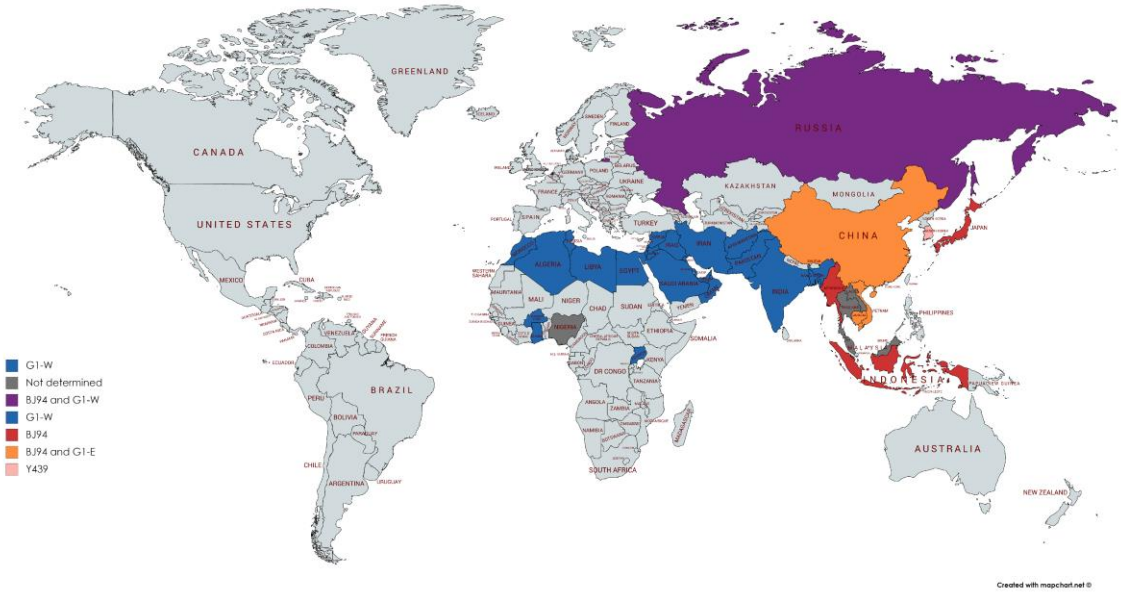


Figure 2. Geographical distribution of Eurasian-like LPAIV H9N2 lineages around the world (Adapted with permission from figure 1 of Peacock et al., 2019, [114], title modified).

2.2. Taxonomy and structure of influenza A viruses

AIVs belong to the family Orthomyxoviridae, genus Influenza virus A [30]. According to the nucleoprotein (NP) and matrix (M) proteins, influenza viruses are classified into 4 types; A, B, C and [147], D [15]. Based on their virulence, two main AI pathotypes are distinguished; highly pathogenic AIVs (HPAIV) and low pathogenic ones (LPAIV). These viruses vary in their pathogenicity to chickens which is reflected, in part, in the amino acid sequence configuration at an endoproteolytical cleavage site in the HA protein.

The genome of AIVs consists of single-stranded RNA with negative polarity distributed across eight separate genome segments. Virions are typically spherical to pleomorphic but can be filamentous also. Individual virions range in diameter from 80 to 120 nm, but filamentous forms can have lengths of up to several hundred nanometers. The

surface is covered by two types of glycoprotein projections: rod-shaped trimers of hemagglutinin (HA) (10–14 nm in length) and mushroom-shaped tetramers (4–6 nm in diameter) of neuraminidase (NA) [30].

The genome of AIV encodes 10 viral proteins and, based on the strain, up to four further proteins are expressed. Viral proteins can be grouped into surface, internal, and nonstructural proteins (Fig. 3). The viral particle contains three surface proteins: HA, NA and matrix-2 (M2) proteins. The HA protein is the major surface protein of the virus, binds to sialic-acid-containing receptors on host cells and is considered the main target of neutralizing antibodies. While the NA viral surface protein removes sialic acid residues from glycoproteins during virus budding from the cell surface, and from assembled virions, it prevents the aggregation of budding virions on the cell surface. The sialidase function of the NA is important also to prevent sticking of virions in mucus membranes covering epithelial cell layers. Antiviral drugs (e.g. oseltaminivir and zanamivir) known as neuraminidase inhibitors target the sialidase function of NA [89]. The surface proteins are the only antigens capable of inducing neutralizing antibodies and are therefore a major target of a protective humoral immune response. Both HA and NA proteins are under selection pressure causing marked sequence and antigenic differences, which resulted in the segregation of 16 and 9 different HA and NA subtypes of AIV [147].

In addition, the transmembrane M2 protein, an ion channel protein, is essential for the uncoating of the virus after attachment. The M2 protein channel promotes the influx of H+ions into the interior of the virions when present in cellular endolysosomes, thereby disrupting protein–protein interactions, specifically including those between the M1 protein and viral ribonucleoproteins (vRNPs). Acidification of the virion also induces steric changes in the HA whereby exposing a fusion domaine that achieves fusion of viral and endolysosomal membranes [33]. Two key residues (His37 and Trp41) within this tetrameric helical channel act as a pH sensor and a proton (H+ion) gate, respectively [115]. Adamantanes inhibit M2 ion channel activity and, as a result, the M1 protein remains associated with the released vRNPs, thereby inhibiting their nuclear import [144].

Internal proteins comprise the three RNA polymerase proteins PA, PB1 and PB2 [76]. The replication of vRNAs and the transcription of viral mRNAs are catalyzed by the

viral polymerase complex, which is composed of the PB2, PB1 and PA subunits. The viral RNA-dependent RNA polymerase activity is encoded by the PB1 protein. The PB2 protein binds to the cap structure of host pre-mRNAs; the endonuclease activity of PA then cleaves the cap structure and the adjacent nucleotides from cellular pre-mRNAs, thereby creating short, capped mRNA primers that are elongated by PB1 for viral mRNA synthesis. All three polymerase subunits, together with the nucleoprotein (NP), are required for efficient influenza virus replication [118].

The nucleoprotein (NP) enwraps genomic RNA, and the matrix-1 (M1) protein forms a stabilizing proteinaceous shell underneath the fragile lipid envelope, which is derived during virion maturation from the host cell surface. Nonstructural protein-2 (NS2) has recently been shown to be packaged in virions and has, aptly, been re-designated as nuclear export protein (NEP). NEP mediates the export of newly synthesized viral RNPs from the nucleus to the cytoplasm. The nonstructural protein-1(NS1) is not packaged into the virus particle, although it is produced in large quantities in infected cells [18, 25, 146, 155]. NS1 is a multi-functional protein that has an integral role in suppressing host antiviral responses.

In 2001 the first so-called "accessory influenza virus protein", PB1-F2, translated from an alternative open reading frame of the PB1 genome segment, was discovered [24]. Subsequently, six more candidate accessory proteins, PB1-N40 [163], PA-X [64], PA-N155 [96], PA-N182 [96], M42 [164] and NS3[127] have been proposed. The function of these novel accessory proteins and their role in the pathogenesis of influenza are still under study. For example, the NS3 protein was predicted in only 33 natural viral strains where it possibly participates in viral transmission between hosts [149]. M42 is restricted to about 0.2% of all influenza A virus strains and probably accomplishes the function of M2 in M2-null viruses [149]. The PA-X, PB1-F2, and PB1-N40 are nonstructural proteins, that are encoded by most influenza A viruses [145]. PB1-F2 is a virulence factor, while PA-X diminishes viral damage leading to higher replication output [41]. PB1-N40 is clearly associated with maintaining the balance between PB1 and PB1-F2 expression. Probably all these proteins play other additional regulatory roles in virus replication [145].

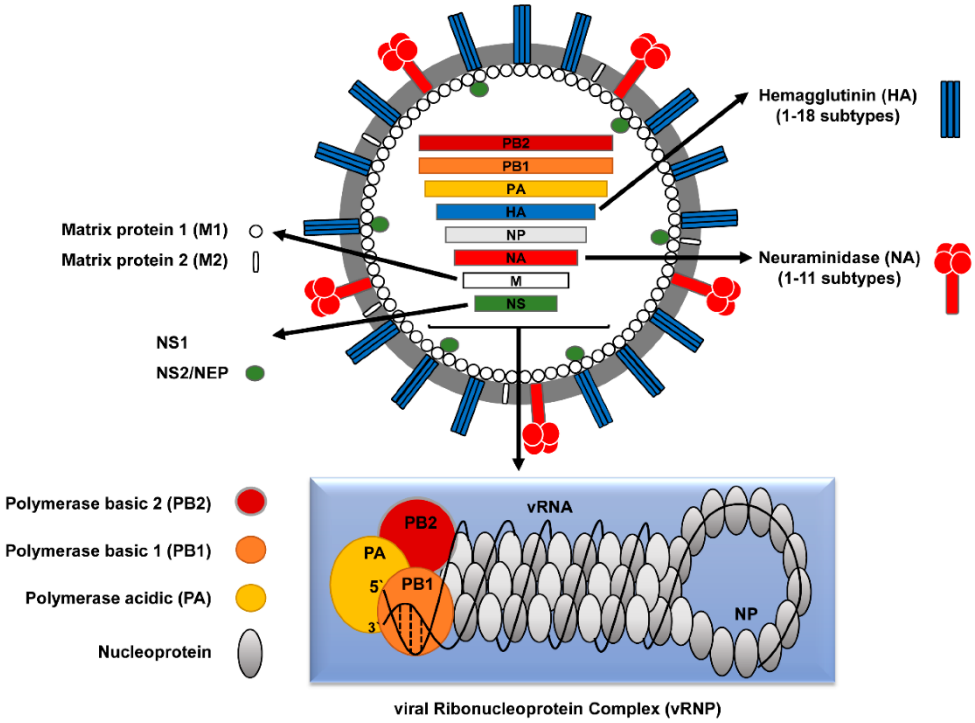


Figure 3. Schematic diagram of the influenza A virus structure showing the location of nine structural viral proteins. (adapted with permission from figure 1 of Mostafa et al., 2018 [95], title modified).

2.3. Viral host and tissue range restrictions

Avian influenza viruses are probably capable to infect most of the avian species. However, they are not able to efficiently replicate in mammals including humans and non-human primates without adaption and to overcome several host range restriction mechanisms in the viral replication cycle [103].

The HA protein plays a major role in determining the host range through binding to sialic acid (SA) receptors at the host cell surface. Internalization into endolysosomes follows; the release of viral ribonucleoprotein complexes into the host cell cytoplasm is achieved through HA-mediated fusion of viral and endolysosomal membranes. NA, the second major envelope glycoprotein displays sialidase activity and removes SA residues, which is helpful in liberating newly budding virions from infected cells. Therefore, for optimized virus replication a well-adjusted balance of HA receptor-binding affinity and antagonistic NA sialolytic activity is pivotal [44]. In general, influenza viruses adapted to humans prefer

binding to terminal sialic acids containing α 2,6 linkage with subterminal galactose, while the HA of AIV has a higher affinity to SA containing α2,3 linkages [86].

The ability of mature virions to successfully initiate replication by attachment to SA residues requires activated HA: The HA protein is synthesized as a precursor (HA0). Functional HA glycoprotein that mediates both attachment to and fusion of viral and cellular membranes requires endoproteolytic cleavage of HA0 into two subunits, HA1 and HA2, by host-specific proteases. The HA0 precursors of LPAIVs are limited to cleavage by tryptic host proteases such as trypsin and other serine proteases. Therefore, these viruses are restricted to replication at sites in the host where such enzymes are found (i.e. the respiratory and intestinal epithelia) [132]. However, the HA0 precursor of HPAIVs can be cleaved by ubiquitous protease(s) of the proprotein-processing subtilisin-like type of which furin is a leading representative [132]. Such feature enables these viruses to replicate systemically throughout the animal organs leading to damage of vital organ functions.

Cleavability by the different protease types is modulated by the amino acids sequence at the HA0 cleavage site. Viruses which encode a mono- or dibasic cleavage site motif, e.g. K/RSSR*GLF (typical for H9 HA of the G1 lineage), are classified as low pathogenic. Viruses that exhibit a polybasic cleavage site motif [R-X-K/R-R*GLF] are expected to be processed by intracellular endoproteoases of the proprotein convertase family such as furin [43, 125]. HP viruses gained the additional basic AA from LP precursor viruses by insertional (polymerase stuttering) or recombinatorial mutation. As such, an HPAI virus can arise from LP precursor viruses. Under field conditions this process has only been confirmed, so far, for AIV of subtypes H5 and H7 [151]. Molecular prerequisites that favor such mutations in H5 and H7 viruses but not in other subtypes are currently not well understood [46]. Legislation however uses the trait of a polybasic HACS to define a highly pathogenic pathotype [105].

