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

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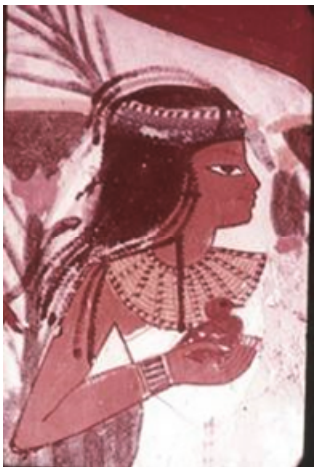
Abbreviations

A/H5N1	Highly pathogenic AIV of H5N1 subtype
AGPT	Agar gel precipitation test
AI	Avian Influenza
AIV	Avian influenza virus
BHK-21	Baby hamster kidney
CEF	Chicken embryo fibroblast
CEK	Chicken embryo kidney
cRNAs	Complementary RNAs
DF-1	Chicken fibroblast
DIVA	Differentiation infected from vaccinated animals
ECE	Embryonated chicken eggs
EID_{50}	Median egg infectious dose
ELISA	Enzyme linked immunosorbent assay
FPV	Fowl Pox Virus
HA	Hemagglutinin
HI	Haemagglutination inhibition test
HP	Highly pathogenic
HVT	Herpes Virus of Turkey
ISA	Infectious Salmon Anemia
LBM	Live bird market(s)
LP	Low pathogenic
M	Matrix
M2e	M2 ectodomain
MDCK	Madin-Darby canine kidney
mRNA	Messenger RNA
NA	Neuraminidase
NDV	Newcastle Disease Virus
NEP	Nuclear export protein
NP	Nucleoprotein
NS	Non-structural
OIE	World Organization for Animal Health
PA	Polymerase acidic
PB	Polymerase basic
PCRs	Polymerase chain reactions
PCS	Proteolytic cleavage site
PPE	Personal protective equipment
QT-35	Quail fibroblast
RBD	Receptor binding domain
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT-PCR	Reverse-transcription polymerase chain reaction
RT-qPCR	Real-time RT-PCR
SA	Sialic acid
SAN	Specific antibody negative
SPF	Specific pathogen free
USA	United States of America
HVT	Turkey Herpesvirus
USSR	Union of Soviet Socialist Republics
Vero	African green monkey kidney
WHO	World Health Organization

Dedication

To my beloved mother and father



Chapter 1

General Introduction

1. Introduction

Prior to the 1990s, avian influenza virus (AIV) was mainly a disease of wild birds, the natural reservoir of the virus and infections were sporadically over spilled to domesticated poultry and very few cases of self-limiting illness in human were reported (225). In 1997, an influenza virus derived in toto from pure avian-origin belonged to the highly pathogenic AIV of H5N1 subtype (A/H5N1) was able to cross avian-human species boundaries without pre-adaptation in "a mixing vessel" host, namely pigs as hypothesized for years. The virus killed 6 out of 18 infected persons in Hong Kong and devastated the poultry industry in the region (229). Since 2003, 63 countries in Asia, Europe and Africa reported the virus in domestic and wild birds to the World Organization for Animal Health "OIE" (162). Whilst most countries have successfully eradicated the disease, the infection remained endemic in poultry in few countries (e.g.: China, Vietnam, Indonesia and Egypt) (82). Another zoonotic and pandemic potential AIV is the H9N2 subtype which causes mild clinical disease in poultry and infrequently transmitted to humans since late 1990s. This virus is endemic in the Middle East and Asia and sporadically reported in Europe and the Americas (87).

Since 2006, infections of poultry in Egypt with A/H5N1 have resulted in destruction of over 100 million birds with total losses of US$ 1 – 3 billions. On the other hand, as of 10 December 2013 a total of 63 human fatalities out of 173 infected persons in Egypt were reported to the World Health Organization (WHO) (224). Vaccination of commercial poultry, year-round nationwide surveillance and culling of infected birds are the main tools for control of A/H5N1 outbreaks in poultry in Egypt. Nevertheless, infections have been repeatedly reported particularly in the backyard sector and live bird markets (LBM) (18). In addition to escape from the repertoire of poultry immune response after vaccination, the virus also has changed gradually toward efficient replication in human (18, 58, 221).

The second AIV in poultry in Egypt is the H9N2 virus which has been isolated for the first time in 2011 and then dominated the poultry industry causing enormous losses in the commercial sector and has very rare incidence in the backyard sector (33). While introduction of A/H5N1 virus into poultry in Egypt in 2006 was linked to wild birds (178), emergence of H9N2 virus remained unresolved although smuggling of poultry from neighbouring countries or wild birds were claimed to be the source of infection. The preliminary analysis of H9N2 genome indicated that the virus carries a genetic signature for adaptation on human (34) (see also publication No. 4 of the present thesis). Many unresolved questions in the epidemiology of AIV, particularly

A/H5N1, in Egypt seek an answer to better understand the dynamic of the infection and effectively control the disease.

Therefore this dissertation aimed at:

1. studying the role of wild birds in transmission of AIV into domestic poultry in Egypt
2. investigating the epidemiological features of A/H5N1 outbreaks in backyard birds, commercial poultry and live bird markets in Egypt
3. genetic characterization of some A/H5N1 viruses of poultry origin
4. isolation and characterization of H9N2 virus from bobwhite quails

2. Review of literature

2.1 Avian influenza virus

Influenza, known also as "Flu", is an acute highly contagious respiratory disease of humans and Avian Influenza (AI) describes the mild or highly fatal disease in poultry; the latter is also known as "bird flu" mostly when the virus transmits from poultry to human (205). The word influenza originated in the fifteenth-century in Italy from the Latin word "influential" which means a disease caused due to unfavorable astrological influences. Nonetheless, the first record of influenza was suggested to be as early as the fifth century B.C as reported by Hippocrates in ancient Greece (139). On the other hand, AI was firstly described in poultry in 1878 by Perroncito in Italy (198).

2.1.1 Virus taxonomy and structure

Avian influenza viruses (AIV) belong to the genus Influenza A Virus in the family *Orthomyxoviridae,* which contains also Influenza B Virus, Influenza C Virus, Thogotovirus and Infectious Salmon Anemia (ISA) Virus (222). They contain a single-stranded ribonucleic acid (RNA) genome composed of eight gene segments encoding at least 11 viral proteins (60). These proteins can be allocated to three categories (Figure 1): surface proteins (hemagglutinin HA; neuraminidase NA; and matrix protein 2 M2), internal proteins (tripartite polymerase PB2, PB1, PA; nucleoprotein NP; matrix protein 1 M1; and nuclear export protein NEP) and non-structural (NS) proteins (NS1 and PB1-F2) (60, 222).

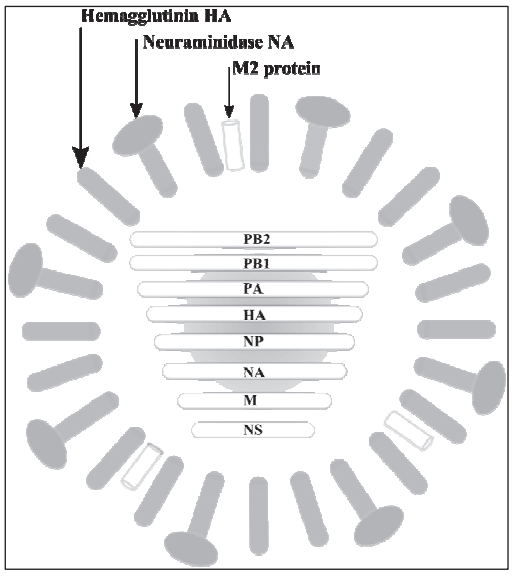


Figure 1: Diagrammatic illustration of an influenza virus and its genome. Virion of influenza is mostly spherical in shape which contains three spikes: HA, NA and M2 proteins anchored in the host-derived lipid bilayer membrane. The genome of AIV consists of eight RNA gene segments.

It is worth to mention that to date, 18 HA and 11 NA subtypes of avian influenza viruses (AIV) have been detected. All AIV subtypes, except H17N10 and H18N11 viruses which have been recently identified in bats (214, 215), are known to infect birds (85). Each infectious influenza virus must carry one HA and one NA subtype and in birds 144 HxNx combinations (e.g.: H1N1, H2N3, H5N1, H9N2, etc.) of AIV are possible (222). Since 1980, a universal nomenclature system for influenza virus has been set by the WHO to include type-specific (A, B or C), the host origin (except for viruses of human origin) (e.g. swine, equine, chicken, duck, turkey, etc.), country, laboratory code if present, year of isolation and HA-NA subtypes. Thus, A/duck/USSR/695/76 (H2N3) is an influenza A virus isolated from duck in the former Union of Soviet Socialist Republics, the isolate number was 695, isolated in 1976, and it had an HA subtype 2 and an NA subtype 3 (35).