2.4. Reassortment and antigenic diversity of avian influenza viruses

The distinctive genomic structure of AIV distributed over eight separate segments offers opportunities for genetic diversification by reassortment. If HA and/or NA segments

are involved, a rapid change of antigenicity, also called "antigenic shift" is possible. Such a "shift" occurred in 2009, when an H1N1 virus with gene segments from North American swine, Eurasian swine, human and avian influenza viruses emerged to infect people and quickly spread in the human population causing a pandemic (Fig. 4). Reassortment affecting genome segments encoding internal proteins may provide genetic markers that allows the virus to cross host barriers [134, 136].

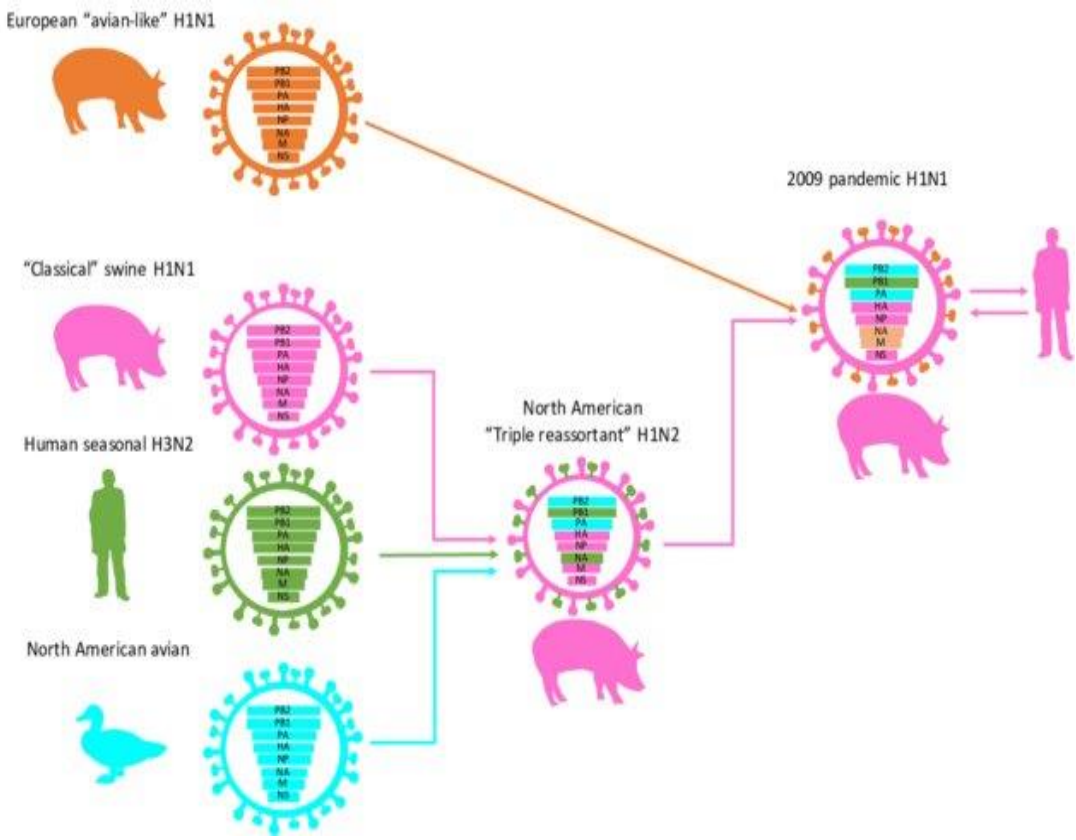


Figure 4. Importance of reassortment in the generation of pandemic H1N1 in 2009derived with permission from figure 4 of the Ph.D thesis of Mancera Gracia, J. C., 2017 [87], http://hdl.handle.net/1854/LU-8526526, title modified).

For avian influenza viruses till now 16 HA and 9 NA subtypes are distinguished that have allowed at least 103 of the possible 144 HA-NA combinations to arise in the field [59]. This indicates a high frequency of reassortment among different HA and NA subtypes.

However, some subtypes are rarely detected, indicating that some restrictions on possible combinations may exist [81].

In addition to reassortment, influenza A viruses have evolved another mechanism for adaptation and immune response escape: "antigenic drift". Incremental accumulation of point mutations affecting critical AA sites in epitopes of the HA and NA surface proteins may lead to escape of virus from antibody-mediated neutralization of infectivity. The HA and NA surface proteins are immunodominant antigens as they are primary targets of the immune system, including the induction of antibodies that can block infection. Antigenic drift proceeds continually over time as the virus replicates under immune selection pressure. This leads to phylogenetic clade shifting and emergence of escape mutant viruses, which finally may cause vaccination failure (https://www.cdc.gov/flu /about/viruses/change.htm).

2.4.1. Reassortment events generating gs/GD HPAIV H5N1

The emergence of the Asian (gs/GD) lineage of HPAI H5N1 viruses since 1996 was a result of reassortment between different avian influenza viruses. Its H5 HA derived from a goose H5N1 virus, the N1 originated from an H6N1 virus and the remaining genes were donated from a H9N2 virus both prevalent in quails (Fig. 5)[48]. This virus and its descendants extensively spread within Asia, to Europe, Africa and North America causing unprecedented outbreaks in poultry and mass mortalities in wild birds in the subsequent years [106]. These viruses also exhibited zoonotic propensity, the first human cases occurred in Hong Kong in 1997 [23].

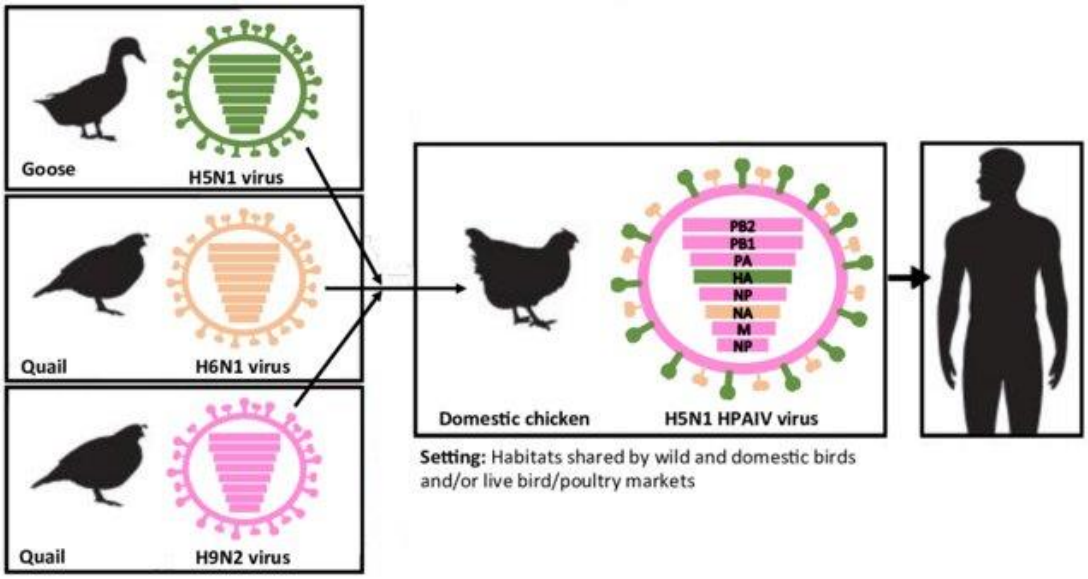


Figure 5. Reassortment between three AI viruses generated HPAIV H5N1, an avian virus with high zoonotic propensity (obtained with permission from figure 5 of the Ph.D thesis of Mancera Gracia, J. C., 2017 [87], http://hdl.handle.net/1854/LU-8526526, title modified).

2.4.2. Reassortment events generating gs/GD HPAIV H5N8

The most recent waves of HPAI hitting large areas of Asia, Europe, Africa and North America were caused by gs/GD-derived viruses of subtype H5N8. This subtype emerged because of reassortment between HPAIV H5N1 clade 2.3.4 viruses with subtype N8 viruses from Eurasia. The first re-assortant reported was found in China: A/duck/Jiangsu/k1203/2010 (H5N8). HPAI H5N8 viruses were then circulating in eastern China and South Korea until late 2013. With the start of 2014, further reassortment with A/duck/Hunan/8–19/2009 (H4N2) and A/environment/Jiangxi/28/2009 (H11N9)-like viruses generated the so-called group B of clade 2.3.4.4 HPAI H5N8 viruses. Subsequent reassortment between HPAIV H5N8 group B viruses and various LPAIVs circulating along the central Asian flyway led to generation of a number of novel HPAIV H5N8 genotypes as shown in Fig. 6 [35].

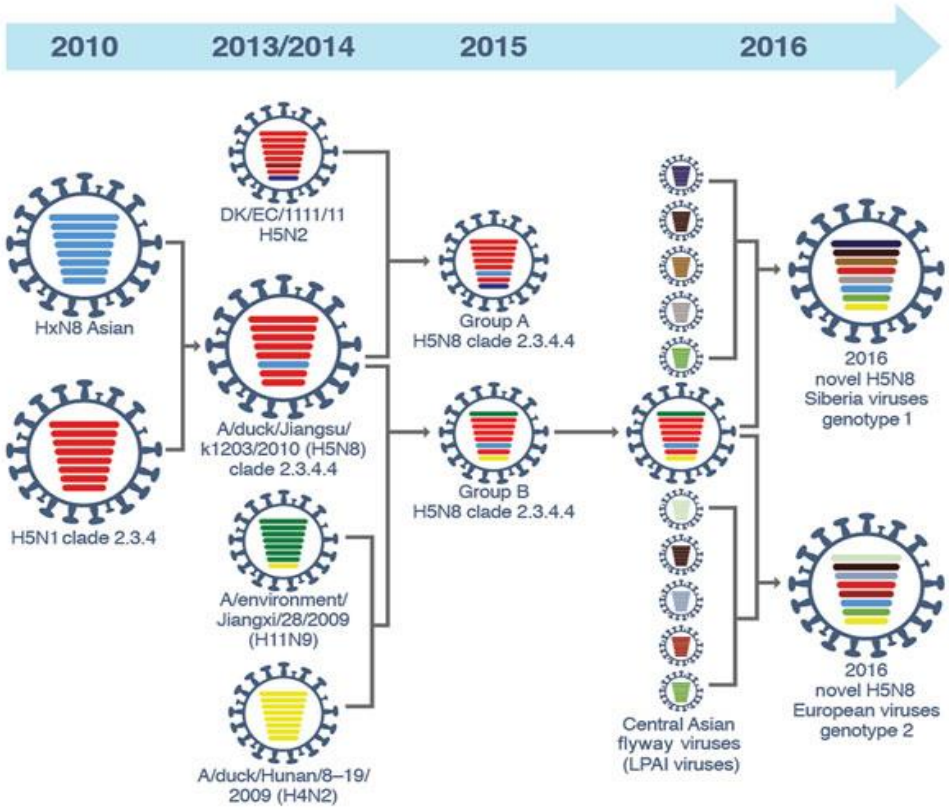


Figure 6. Impact of reassortment in the emergence of novel HPAI H5N8 viruses isolated from China, Siberia and Europe until 2016 (obtained with permission from figure 2 of El-Shesheny et al., 2017 [35], title modified).

2.4.2. Reassortment events generating HPAIV H5N2

Concurrent with the spread of HPAIV H5N8 clade 2.3.4.4 another novel reassortment HPAIV H5N2 of clade 2.3.4.4a was detected as the cause of outbreaks in poultry farms in British Columbia, Canada, 2014. The virus subsequently was detected in wild waterfowl in Oregon, U.S.A., and spread to various poultry holdings in the US and in Canada [63, 113]. Genomic analysis suggested that five segments (PB2, PA, HA, M, and NS) of this virus were donated by a Eurasian HPAI H5N8 virus and the remaining three segments (PB1, NP and NA) derived from North American lineage waterfowl viruses [113].

In Egypt, a novel re-assortant HPAIV H5N2 clade 2.3.4.4b was detected in 2019 in a commercial healthy duck flock (Fig. 7) [49]. The virus is a re-assortant comprising seven

gene segments of Egyptian H5N8 viruses and the NA (N2) of Egyptian LPAI H9N2 viruses (Fig. 7).

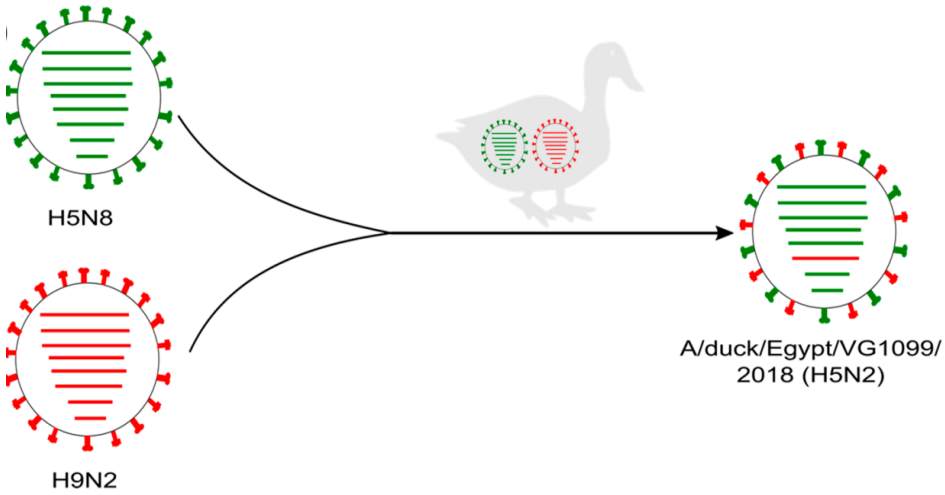


Figure 7. Origin of a novel re-assortant HPAI H5N2 virus of clade 2.3.4.4b detected in a duck flock in Egypt in 2019 (obtained with permission from figure 1C of Hagag et al., 2019 [49], title modified).

2.4.2. Role of LPAIV H9N2 in the emergence of HPAIVs

The LPAI H9N2 virus is well known as a donator of gene segments to other AIV subtypes, often resulting in the generation of new reassortment HPAI viruses some of which may infect human beings [60]. The extended spread and frequent co-circulation of H9N2 viruses with other subtypes of influenza A virus likely increases the risk of emerging new re-assortant viruses. The HPAI H5N1 virus that has caused the first human H5N1 infections in Hong Kong in 1997 already harboured six internal genes from clade G1/97-like H9N2 viruses that circulated at that time in poultry in southern China [48]. Similarly, H9N2 was also involved in the generation of other zoonotic LP and HPAIV in China: The novel highly zoonotic LPAI H7N9 virus which emerged in 2013 in China contained six internal gene segments of H9N2 clade G57. Since 2007, genotype 57 (G57 or S) has been prevalent and became the dominant lineage in eastern China, which bears the backbone of F/98-like viruses by integrating PB2 and M genes of G1-like H9N2 viruses [47]. Recent research demonstrated that G57 had the ability to donate gene segments to other highly pathogenic

AIVs like H5N2, the H7N9/2013, and H10N8, which have caused human infections and deaths [117]. Chinese H7N9 viruses represent some of the most serious zoonotic AI viruses, that have caused 1,568 confirmed infected human cases with 616 deaths mainly in China [42, 61].

In a reverse fashion, HPAIV of the gs/GD lineage also donated segments to H9N2 viruses: G1-like H9N2 viruses circulating in Bangladesh harboured internal genes (M and NS) that originated from HPAIV H5N1 clade 2.2.2 [111]. Other Bangladeshi H9N2 viruses shared internal genes with HPAIV H5N1 clade 2.3.2.1a (PB1) and with the Pakistani HPAIV Pak/H7N3 (NS and NP) [93, 112, 129].

In Egypt, novel re-assortant LPAI H9N2 viruses were detected in pigeons [66]. Molecular analysis indicated the presence of five new re-assortant internal gene segments (PB2, PB1, PA, NP and NS) derived from various wild bird AIV in the "classical" background of HA, NA and M genes of Egyptian H9N2 viruses which circulated since 2010 in poultry farms [66].

2.5. Avian influenza in Egypt

2.5.1. History and current situation

In the last decade, the poultry industry in Egypt faced continuous challenges by enzootic circulation of various AIV sub- and pathotypes including HP H5N1, H9N2 and HP H5N8 being reported since 2006, late 2010 and late 2016, respectively. The infections were declared endemic in Egypt soon after their incursions despite the efforts paid for AI prevention and control [70, 133] (Fig.8).

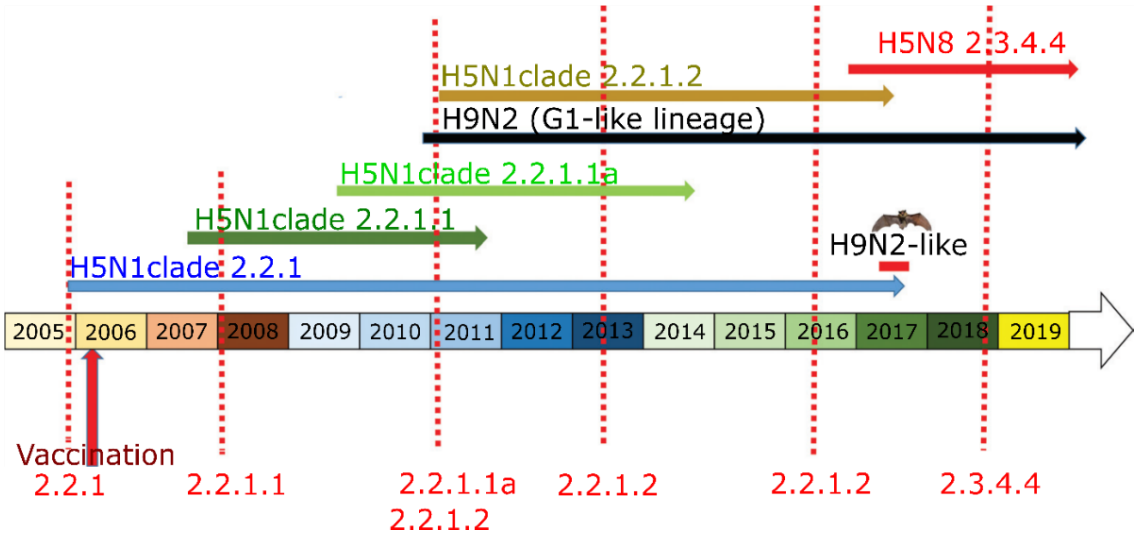
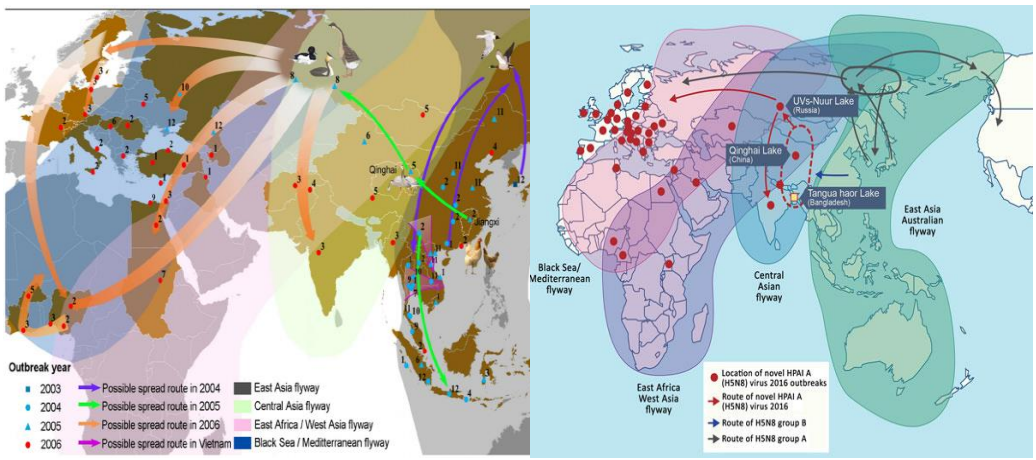


Figure 8. Overview of the history of the avian influenza situation in Egypt since the first introduction of an HPAIV in 2005 (Obtained with permission from figure 1 of El-Shesheny et al., 2020 [36], title modified).

2.5.1.1. Geo-ecological framework

The geographical position of Egypt in northeastern Africa puts the country at a hinge between Africa and Eurasia. Migratory wild birds along the East Africa-West Asia and the Mediterranean-Black Sea flyways make use of this transcontinental connection. In addition, Egypt is part of the more regional Rift Valley-Red Sea migratory flyway [157]. Locally rich natural resources of water and feeding create several stop over sites attractive for migratory birds in Egypt. This explains the pivotal role of migratory birds in transferring AIV to Egypt [101] (Fig.9).



A **B**

Figure 9. Global movement of migratory wild birds and their putative role in transferring HPAIV to Egypt. (A) Introduction of HPAIV H5N1 into Egypt in 2006. Numbers at triangles and dots indicate the months of occurrence of cases [77]. (B) Incursion of HPAI H5N8 viruses during 2016 into Egypt. Read arrows and dots depict HPAI H5N8 clade 2.3.4.4b virus presence. Dashed circles indicate putative sites of reassortment between HPAI H5N8 viruses and low pathogenicity AIV circulating along the Central Asian flyway [35] (obtained with permission from (A) figure 7 of Liang et al., 2010 [77] and (B) figure 1 of El-Shesheny et al., 2017 [35], title modified).

2.5.1.2. Introduction and spread of HPAIV H5N1 in Egypt

HPAI H5N1 virus of clade 2.2.1 was the first HPAIV to be detected in Egypt in 2005 (Figs. 9, 10 and 12). A Eurasian teal (*Anas crecca*) trapped in the Damietta region of northern Egypt in December 2005 proved to be virus-positive, and subsequently antigenically similar HPAI H5N1 viruses were detected in domestic birds since February 2006 [122]. Despite intense efforts of the Egyptian authorities including trade restrictions and blanket vaccination campaigns, an enzootic entrenchment of the virus in local poultry populations could not be prevented. In fact, patchy vaccination coverage and antigenically ill-matched vaccines created an environment of imperfect immune pressure in vaccinated birds that fostered the selection of virus variants which escaped from vaccine-induced immune defense mechanisms [3]. Clinically silent spread of virus in vaccinated flocks further promoted its

spread. In early 2007, a new antigenic-drift variant of HPAIV H5N1, termed clade 2.2.1.1, was detected (Figs. 9, 10 and 12), which was primarily isolated from vaccinated commercial poultry. These viruses dominated during 2008-2010 despite the massive vaccination program starting in Egypt. Clade 2.2.1.1 emerged in late 2007 and stayed until 2009; a second variant clade, termed 2.2.1.1a, emerged in 2008 and remained until 2011. These variant clusters have not been detected (Fig. 8) after 2014 and are believed to be extinct [12]. It has been speculated that the escape variant viruses had severe fitness deficits compared to the parental virus and another lineage that evolved from it [7, 37, 119].

However, clade 2.2.1 viruses continued to circulate und evolve. Since 2014-2015, more and more outbreaks of HPAIV H5N1 were reported caused by a newly emerged HPAIV H5N1 of clade 2.2.1.2 [13, 99] (Fig. 10), which was probably established and mainly circulated in non-vaccinated backyard birds and small-scale poultry farms. This clade caused huge economic losses in poultry farms. The variant also showed high zoonotic propensity and was transmitted to humans. As a result Egypt reported the highest number of human infections with HPAIV H5N1 worldwide [159]. The majority of infected humans were infected by viruses of this clade [168] and they continue to circulate in poultry in Egypt to this date [71, 130].

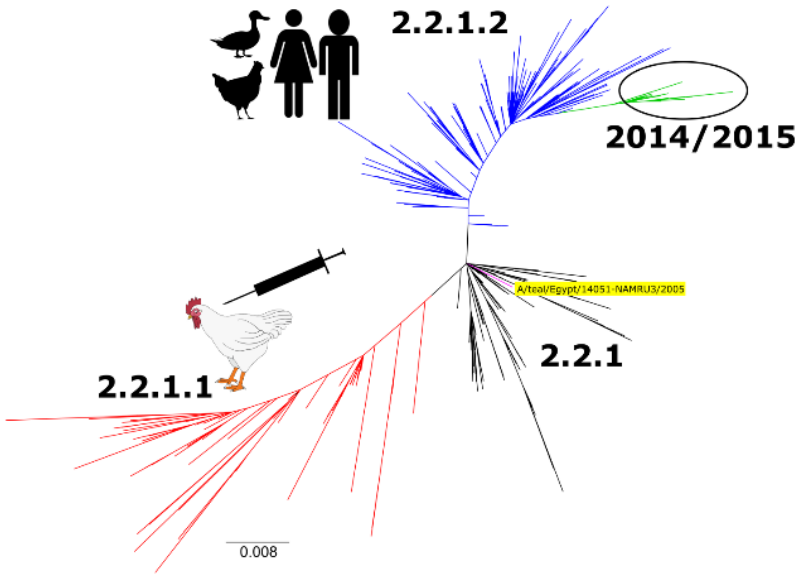


Figure 10. Phylogenetic analysis of the HA1 gene of Egyptian HPAI H5N1 viruses from 2005 to 2015. Clade 2.2.1 viruses (black) depict the first wave of virus replication in Egypt; yellow color highlights the first HPAI H5N1 virus isolated from a wild bird in Egypt in 2005. Clade 2.2.1.1 variant viruses (red) were commonly isolated from vaccinated commercial chickens; these viruses were antigenically distinct and escaped vaccine control measures using vaccine strains derived from clade 2.2.1. Clade 2.2.1.2 viruses depicted in blue were commonly isolated from backyard poultry and human cases. Viruses from the same clade continued to circulate in 2014/2015 (green) (obtained with permission from figure 1 of Abdelwhab et al., 2016 [7]).