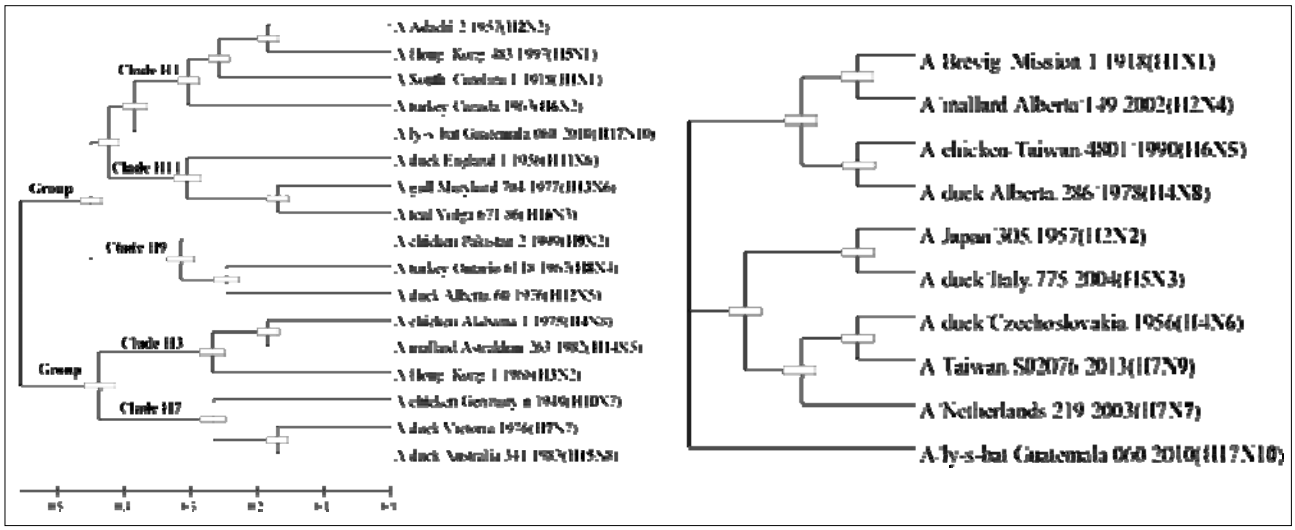


Figure 2: Phylogenic relatedness of influenza virus HA and NA subtypes. Maximum-likelihood trees were generated by comparing the nucleotide sequences of HA (left) and NA (right) genes using MEGA version 5.2 software (209) and allocated into groups and clades according to Palese and Shaw (165).

An influenza virion has a spherical or pleomorphic morphology with typical 80 – 120 nm length or rarely filamentous shape with up to 300 nm. The outer surface of the virus consists of a host cell–derived bilayer lipid membrane containing three types of spikes (Figure 1): the HA appears as "rod-shaped" trimmers, the NA appears as "mushroom-shaped" tetramers and the M2 appears as channels (222). Generally, the HA is more abundant and the HA-NA ratio is different according to the virus strain and it was estimated to be 4 HA to 1 NA (50, 165) (e.g.: 800 HA to

200 NA (67)) or 10 to 1 (146). Meanwhile, the HA:M2 ratio was estimated to be 10-100:1 (50). Underneath the viral envelope there is a matrix of M1 protein which encloses the virion core in addition to other internal proteins (165).

The HA protein is encoded by segment 4. It is the most important protein in AIV where pathogenicity, immunogenicity, antigenicity and receptor-binding of the virus is mainly determined by HA (196). The immature HA protein is known as HA0. For the virus to be infectious the HA0 must be cleaved into HA1 and HA2 parts by protease enzymes in the host cell. The protein is formed from stalk and head domains; the stalk domain constructed by both HA1 and HA2 while the head domain is formed exclusively by the HA1. The HA1 contains five immunogenic epitopes (designated A, B, C, D and E) mostly on the exposed part of the distal proximity of the head domain. Therefore, HA1 plays the major role in the immunogenicity of AIV (117). It also contains the receptor binding domain (RBD) which formed by about 13 amino acids (68). These amino acids form a pocket-like structure at the center of the head domain which bind with the sialic acid (SA) receptors on the host cell. Human influenza viruses bind to 2,6 SA linked carbohydrate side-chain and AIV prefers binding with 2,3 SA linked carbohydrate side-chain (177). Thus, the HA determines the host range of influenza viruses (90). It is worth pointing out that some animals contain both 2,3 and 2,6 SA receptors such as pigs and quails acting as intermediate host "or mixing vessel" for the generation of novel reassortant viruses (157, 213). Between the HA1 and HA2 domains there is a connecting peptide known as the proteolytic cleavage site (PCS) formed usually from Arginine-X-X-Arginine*Glycine (R-X-X-R*G) motif for the H5 viruses and Lysine-X-R*G (K-X-R*G) or R-X-T-R*G for the avirulent viruses (196). The existence of multiple basic amino acids (e.g. R or K) indicates that the virus is highly pathogenic AIV (HPAIV) to chickens, whereas monobasic amino acids PCS motif is characteristic to the low pathogenic AIV (LPAIV). This alteration of PCS results in HA cleavability by ubiquitous host furin-like proteases not only in the respiratory and digestive tracts but also throughout the host inducing systemic infection. Also, virulence markers either in the vicinity of the PCS or beyond the HA exist (48). The HA2 domain contains the transmembrane region anchored in the viral membrane and the fusion peptide required for virus entry and successful release of the AIV genome into the host cell (187). Both HA1 and HA2 contain several N-linked glycosylated amino acids, predicted sequence motif is asparagine-X-serine or threonine "N-X-S/T", where X can be any amino acid except proline. Changing of the glycosylation pattern of the HA is a viral strategy to evade the host immune response, alter receptor-binding specificity, mask important antigenic sites, modulate virulence and/or enhance the adaptation of influenza virus (32, 127).