2.5.1.2. Introduction and spread of LPAIV H9N2 in Egypt

During 2010-2011, LPAIV H9N2 has been reported for the first time in chickens and commercial quails in Egypt [1, 38, 92]. Until now, there is no clear understanding of the introduction route of LPAIV H9N2 into Egypt [101]. The virus spread widely into poultry farms. All LPAI H9N2 viruses circulating in Egypt were shown to belong to the G1-B lineage (Fig. 11). Subsequent huge economic losses were registered in the Egyptian poultry industry because of the associated clinical complications resulting from co-infection of LPAIV H9N2 with other viral, bacterial and/or parasitic infectious agents [51, 124, 130]. H9N2 outbreaks peaked in 2011 and often co-infection with HPAIV H5N1 was detected

[70]. In 2014, a novel re-assortant H9N2 virus was detected in pigeons in Egypt. This virus carried PB2, PB1, NP and NS genome segments that originated from AIV of wild birds within or outside of Egypt [66]. This virus is considered the first reassortment affecting enzootic AIV in Egypt. Given the long period of co-circulation of HPAIV H5N1 and LPAIV H9N2 in Egypt, it is very astonishing that no evidence for further natural reassortment was detected [100]. The two H9 G1-B genotypes are prevalent in Egyptian poultry until to date.

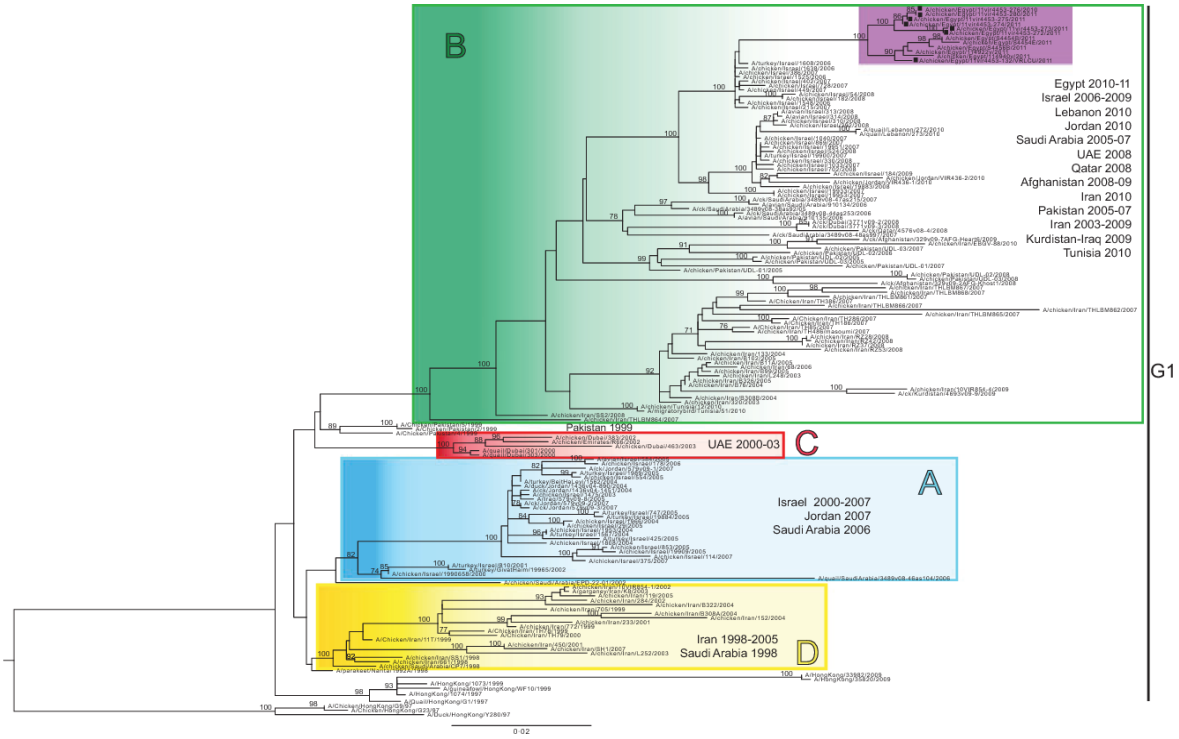


Figure 11. Maximum likelihood phylogenetic tree for the HA gene segment of LPAI H9N2 viruses. The tree includes H9N2 sequences from Middle Eastern, Central Asian and African countries. The Egyptian H9N2 sequences are highlighted in purple. G1 lineage groups are colored blue- group A, green- group B, red- group C, and yellow- group D. The numbers at the nodes represent bootstrap values (obtained with permission from figure 1 of Monne et al., 2012 [92], title modified).

Moreover, most recently through AI surveillance in Egypt, isolation and characterization of an influenza A virus from Egyptian fruit bats was reported [67]

(Fig.8). The isolated virus had affinity to avian-like receptors; also, it was able to infect mice. Genomic analysis of the HA gene suggested a common ancestry with other H9 viruses, and the virus showed a low level of cross-reactivity with serum raised against avian H9N2 viruses [67].

2.5.1.3. Introduction and spread of HPAIV H5N8 in Egypt

Gs/GD HPAIV H5N8 of clade 2.3.4.4b originated in Far East China, and spread across Central Asia into Europe and Africa [32, 68, 74, 126, 135]. In Egypt, this virus was first detected in two Eurasian coots in the Mediterranean Damietta region in November 2016, then in two apparently healthy Eurasian teals sampled in Port Said in northern Egypt in December 2016 [68, 108, 126]. In early 2017, HPAI H5N8 virus was isolated from oropharyngeal swabs of ducks sampled at backyard and commercial farms suggesting multiple introductions into the poultry farming system of Egypt [8, 167]. Thus, it is highly likely that this most recent HPAIV incursion was by wild migratory birds. Migratory birds not only played a role in the introduction of those endemic HPAIVs into Egypt, but also seem to be involved in the donation of new re-assortant gene segments to circulating endemic HPAI H5N8 viruses. The presence of typical wild bird AIVs including LPAIV H7N9, H7N3, H10N6, and H3N6, was confirmed in wild aquatic birds hunted or captured and sold at live bird markets in two locations along the Mediterranean Coast of Egypt between 2014 and 2016 [71]. Gene segments of some of those viruses, in particular of the H7N3 virus, were detected in HPAI H5N8 viruses circulating in domestic farms in Egypt [71]. All HPAI H5N8 viruses detected in Egypt up to date belong to clade 2.3.4.4b (Fig.12).

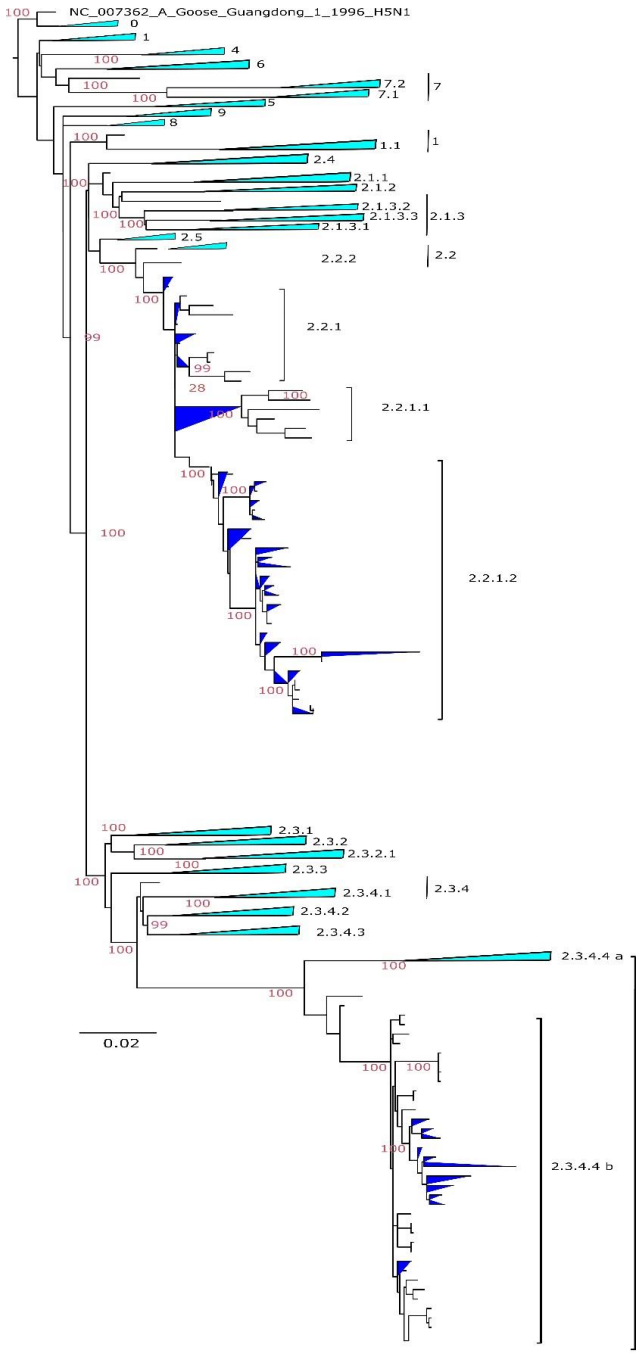


Figure 12. Evolution of the gs/GD-like HPAIVs of subtype H5 from 1996 to 2020. The phylogenetic tree was modified from figure 1 of Smith et al., 2015 [135], who attempted to clarify the nomenclature of gs/GD viruses. The designation of clades is indicated to the right of the tree. Egyptian HPAIVs are highlighted by the blue color.

2.5.2 Challenges for poultry production in Egypt

Currently, there are three different AIV subtypes enzootically circulating in Egypt: HP H5N1 (clade 2.2.1.2), HP H5N8 (2.3.4.4b) and LPAIV H9N2 (G1-B). In addition, some newly detected re-assortant HP viruses such as the H5N2 must be expected in the field [116]. So far, all control measures failed to stop the spread and circulation of AIVs in poultry farms. As such, "The current endemic situation of HPAI in Egypt is affecting not only the commercial flocks but also rural households through most of the governorates" the OIE justly stated in 2015 [107]. Several factors have been identified that contributed to the current situation:

2.5.2.1. Backyard and mixed species rearing

In Egypt, different types of poultry production systems are identified. They range from high industrial standards (so-called sector 1 and 2) to medium-size (sector 3) and backyard-rearing systems (sector 4). Usually, the high density sector 1 and 2 production systems have relatively less problems in disease control and biosecurity, while the non-registered and non-regulated small- to medium-scale farms, and backyard premises (i.e. house-hold and roof-top rearing) represent the actual threat for disease spread and control in Egypt [101]. In addition, breeding and rearing of quails, pigeons and waterfowl as domestic birds together with other poultry species is an important factor in persistence and spread of infections in the country. In addition, clinically inapparent infected free-ranging ducks and geese played a pivotal role in maintaining and spreading of AIV [11].

2.5.2.2. Biosecurity limits in control of AI in Egypt

Many authors have investigated risk factors at the farm level and reported significant factors linked to biosecurity concerning isolation (geographic, movement of large industrial enterprises out of the Nile delta into adjacent desert areas), sanitation and traffic control [22]. One of the most important factor in failure of control of AIV is associated with the widespread habit of live bird trading in the country. Uncontrolled transport of live birds between governorates and lack of biosecurity measures at live bird markets are hallmarks of

these problems [53]. In household and backyard poultry production systems, the improper disposal of dead birds and wastes is another important factor in failure of disease control [131]. Insufficient biosecurity measures also includes the movement of staff between farms. External injectors (i.e. for vaccination or in some cases for medication purposes), part-time day farm workers, visiting veterinary practitioners, feed delivery, egg and litter collecting are important drivers of transfer and spread of infections [169].

2.5.2.3. Role of seasons and climatic changes in AI spread in Egypt

In the winter season, in order to maintain sufficient temperatures in the poultry houses, there is a lack of good ventilation, leading to increased humidity and ammonia levels. This physically strains the respiratory tract of poultry and increases risks for AIV and other co-infections to provoke more severe clinical disease [51]. In summer, in contrast, high ambient temperatures ranging from 35-45°C pose a severe stress factor for intensively reared poultry. Several studies focused on such factors and provided evidence for a higher tenacity of enzootic AIV in Egyptian climates [39, 97].

2.5.2.4. Impact of co-infections in AI in Egypt

Co-circulating of AIV, bacterial and viral co-pathogens have been identified as one of the most important challenges for poultry production in Egypt. The etiology of poultry respiratory diseases in Egypt is complex and often involved more than one pathogen at the same time [166]. Different viral pathogens such as velogenic Newcastle disease virus (NDV) and Infectious bronchitis virus (IBV) were identified to play a crucial potentiating role in combination with AIV in the so-called respiratory disease complex syndrome. Frequently, these co-infections are associated with high mortality rates in broiler chicken flocks [51, 124]. These pathogens are of major significance and have a great economic impact [120]. Presence of different serotypes and multiple variants of IBV complicate control measures and have led to the use of different types of live-attenuated IBV vaccines in Egypt [2]. However, the live-attenuated IBV vaccines themselves (specially those based on variant strain 4/91) are able to exacerbate the manifestation of AIV H9 infections, as shown in

experimental infection studies in broiler chickens [50, 52]. IBV infection provokes ciliostasis in the host's respiratory epithelia and may therefore facilitate co-infection with other co-localized pathogens such as AIV [28]. Moreover, during co-infection with IBV and H9N2, IBV could provide a trypsin-like serine protease which is necessary for HA cleavability [79] and thus enhances the pathogenicity of H9N2 in the affected flocks [52] (Fig. 13). In addition, bacterial co-pathogens such as *Escherichia coli* and *Haemophilus paragallinarum*, may provide endoproteases able to enhance the infectivity of H9N2 by fostering cleavage of precursor HA proteins [50, 110].

Simultaneously, the widespread presence of velogenic NDV is no less complicated compared to IBV. Egypt is endemic for ND with continuous long lasting outbreaks causing significant losses in the poultry industry [51]. Recently, with the new introduction of NDV genotype VII in 2011-2012 numerous outbreaks of ND in all chicken production sectors were reported, despite of intensive vaccination attempts with homologous ND genotype VII vaccines [91, 109, 121]. Vaccine failure seem to be associated, at least in part, with co-infections of IBV and/or AIV [51, 124, 130].

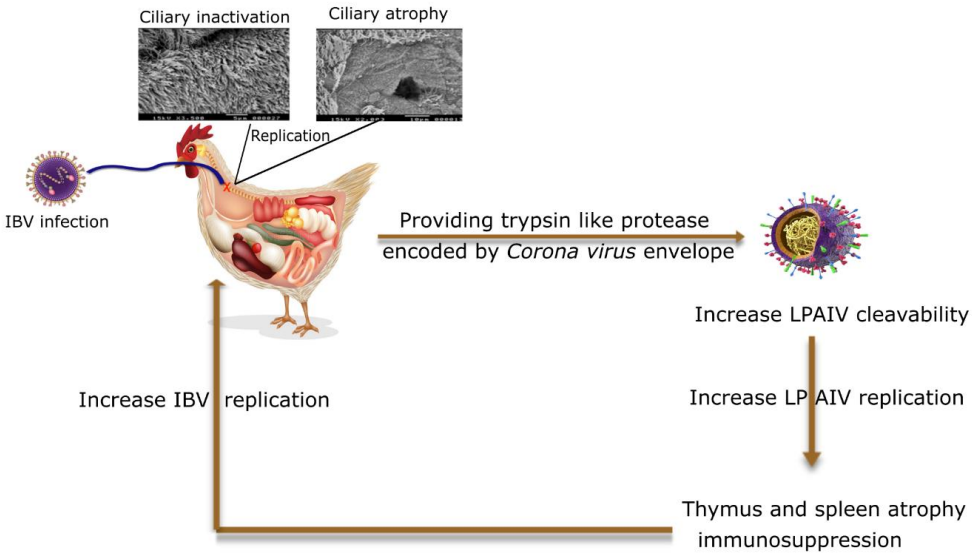


Figure 13. Synergistic pathogenic effects during co-infections of chickens with LPAIV (H9N2) and IBV designed here from data available in Hassan et al., 2017 [52].

2.5.3. Diagnostic challenges

In several poultry species, especially in chickens and turkeys, the case history, clinical manifestations, and mortality can be used for a suspect diagnosis of HPAI, but none of the signs and lesions are pathognomonic, and the etiology must be confirmed using laboratory diagnostic tests [142]. In parallel with the growing challenges of enzootic HPAIV infections since the early 2000's new rapid diagnostic tools have been developed to detect viral antigens or viral nucleic acids including but not limited to rapid chromatographic assays and real time reverse transcriptase polymerase chain reaction (RT-qPCR) [26, 139]. Considering a high mutation rate and reassortment of AIV and changing patterns of co-infections with further viruses and bacterial agents, frequent updates of diagnostic tools are required to ensure their fitness for purpose. The real-time RT-qPCR is particularly suited to detect and characterize the virus directly in field samples without the need of virus isolation providing a fast and reliable tool for epidemiological mapping of circulating disease agents and especially for AIV [56, 138]. It is much faster than the classic method of virus isolation but requires an expensive laboratory setup, special equipment and skilled personnel.

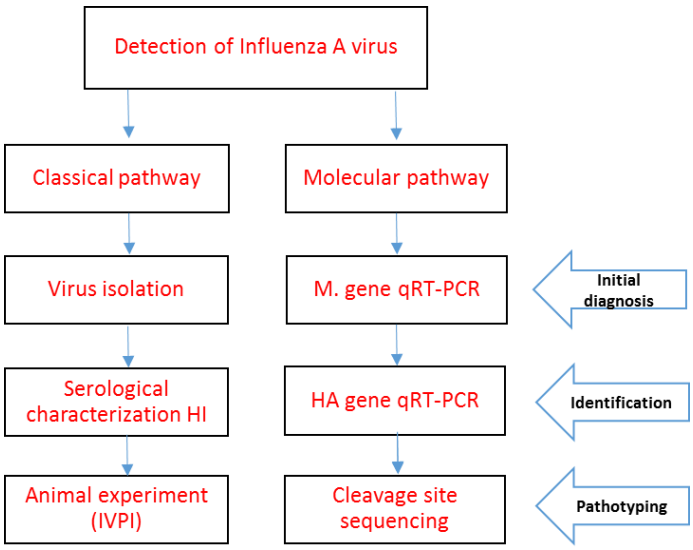


Figure 14. Schematic protocol detailing classical and modern molecular methods used for AI diagnosis (designed here from data available in the OIE Manual of Diagnostic Tests and Vaccines, Avain influenza diagnosis, 2018 [105].

2.5.4. Zoonotic challenges

Regardless of multiple efforts to control AIV, many countries still suffer from enzootic AI in poultry populations. China, Indonesia, Vietnam and Egypt continuously experience outbreaks in poultry, at times along with human infections [65].

The zoonotic nature of HPAI H5N1 viruses of the gs/GD-lineage was first observed in 18 human patients in Hong Kong in 1997. Severe pulmonary disease resulted in six deaths [23]. Along with the spread of these viruses across Asia to European and African countries, more human cases of infection were experienced. According to the World Health Organization (WHO) [160], a total of 861 human infections with 454 fatal cases of an HPAIV H5N1 infection were reported from 16 Countries and Territories so far. The vast majority of human H5N1 cases were associated with close contact to infected live or dead poultry, or H5N1-contaminated environments [72]. In a minority of cases stuttering human-to-human transmission, e.g. in nursing situations or in small family clusters, was suspected [165].

For an effective transspecies transmission of AIV from poultry to humans adaptive changes in receptor binding efficacy are required firstly. Receptor binding sites (RBS) in the HA glycoprotein need to mutate from its avian-optimized configuration to increased binding preference for α2-6 SA, the human receptor type [87, 148]. Mutations mainly in two key AA in the RBS were important during the evolution of the H5N1 viruses isolated from human infected cases [23, 141]. Key amino acids at positions Q226 and G228 (H3 HA numbering) affect the SA preference of the HA of several subtypes [27, 165]. Progressive mutations Q226L and G228S enables AIV to skew their SA linkage preference toward human receptors. A plethora of further adaptive mutations in other internal gene segments like in PB2, NP and NA play a role in adapting the virus better to the mammalian cellular machinery and to allow for more unimpeded replication [143]. Egyptian HPAI H5N1 viruses revealed binding affinity to both avian and human-like receptors [123]. Specially in clade 2.2.1.2 viruses were capable of increased replication in human cells while maintaining the ability for replication in avian cells [153]. However, key mutations at positions 226 and 228 were not detected in clade 2.2.1.2, but other four characteristic mutations were common (D43N,

S120[D,N], [S,L]129Δ and I151T) and indicated the extensive evolution of Egyptian H5N1 HPAI virus towards human hosts [13].

Egypt reported 41.8% of all globally confirmed human cases of HPAIV H5N1, i.e. 359 cases with 120 deaths. It holds the second rank following Indonesia with respect to case fatality, 33 versus 84% [159]. From 2006 – 2014, 210 human H5N1 cases were reported in Egypt, however, the incidence peak of reported human infection was in 2015 [162]. Within a short period of several months along with the emergence of the new H5N1 clade 2.2.1.2, 136 human cases occurred [13]. Egyptian clade 2.2.1.1 H5N1 viruses, present from 2008-2010 in Egypt, in contrast were highly adapted to (vaccinated) chickens and replicated at lower levels in human lung cells than viruses of clade 2.2.1.2 [154]. This may partially explain the grossly absence of incidence human infected cases with clade 2.2.1.1 viruses in Egypt. Egypt reported 173 infected human cases by clade 2.2.1 from 2006 until 2013 versus 173 infected human cases with clade 2.2.1.2 during only 2014-2015 [159]. Moreover, absence of human cases with clade 2.2.1.1, indicating that most avian-to-human transmissions were attributed to the avian viruses of clade 2.2.1 and 2.2.1.2 [90].

With the introduction of HPAIV H5N8 since 2016 and increased numbers of H5N8-infected farms a reduction in numbers of human cases in Egypt has been noted (2016: n=10, 2017: n=3, no cases in 2018 and 2019) [159] (Fig.15). Although this may be purely co-incidental and signal underreporting or more effective barriers at the poultry-human interphase, clade 2.3.4.4b viruses are known to have grossly reduced zoonotic propensity versus clade 2.2.1.2 H5N1 [13].

In addition, the LPAIV H9N2 also possesses zoonotic potential. However, upon transmission to humans mostly self-limiting respiratory infections are caused but multiple organ failures leading to death has been reported from immunocompromised patients [102]. Egypt reported three cases of human infection with influenza A (H9N2) viruses until 2014 [161].

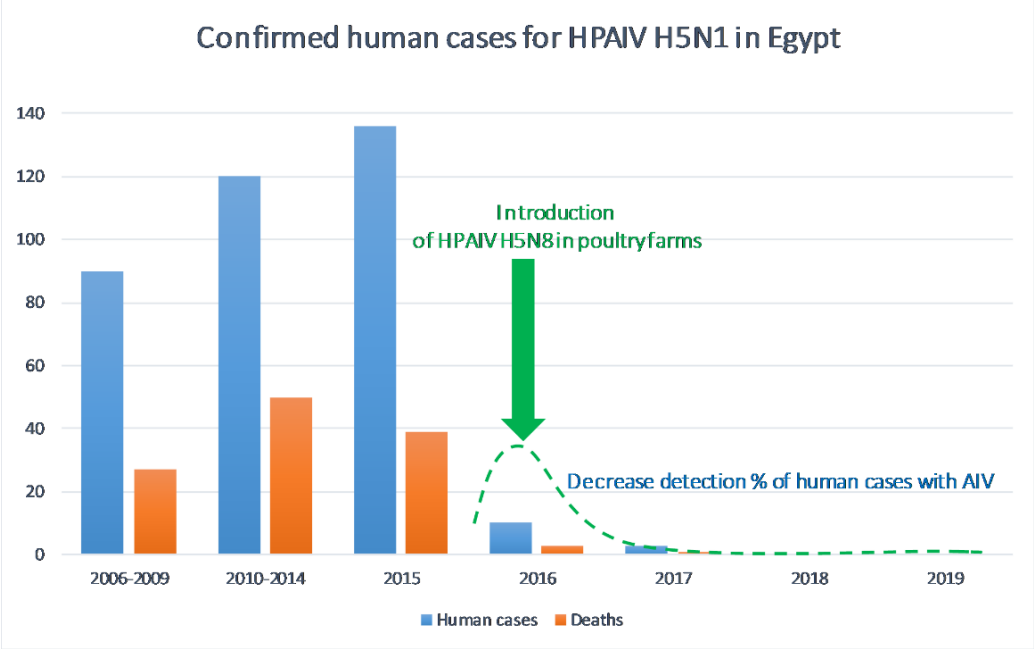


Figure 15. Human cases of HPAIV H5N1 infection (blue) in relation fatal cases (orange) since the introduction of HPAIV H5N1 into Egypt until 2019. Note the decline in reported human cases along with the new introduction of HPAIV H5N8 in 2016 into Egypt (designed here from data available from WHO/GIP reports as of 27 September 2019 [159]).

3. Summary of the current dissertation

3.1. Publication 1

Kareem E. Hassan, Magdy F. El-Kady, Azza A. A. EL-Sawah, Christine Luttermann, Rokshana Parvin, Salama Shany, Martin Beer, and Timm Harder**. Respiratory disease due to mixed viral infections in poultry flocks in Egypt between 2017 and 2018: Upsurge of highly pathogenic avian influenza virus subtype H5N8 since 2018**. Transboundary and Emerging Diseases. 2019; e.p. 1– 16. https://doi.org/10.1111/tbed.13281.

In this publication, our analyses focussed on 39 poultry farms suffering from respiratory manifestation and high mortality in six Egyptian governorates during 2017–2018. Real-time RT-PCR (RT-qPCR) substantiated the co-presence of at least two respiratory virus species in more than 80% of the investigated flocks. The percentage of HPAIV H5N1-positive holdings was fairly stable in 2017 (12.8%) and 2018 (10.2%), while the percentage of HPAIV H5N8-positive holdings increased from 23% in 2017 to 66.6% during 2018. The proportion of H9N2-positive samples was constantly high (2017: 100% and 2018: 63%), and H9N2 co-circulated with HPAIV H5N8 in 22 out of 39 (56.8%) flocks. Analyses of 26 H5, 18 H9 and 4 N2 new sequences confirmed continuous genetic diversification. In silico analysis revealed numerous amino acid substitutions in the HA and NA proteins suggestive of increased adaptation to mammalian hosts and putative antigenic variation. For sensitive detection of H9N2 viruses by RT-qPCR, an update of HA and NA primers and probe sequences was conducted.

3.2. Publication 2

Kareem E. Hassan, Jacqueline King, Magdy El-Kady, Manal Afifi, Hassanein H. Abozeid, Anne Pohlmann, Martin Beer, and Timm Harder. **Novel Reassortant Highly Pathogenic Avian Influenza A (H5N2) Virus in Broiler Chickens, Egypt.** Emerging Infectious Diseases. 2020; 26(1):129-133. https://dx.doi.org/10.3201/eid2601.190570.

In this publication, we investigated 11 commercial broiler farms reporting respiratory clinical signs among chickens by using the Riems Influenza A Typing Assay. We detected co-presence of avian influenza viruses subtypes H5 and H9 with N2 (8 farms) as well as

H5N2 only (3 farms). We selected 8 samples representing H5N8, H9N2, and H5N1 from 2017–2018, plus a single positive H5N2 sample from 2019, for full-genome sequencing. Sanger- and next generation sequencing results identified various reassortants new to Egypt during early 2019. Full genome sequencing also confirmed presence of a novel reassortant highly pathogenic avian influenza A (H5N2) virus in three chicken farms in Egypt for the first time. The virus carried genome segments of a pigeon H9N2 influenza virus detected in 2014; a nucleoprotein segment of contemporary chicken H9N2 viruses from Egypt, and hemagglutinin derived from the 2.3.4.4b H5N8 virus clade. In addition, full genome sequencing detected novel reassortant LPAIVs H9N2 in chicken farms in Egypt for the first time. This virus was a result of reassortment between the classical LPAI H9N2 viruses circulating in Egyptian poultry farms since the virus introduction into Egypt and novel reassortant LPAI H9N2 detected in pigeons in Egypt at 2014.

3.3. Publication 3

Kareem. E. Hassan, Noha Saad, Hassanein H. Abozeid, Salama Shany, Magdy. F. El-Kady, Abdelsatar Arafa, Azza A. A. EL-Sawah, Hafez. M. Hafez, Martin Beer, Timm Harder. **Genotyping and reassortment analysis of highly pathogenic avian influenza viruses H5N8 and H5N2 from Egypt reveals successive annual replacement of genotypes.** Infection, Genetics and Evolution. 2020; P. 104375. ISSN 1567-1348.
https://doi.org/10.1016/j.meegid.2020.104375

In this study, we focussed on the analysis of 32 duck flocks, four broiler chicken flocks, and a single turkey flock, suffering from respiratory manifestations with moderate to high mortality reared in two Egyptian governorates during 2019. By RT-qPCR, HP H5N8 was detected in 21 of the 37 investigated flocks with mixed infection of H9N2 in two of them. Failure to detect any HP H5N1 viruses in the 21 HP-positive farms is in line with our previous observation of a substantial decline of HP H5N1 versus HP H5N8. Full hemagglutinin (HA) sequencing of ten samples and full genome sequencing of three additional samples revealed presence of a single, homogenous genotype. Remarkably, amino acid substitutions in the Matrix (M2) and the Neuraminidase (NA) proteins conferring resistence to antivral drugs Amantadine and Oseltamivir were detected. Also, systematic reassortment analysis of all

publicly available Egyptian whole genome sequences of HP H5N8 (n=23), reassortant HP H5N2 (n=2) and LP H9N2 (n=53) viruses revealed presence of at least seven different genotypes of HPAI H5Nx viruses of clade 2.3.4.4b in Egypt since 2016. For H9N2 viruses, at least two genotypes were distinct. Heat mapping and tanglegram analyses suggested that several internal gene segments in both HP H5Nx and H9N2 viruses originated from AIV circulating in wild bird species in Egypt. Based on the limited set of whole genome sequences available, annual replacement patterns of HP H5Nx genotypes emerged and suggested selective advantages of certain genotypes since 2016.

CHAPTER 2: Results

2.1. Respiratory disease due to mixed viral infections in poultry flocks in Egypt between 2017 and 2018: Upsurge of highly pathogenic avian influenza virus subtype H5N8 since 2018

Kareem E. Hassan, Magdy F. El-Kady, Azza A. A. EL-Sawah, Christine Luttermann, Rokshana Parvin, Salama Shany, Martin Beer, and Timm Harder.

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

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2.3. Genotyping and reassortment analysis of highly pathogenic avian influenza viruses H5N8 and H5N2 from Egypt reveals successive annual replacement of genotypes

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CHAPTER 3: General discussion

Discussion

Avian influenza is considered one of the most important diseases in the OneHealth field causing continuous threats for poultry and human health during the past decades [142]. The continuous emergence of new (HP) AIV subtypes had a massive negative impact on poultry production and international trade [7]. This is partly due to the very high death toll of HPAI in poultry populations causing huge economic losses, and partly because of the ability of some AI viruses to cross species barriers and invoke severe and often fatal illness in human beings [106].

Egypt is considered one of the countries that has been affected harshly by HPAI since the first incursion of virus in 2006 [11]. All efforts to eradicate and control AI in Egypt since then were unsuccessful. Owing to failure of HPAI control Egypt is classified as a country endemically infected with HPAIV [6]. The AI situation in Egypt has been characterized by a continuous emergence of variants (HP H5N1 clade 2.2.1.1), introduction of new subtypes (HP H5N8 clade 2.3.4.4b), and co-circulation of different AI subtypes (HP H5, H9) [5, 9, 11, 27].

Here, the main objectives of this study (see page 4 of this thesis) aim at providing an update of the epidemiological situation regarding AIV including molecular characterization (chapters 2.1, 2.2 and 2.3), and genotyping and reassortment analysis (chapter 2.2 and 2.3) of AIV subtypes circulating in poultry in Egypt between 2017-2019. In addition, an assessment was attempted of the role of co-infections with different AIV subtypes and/or other respiratory viruses such as IBV and NDV (chapter 2.1). As a prerequisite for such work improved diagnostic tools were developed and evaluated for AIV detection (chapters 2.1).

3.1. Respiratory disease due to mixed viral infections in poultry flocks in Egypt between 2017 and 2018: Upsurge of highly pathogenic avian influenza virus subtype H5N8 since 2018

3.1.1. Co-circulation of avian respiratory viruses

This study confirmed the co-circulation of three different AIV subtypes, HPAIV H5N1, HPAIV H5N8 and H9N2 to be an ongoing problem (chapter 2.1). In contrast to

previous studies focussing on the years 2016 and 2017 [130], however, a gain in numbers of HPAIV H5N8-positive farms (35 of 39 holdings examined) was observed. Conversely, the ratio of HPAIV H5N1 infected farms declined (9 of 39 holdings, in 2018) whereas until beginning of 2017 HP H5N1 dominated the situation [14]. Full genome sequence analysis of two HPAI H5N1 viruses from 2017 suggested a stable genomic structure compared to older strains in the country, and no differences in the HA protein of clade 2.2.1.2 were seen in phylogenetic analyses compared to HPAI H5N1 viruses isolated since 2015 (Chapter 2.2). In a second field investigation study that had been launched in 2019 (Chapter 2.3) the HP H5N1 virus was no longer detected in any of 27 positive AI farms although all farms were positive for HP H5N8. The reasons for the gain of H5N8 positive farms are not clear but it must be considered that all vaccination in Egypt against HPAIV, until late 2018, was carried out with vaccines originally designed against clade 2.2.1-like H5N1 viruses [69]. It may be speculated that under field conditions, antigenically distinct clade 2.3.4.4b viruses are not sufficiently controlled by such vaccine-induced immunity and therefore gained an advantage with respect to a lack of herd immunity against clade 2.3.4.4b viruses. However, no representative serological data are available to support this assumption.

In addition to the upsurge of HP H5N8 infections, combinations of co-infection of three different AIV subtypes and other respiratory pathogens were detected in 32 out of 39 (82.1%) farms examined here (Chapter 2.1). Single infections were registered for only 17.9% of farms (7/39, three and four times H9N2 or H5N8, respectively). The percentage of H9N2-positive samples was high (27/39), and co-presence with HPAIV H5N8 was seen most often, that is in 56.4% (22/39) of the farms. In addition, IBV-positive samples were frequently detected, especially during the winter season 2017/18 (Chapter 2.1). Occasionally, also vNDV was detected in co-infections including two duck farms. The latter finding was unexpected but seems to be in line with a reported gradual increase of vNDV cases in commercial duck farms in recent years [10, 31, 88] (Chapter 2.1).

In conclusion, multifactorial synergistic actions of several avian viral pathogens were shown in this study to contribute to a clinically diffuse but loss-making poultry respiratory syndrome in Egypt with (HP) AIV, IBV and vNDV being the most frequently detected viruses (Chapter 2.1) similar to what was reported previously [84, 120].

3.1.2. Viral sequence and phylogenetic analyses

Sequence and phylogenetic analyses of the HA sequences showed that both H5 and H9 viruses examined here fitted the tips of the respective cluster branches as a result of ongoing genetic drift (Chapter 2.1). This drift was particularly evident for the HPAIV H5N8 HA sequences of clade 2.3.4.4b (chapter 2.1, Figure 3a). It is noticed also that viruses originating from duck holdings sampled mainly in 2017 were distinct from younger H5N8 viruses collected mainly from gallinaceous poultry in 2018. HP H5N8 virus clusters of 2018 were marked by amino acid substitutions M/I175L and/or E268G in addition to S155D (chapter 2.1, Figure 4a). These data together also indicate the existence of separate introductions and continued co-circulation of different H5N8 virus lineages in gallinaceous poultry and waterfowl flocks in Egypt [8, 167]. For H5N1 viruses, continuous circulation of clade 2.2.1.2 with unaltered deduced protein sequences was shown until end of 2018.

The HA sequence and phylogenetic analysis of recently detected H9N2 viruses showed that circulating viruses still belong to the G1-lineage closely related to H9N2 viruses detected in Egypt since 2015 with HA signature mutations M40K/R , V194I, V411I and K483M/I (chapter 2.1, Figure 4b). Several coding mutations were delineated in the NA haemadsorption site: R372A, I402N and R403W (chapter 2.1, Figure 4C); substitutions in these sites had been associated with adaptation to mammalian hosts [94]. The NA of the four H9N2 viruses under study has eight potential N-linked glycosylation sites similar to ancestral H9 G1 strains. This differs, however, from all previous Egyptian H9 strains that had only seven potential N-linked glycosylation sites. An additional site at 402 was identified (S/G402N). The same site was also present in an H9N2 G1 virus of human origin (A/HK/1073/99)[78]. It is interesting to note that the global number of human H9N2 G1 infections has recently been increasing [158].

3.2. Novel reassortant avian influenza viruses in Egypt

A first suspicion that a new HPAIV H5N2 reassortant may have emerged in Egyptian poultry came from the examination of a duck sample (AR-542/18, collected in January 2018), which was positive for both H5 and N8 and for N2 but negative for H9. Examination of this sample by RITA confirmed that no other HA subtype except H5 was

present (Chapter 2.1) HPAI H5N2 reassortant viruses carrying the H5 of the 2.3.4.4 clade have been described in domestic birds in Canada 2014 [113] and in wild birds in China [83]. While low pathogenic H5N2 virus has been detected in wild birds in Egypt (A/Shoveler/Egypt/20313-NAMRU3/2003, H5N2) [137], the presence of such viruses in poultry in Egypt has not been confirmed so far. Nevertheless, the generation and spread of reassortants between the two HPAIV H5 virus clusters in Egypt with H9N2 viruses would not come as a surprise and must be considered in all surveillance efforts [100].

3.2.1. Detection of novel reassortant H5N2 viruses in chickens

For further investigations on the suspected presence of reassortants new samples were collected during early 2019 from 11 commercial broiler chicken farms in Egypt reporting respiratory clinical signs [57] (Chapter 2.2). We detected co-presence of AIV subtypes H5, H9 and N2 on 8 farms as well as H5N2 only on three farms. Sanger- and next-generation sequencing confirmed the existence of an HPAIV H5N2 reassortant in samples of the latter three farms (Chapter 2.2). At the same time in March 2019 the Egyptian Ministry of Agriculture announced the detection of another new HPAIV H5N2 detected in seemingly healthy ducks in the Dakahlia governorate [49]. The duck reassortant virus carried a neuraminidase N2 segment of chicken H9N2 viruses in the background of an HPAI H5 clade 2.3.4.4b virus. Our data confirm the presence of another, different HPAIV H5N2 reassortant and its occurrence in chickens in different geographic regions of Egypt (Chapter 2.2). The HA of the latter reassortant was derived from HPAIV H5 subtype of clade 2.3.4.4b whereas four genome segments (PB1, PB2, PA, and NS) originated from a novel reassortant H9N2 viruses first detected in pigeons in Egypt in 2014. The NP, NA and M segments were likely acquired from H9N2 viruses circulating in chickens in Egypt since 2010, although M and NA segments are identical in the pigeon and chicken H9N2 viruses (Chapter 2.2, Figure 1).

It is interesting to note that only HPAI viruses of clade 2.3.4.4b were active in reassortment events in Egypt whereas up to date no reassortants involving HPAI H5N1 viruses have been reported from the field in Egypt. Given the current decline of HPAIV H5N1 and the expansion in poultry populations of HPAIV H5 2.3.4.4b the emergence of further reassortant strains is becoming more likely and, in fact, has been documented in further studies carried out in the frame this thesis:

3.2.2. Detection of multiple reassortant H5N8 viruses

Full genome sequencing of three H5N8 viruses isolated from duck and turkey holdings during 2017 and 2018 revealed the presence of two different reassortants (Chapter 2.2). For the HP H5N8 virus from the turkey holding all genome segments except PB1 showed high homology to Russian HP H5N8 viruses detected in 2017, while the PB1 was related to Chinese HP H5N8 viruses of 2016. The HP H5N8 viruses from duck holdings harboured PB2, PB1 and NP segments that were more closely related to an H3N6 virus from Bangladesh, an H7N7 virus from Georgia and HP H5N8 viruses from Russia, respectively, while other segments were related to Chinese HP H5N8 viruses (Chapter 2.2, Figure 1). Presence of these and other reassortant H5N8 viruses suggests a continuous introduction of new HP H5N8 reassortants besides on-going virus circulation and endemic reassortments in Egypt [8, 126, 167].

Retrospective analysis of whole-genome sequences of HP H5N8 viruses including 23 complete genomes of HP H5N8 sampled in Egypt during the period 2016–2020 with three new HP H5N8 complete sequences of 2019 established in this study in addition to two reassortant HP H5N2 virus genomes revealed annual patterns of replacement of dominant genotypes (Chapter 2.3, Figure 1A). In 2016, the year of introduction, HP H5N8 was mainly detected in wild birds [126, 167]. Similar to the situation described in Europe, at least two genotypes were simultaneously circulating. When the focus of virus circulation shifted from wild birds to poultry, another genotype took over, carrying a new set of internal segments. The same process was observed in 2018/2019 (Chapter 2.3, Figure 2 A).

Based on the host species and on closely related wild bird sequences from Russia, Korea, Mongolia, India and Germany, genotypes-1 and -2 are likely to be direct introductions via infected wild birds. Similarly, genotype-5 viruses in Egypt have closely related ancestral genomes detected earlier in western Europe (Chapter 2.3, Figure 1A). A substantial proportion of sequences available for analysis here comes from duck holdings, which often have a much closer connection to aquatic wild bird habitats in Egypt enabling reassortment in unknown avian populations within Egypt. In particular, viruses of genotypes -3 and -4 are unique to Egypt (Chapter 2.3, Figure 1A). It is interesting to note that these genotypes carried genome segments that were very closely related to those present in LP

H7N9, and H7N3 and H3N6 viruses found in Egyptian wild birds earlier or at the same time [8, 71, 101, 167].

3.2.3. Detection of novel reassortant H9N2 viruses in chickens

Presence of novel reassortant LPAI H9N2 viruses in chicken holdings in Egypt was detected in this study for the first time. PB2, PB1, PA and NS gene segments were derived from reassortant H9N2 viruses detected in pigeons in Egypt in 2014 [66], while the remaining segments were obtained from H9N2 virus circulating in poultry farms in Egypt since 2010 (Chapter 2.2, Figure 1). Phenotypic characteristics of this reassortant affecting virulence, zoonotic propensity and further reassortment behaviour have not been analysed here. However, a retrospective analysis for all available full genome H9N2 sequences (52 complete genomes of H9N2 viruses sampled from Egypt during the period 2010–2020, Chapter 2.3, Figure 1B) showed, that new reassortant H9N2 viruses recently detected in chickens in 2019 more related to reassortant H9N2 detected in pigeons in 2014 (Gt-2) [66], but formed a new genotype (Gt-3) that differed from classical H9N2 circulating in Egypt since 2010 (Gt-1). The new chicken origin reassortant HP H5N2 derived its main genome cassette from H9N2 G-3 viruses (Chapter 2.3, Figure 4).

3.3. Diagnostic challenges and novel solutions

Avian influenza evolution is fast, with reassortation and point mutations shaping central biological characteristics such as host tropism, virulence, and immune escape. However, escape from molecular diagnostic tools may be another sequel of these processes. Due to the error-prone replication of IAV, genetic drift occurs and mutations may also affect primer and probe binding sites used in diagnostic RT-PCRs. In Egypt, such difficulties in diagnosis have been reported [5] in particular with viruses of the escape mutant lineage of clade 2.2.1.1 [7, 55]. The consensus sequence of this clade revealed multiple single nucleotide substitutions in the target regions of primers and probes of the H5 gene. A modified RT-qPCR assay re-established sensitivity for the detection of clade 2.2.1.1 viruses in Egypt [5]. Therefore, continuous updating and improving such molecular diagnostic tools is of utmost importance. Presence and circulation of multiple AI subtypes in Egypt with possibilities of emerging reassortants is one of the most important diagnostic challenges in

Egypt besides the presence of different subtypes detected in wild migratory birds. Sampling, sample transfer, preservation and processing are also considered problematic in hot climates like in Egypt [45].

3.3.1. Diagnostic challenge for H9 and N2 detection

Several Egyptian poultry samples examined here yielded highly positive M-PCR signals but produced very flat H9 and N2 amplification signals when using previously published RT-qPCRs (Chapter 2.1). By sequence analyses these samples were shown to contain H9N2 viruses with several mutations present in the primer binding regions of the HA and NA genome segments. Redesigned sets of primers and probes re-established sensitivity and specificity of the RT-qPCRs for H9 and N2 (Chapter 2.1). This example emphasizes that diagnostic RT-qPCR tools must be checked regularly to be fit for the purpose of proper surveillance.

3.5. Conclusions

1. Increased detection of avian-adapted HPAIV H5N8 and decreased circulation of potentially zoonotic HPAIV H5N1 was noted. This might have contributed to the absence of human HPAI H5 cases in Egypt since 2018.
2. Co-circulation of respiratory pathogens such as IBV and ND with different AI subtypes in Egypt led to exacerbated clinical signs (poultry respiratory syndrome), complicated intervention, and control procedures.
3. The continuous high detection rate of LPAIV H9N2 challenges the efficacy of vaccines and vaccination strategies against this virus.
4. Phylogenetic analyses suggest on-going genetic drift for H5N8 and H9N2 viruses.
5. The incursion of clade 2.3.4.4b viruses into Egypt in 2016 not only added another antigenically distinct HPAI virus, but also signalled an increased reassortment risk.
6. Detection of novel reassortant HPAI H5N2 and LPAI H9N2 viruses in chickens re-emphasizes the need for a long-term solution in combatting AIV infections in poultry, also to prevent the emergence of further potentially zoonotic influenza viruses.

7. Systematic reassortment analysis of all publicly available Egyptian whole genome sequences of HP H5N8, reassortant HP H5N2 and LP H9N2 viruses revealed presence of at least seven different genotypes of HPAI H5Nx viruses of clade 2.3.4.4b in Egypt and three distinct genotypes of LP H9N2 G1 viruses
8. Heat mapping and tanglegram analyses suggested that several internal gene segments in both HP H5Nx and H9N2 viruses originated from avian influenza viruses circulating in wild bird species in Egypt.
9. Amino acid substitutions in the Matrix (M2) and the Neuraminidase (NA) proteins of H5N8 viruses circulating in 2018-2019 suggested presence of antiviral resistance against both Amantadine and Oseltamivir which apparently was acquired during circulation in Egypt.
10. A continuous updating of diagnostic tools is urgently needed to counterbalance the emergence of new reassortant and mutant AIV.

3.6. Recommendations

Combatting the risks of human exposure to zoonotic animal viruses is based on draining the reservoirs of these viruses in animal populations [152]. In Egypt, this would mean to disrupt and terminate the enzootic circulation cycles of AIV in poultry. Progress in this respect can be expected only from a concerted national control strategy in cooperation between the Ministries of Agriculture (veterinary authorities), Health, Interior Affairs and the Presidential authority. Strategic targets for consideration are suggested as follows:

1. Transfer of most poultry farms into desert areas and away from areas with dense human population whereby securing adequate safety distances.
2. Provision of good infrastructure for these farms.
3. Strictly prohibit any poultry farming without licence from the competent veterinary authority.
4. Requirement of certified specific biosecurity instructions and continuous veterinary campaigns on farms to check the biosecurity measures in place.
5. Continuous awareness campaigns in media about the dangers of backyard and household rearing of poultry.

6. Encouragement of public-private partnerships for building poultry abattoirs in different governorates as an advanced step towards prohibiting live birds markets.
7. Provide advanced outlets for frozen poultry meat from national production under veterinary authority supervision.
8. Prohibit uncontrolled live bird markets and transfer of live birds from one place to another.
9. Provide special well-prepared vehicles for transfer of poultry to slaughter houses.
10. Issue new licences for hatcheries with specific biosecurity measures and provide similar vehicles for transfer of one-day-old chicks to farms.
11. Reorganize the issuing of vaccine licences and re-evaluate the efficacy of vaccines especially that of live-attenuated ones.
12. Ensure continuous surveillance and full genome sequencing of circulating viruses from poultry and wild birds; provide data in public epidemiological maps.
13. Strict supervision of the use of pharmaceutical drugs in poultry. Antivirals are for human medication only.
14. Use of updated and approved diagnostic tools that are regularly evaluated to be fit for purpose in the Egyptian setting.
15. Provide updated compensation regulations for notifiable outbreaks of avian diseases including backyard and household poultry rearing systems.

CHAPTER 4: Summary

Summary

Since 2006, Egypt is facing huge challenges related to the spread of avian influenza viruses. This is partly due to the very high death toll of HPAI in poultry populations causing huge economic losses. The ability of some AI viruses to cross species barriers and invoke severe and often fatal illness in human beings causes further alert. Sequential introduction of new viruses and enzootic co-circulation of different AI subtypes and clades as well as presence of further avian respiratory pathogens, such as IBV and NDV, are at the basis of these problems. All previous efforts to eradicate and control AI in Egypt were unsuccessful. The main objectives of this study aim at providing an update of the epidemiological situation regarding AIV including molecular characterization, and genotyping and reassortment analysis of AIV subtypes circulating in poultry in Egypt between 2017 and 2019. In addition, an assessment was attempted of the role of co-infections with different AIV subtypes and/or other respiratory viruses such as IBV and NDV. As a prerequisite for such work, improved diagnostic tools were developed and evaluated for AIV detection.

In Chapter 2.1, various combinations of co-infections in poultry flocks with three different AIV subtypes and other respiratory pathogens such as IBV and NDV were detected in 32 out of 39 farms examined. The percentage of AIV H9N2-positive samples was high (27/39), and co-presence of HPAIV H5N8 was detected most often (22/39). The previously dominating HPAIV subtype H5N1 was only infrequently detected. Sequence and phylogenetic analyses of the hemagglutinin gene showed that both H5 and H9 viruses were located at the tips of the respective cluster branches indicating ongoing genetic drift. Several coding mutations in the neuraminidase N2 hemadsorption site suggested some adaptation to mammalian hosts. Sensitive detection of H9N2 viruses by RT-qPCRs required an update of diagnostic tools.

Examination of further Egyptian poultry samples collected in early 2019 from 11 commercial broiler chicken farms revealed the presence and circulation of new reassortant AI viruses (Chapter 2.2). This affected HPAIV H5N8 of which two different reassortants were characterized that had not been reported in Egypt before. Also, novel reassortant H9N2 viruses were detected in chicken holdings for the first time in Egypt. Most importantly, however, a novel reassortant HPAIV H5N2 was found on three chicken holdings in different areas of Egypt. The HA gene segment was donated from HPAI H5 clade 2.3.4.4b viruses whereas four

genome segments (PB1, PB2, PA, and NS) originated from another novel reassortant H9N2 viruses first detected in pigeons. Other segments (NP, NA, M) were acquired from H9N2 viruses circulating in chickens in Egypt since 2010. The formation of new reassortants and subtype may cause further problems in diagnosis and control of these viruses.

Real-time RT-PCR investigations substantiated the presence of HP H5N8 in 21 of a further 37 investigated flocks sampled in 2019 (Chapter 2.3). HPAIV H5N1 was detected no longer in any of those holdings. Full-genome sequencing of three H5N8 viruses revealed presence of amino acid substitutions in the M2 and NA proteins conferring resistence to antiviral drugs Amantadine and Oseltamivir. Apparently, these mutations were acquired during circulation in Egypt. Systematic reassortment analysis of all publicly available whole genome sequences of HP H5N8 (n=23), reassortant HP H5N2 (n=2) and LP H9N2 (n=53) viruses from Egypt revealed presence of at least seven different genotypes of HPAI H5Nx viruses of clade 2.3.4.4b in Egypt since 2016. For H9N2 viruses, at least three genotypes were distinguishable. Heat mapping and tanglegram analyses suggested an annual replacement pattern of HP H5 genotypes. Several internal gene segments in both HP H5Nx and H9N2 viruses originated from AIV circulating in wild bird species in Egypt indicating local reassortment processes.

Constant introduction of new AIV strains, reassortment leading to the generation of new subtypes and on-going genetic drift in AIV in Egypt called for a continuing update of molecular diagnostic tools. Based on the conclusions of this study recommendations for improved regulations and control of notifiable avian diseases in Egypt are provided.

CHAPTER 5: Zusammenfassung

Aviäre Influenza-Infektionen in Geflügelfarmen in Ägypten, eine ständige Herausforderung: Aktuelle Probleme im Zusammenhang mit Pathogenese, Epidemiologie und Diagnose

Zusammenfassung

Ägypten steht seit 2006 vor großen Herausforderungen im Zusammenhang mit der Verbreitung von Aviären Influenzaviren (AIV). Dies ist teilweise auf die sehr hohe Mortalität der hochpathogenen aviären Influenza (HPAI) in Geflügelpopulationen zurückzuführen, die enorme wirtschaftliche Verluste verursachen. Die Fähigkeit einiger AI-Viren, Artenbarrieren zu überwinden und beim Menschen schwere und nicht selten tödliche Infektionsverläufe hervorzurufen, stellt eine Bedrohung der öffentlichen Gesundheit dar. Die sequentielle Einführung neuer Viren und die enzootische Kozirkulation verschiedener AI-Subtypen und -Kladen sowie das Vorhandensein weiterer aviärer Atemwegserreger wie das infektiöse Bronchitis Virus (IBV) sowie das Newcastle Virus (NDV) sind die Grundlage dieser Probleme. Alle bisherigen Bemühungen zur Ausrottung und Kontrolle der AI in Ägypten waren erfolglos. Die Hauptziele der hier vorgelegten Untersuchungen sind die Aktualisierung der Kenntnisse der epidemiologischen Situation in Bezug auf AIV, einschließlich der molekularen Charakterisierung sowie der Genotypisierung von AIV-Subtypen, die zwischen 2017 und 2019 bei Geflügel in Ägypten zirkulierten. Darüber hinaus wurde versucht, die Bedeutung von Koinfektionen mit verschiedenen AIV-Subtypen und anderen Atemwegsviren zu bewerten. Als Voraussetzung für solche Arbeiten wurden auch verbesserte Diagnosewerkzeuge entwickelt und für die AIV-Erkennung und Subtypisierung evaluiert.

In Kapitel 2.1 wurden in 32 von 39 untersuchten Geflügelbetrieben verschiedene Kombinationen von Koinfektionen mit drei verschiedenen AIV-Subtypen und anderen respiratorischen Pathogenen wie IBV und NDV nachgewiesen. Der Anteil AIV H9N2-positiver Proben war hoch (27/39), und das gleichzeitige Vorhandensein von HPAIV H5N8 wurde am häufigsten festgestellt (22/39). Der zuvor dominierende HPAIV-Subtyp H5N1 wurde dagegen nur selten nachgewiesen. Sequenz- und phylogenetische Analysen des Hämagglutinin-Gens zeigten, dass sich sowohl H5- als auch H9-Viren an den Spitzen der jeweiligen Cluster-Zweige befanden, was auf eine anhaltende genetische Drift hinweist. Der sensitive Nachweis von H9N2-Viren durch RT-qPCRs erforderte eine Aktualisierung der Diagnosetools. Mehrere kodierende Mutationen im Hämadsorptionsbereich der N2 Neuraminidase deuteten auf eine zunehmende Anpassung dieser H9N2-Viren an Säugetierwirte hin.

Die Untersuchung weiterer ägyptischer Geflügelproben, die Anfang 2019 von 11 kommerziellen Broiler-Hühnerfarmen entnommen wurden, ergab Hinweise auf die Verbreitung neuer, reassortierter AI-Viren (Kapitel 2.2). Dies betraf HPAIV H5N8, von dem zwei verschiedene Reassortanten charakterisiert wurden, die zuvor in Ägypten nicht nachgewiesen worden waren. Außerdem wurden in Ägypten erstmals neuartige H9N2 Reassortanten in Hühnerbetrieben nachgewiesen. Am wichtigsten ist jedoch, dass in drei Hühnerbetrieben in verschiedenen Gebieten Ägyptens eine neuartige HPAIV H5N2 Reassortante gefunden wurde. Das HA-Gensegment stammte von HPAI-H5N8-Klade-2.3.4.4b-Viren, während vier Genomsegmente (PB1, PB2, PA und NS) von anderen neuartigen H9N2-Viren stammten, die erstmals 2104 in Ägypten in Tauben nachgewiesen wurden. Andere Segmente (NP, NA, M) wurden von H9N2-Viren erworben, die seit 2010 in Hühnern in Ägypten zirkulieren. Die fortgesetzte Bildung neuer Reassortanten und Subtypen verursacht erhebliche Probleme bei der Diagnose und Kontrolle dieser Viren.

Echtzeit-RT-PCR-Untersuchungen belegen das Vorhandensein von HP H5N8 in 21 von weiteren 37 untersuchten Herden, die 2019 beprobt wurden (Kapitel 2.3). HPAIV H5N1 wurde in keinem dieser Betriebe mehr nachgewiesen. Die vollständige Genomsequenzierung von drei H5N8-Viren zeigte das Vorhandensein von Aminosäuresubstitutionen in den M2- und NA-Proteinen, die Resistenz gegen die Virustatika Amantadin und Oseltamivir vermitteln. Anscheinend wurden diese Mutationen im Zuge der Verbreitung dieser Viren in Ägypten erworben. Eine systematische Analyse aller öffentlich verfügbaren Gesamtgenomsequenzen von HP H5N8 (n = 23), HP H5N2 Reassortanten (n = 2) und niedrigpathogenen H9N2-Viren (n = 53) aus Ägypten ermittelte sieben verschiedene Genotypen von HPAI H5Nx-Viren der Klade 2.3.4.4b seit 2016. Heatmap- und Tanglegramm-Analysen deuteten auf eine jährliche Ersetzung der jeweils prävalenten HP H5-Genotypen hin. Für H9N2-Viren waren mindestens drei Genotypen unterscheidbar. Mehrere interne Gensegmente sowohl in HP H5Nx- als auch in H9N2-Viren stammten von AIV, die in Wildvogelarten in Ägypten zirkulierten, was auf lokale Reassortierungsprozesse hinweist.

Die fortgesetzte Einführung neuer AIV-Stämme, deren lokale Reassortierung, die zur Erzeugung neuer Subtypen führte sowie die anhaltende genetische Drift der AIV, erforderte eine kontinuierliche Aktualisierung der molekulardiagnostischen Instrumente. Basierend auf den Ergebnissen dieser Studie werden Empfehlungen für verbesserte Kontrollmaßnahmen anzeigepflichtiger Geflügelkrankheiten in Ägypten gegeben.

CHAPTER 6: References

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CHAPTER 7: Curriculum vitae

Vita

For reasons of data protection, the curriculum vitae is not published in the electronic version

CHAPTER 8: Publication list

List of publications

1. Ibrahim Moharam, Hesham Sultan, **Kareem Hassan**, Mahmoud Ibrahim , Salama Shany, Awad A. Shehata, Mohammed Abo-ElKhair, Florian Pfaff, Dirk Höper , Magdy EL Kady, Martin Beer, Timm Harder, Hafez Hafez , Christain Grund., 2020. Emerging infectious bronchitis virus (IBV) in Egypt: Evidence for an evolutionary advantage of a new S1 variant with a unique gene 3ab constellation. Infections, Genetics and Evolution.85, 104433, ISSN 1567-1348. https://doi.org/10.1016/j.meegid.2020.104433.
2. **Hassan, K.E,** Noha Saad, Hassanein H. Abozeid, Salama Shany, Magdy. F. El-Kady, Abdelsatar Arafa, Azza A. A. EL-Sawah, Hafez. M. Hafez, Martin Beer, Timm Harder. Genotyping and reassortment analysis of highly pathogenic avian influenza viruses H5N8 and H5N2 from Egypt reveals successive annual replacement of genotypes. Infection, Genetics and Evolution 2020; P. 104375. ISSN 1567-1348. https://doi.org/10.1016/j.meegid.2020.104375.
3. **Hassan, K.E.,** King J, El-Kady M, Afifi M, Abozeid HH, Pohlmann A, Beer M, T., Harder., 2020. Novel Reassortant Highly Pathogenic Avian Influenza A (H5N2) Virus in Broiler Chickens, Egypt. Emerging Infectious Diseases. 26, 129-133. https://dx.doi.org/10.3201/eid2601.190570.
4. **Hassan, K.E**., El-Kady, M.F., El-**Sawah**, A.A.A., Luttermann, C., Parvin, R., Shany, S., Beer, M., Harder, T., 2019. Respiratory disease due to mixed viral infections in poultry flocks in Egypt between 2017 and 2018: Upsurge of highly pathogenic avian influenza virus subtype H5N8 since 2018. Transboundary and Emerging Diseases. p.1-16. https://doi.org/10.1111/tbed.13281.
5. Parvin, R., Schinkoethe, J., Grund, C., Ulrich, R., Bönte, F., Behr, K.P., Voss, M., Samad, M.A**., Hassan, K.E.,** Luttermann, C., Beer, M., Harder, T., 2020. Comparison of pathogenicity of subtype H9 avian influenza wild-type viruses from a wide geographic origin expressing mono-, di-, or tri-basic hemagglutinin cleavage sites. Veterinary Research. https://dx. doi: 10.1186/s13567-020-00771-3.
6. **Hassan, K.E.,** Ali, A., Shany, S.A.,El-Kady, M.F .Experimental co-infection of infectious bronchitis and low pathogenic avian influenza H9N2 viruses in commercial broiler chickens. Research in Veterinary Science. Volume, December 2017, Pages 356-362. https://doi.org/10.1016/j.rvsc.2017.06.024.
7. Mahmoud Naguib, Magdy El-Kady, Dörte Lüschow, **Hassan, K.E.,** Abdelsatar Arafa, Ali EL-Zanaty, Mohamed Hassan, Hafez M. Hafez, Christian Grund, Timm Harder

(2017), New real time and conventional RT-PCRs for updated molecular diagnosis of infectious bronchitis virus infection (IBV) in chickens in Egypt associated with frequent co-infections with avian influenza and Newcastle Disease viruses .Journal of virological methods.Volume245, July2017, Pages 19–27.
http://dx.doi.org/10.1016/j.jviromet.2017.02.018.

8. **Hassan, K.E.,** Ali, A., Shany, S.A., El-Sawah, A., El-Kady, M.F. Dahshan;.A.M (2016). Prevalence of Avian Viruses in Respiratory Disease Outbreaks in Broiler Flocks in Egypt. Poultry science journal (June 2016) 95(6): 1271-1280.
http://dx.doi.org/10.3382/ps/pew068.

CHAPTER 9: Acknowledgment

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CHAPTER 10: Declaration of authorship

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, 17.07.2020 Kareem Hassan