The NA protein, known also as sialidase, is a glycosylated transmembrane protein encoded by segment 6. It is formed from a stalk connected to head domain. The main function of the NA is to release the progeny virions via cleavage of their binding with the infected cell surface SA as well as from extracellular inhibitors (e.g. mucous) (186). The head domain harbors the sialidase active sites which are a target for the common NA inhibitors (143). Deletion in the stalk domain of the NA has been found to be an adaptation genetic marker of wild-bird origin AIV on terrestrial birds (138). Also, NA protein plays a role, although less important than HA, in the protection against homologous influenza virus infection (208). Moreover, meticulous HA-NA functional balance is thought to be crucial for a successful influenza virus replication cycle (219).

The third surface protein is M2, ion-channel protein, encoded by segment 7. This protein is essential for uncoating of influenza virus immediately after internalization. It is also an important target for antivirals (9, 80). While many researchers found that M2 is immunogenic and can be a target for development of universal influenza vaccines (204), others reported contradictory results (154). Furthermore, due to the scarce number of M2 on AIV, detection of antibodies against the M2 ectodomain (M2e) considered a valuable tool to differentiate between vaccinated and infected birds "DIVA" (125).

Segment 7 also encodes the M1 protein which encapsulates the viral genome. It is thought that M1 bind to the cytoplasmic tails of HA, NA and M2 from one side and on the other side interact with the ribonucleoprotein "RNP" (NP, PB2, PB1 and PA proteins). It plays a vital role in virus assembly and pudding (176). The NP is encoded by segment 5. It associates with the polymerase subunits (PB2, PB1 and PA) to form the RNP complex which encapsidates all viral gene segments. Generally, it has been stated that RNPs control replication, transcription, intracellular transport and packaging of viral RNA. The NP is a multifunction viral protein that interacts viral proteins (e.g. PB2, PB1, or M1) and host proteins. Proven functions of the influenza NP are to wrap, facilitate folding, replication and transcription of viral RNA (234) in addition to nuclear transport of parent RNPs during the early stage of infection and nuclear export and packaging of nascent RNPs (61). Segments 1, 2 and 3 are the longest influenza genome segments that encode PB2, PB1 and PA proteins, respectively. The three polymerase proteins have multiple enzymatic activities required for transcription and replication of viral RNA in the nucleus of infected cells (84). NEP, previously referred to as non-structural protein 2 (NS2), is encoded by segment 8. A primary function of the NEP is the nuclear export of RNP from the infected cell nucleus as well as assembly of progeny virions. It was also proposed that NEP regulates transcription and replication of influenza virus in infected cells (175).

NS1 is a non structural protein encoded also by segment 7. The major role of the NS1 is to antagonize the host interferon system to allow optimal replication of influenza virus (96). Nevertheless, NS1 is a multifunctional protein that influences influenza virus replication, adaptation, virulence and host-range (11). PB1-F2 is the second non-structural protein encoded by PB1 gene segment, by alternative (+1) open reading frame and is involved in apoptosis of infected cells and increased pathogenicity of influenza A virus in mice (93).

2.1.2 Virus replication

Stages of influenza virus replication have been reviewed in details (50, 165, 222). Briefly, those stages include attachment, fusion and uncoating, RNA synthesis, packaging and budding. Infection of the host is common via nasal, ocular and/or oral routes. Firstly, the viral HA **attaches** to the SA receptors on the host cell membrane through the RBD. Then, the virus enters the host cell by receptor-mediated endocytosis into a vesicle or endosome. Cleavage of the HA occurs extracellular (in case of LPAIV) or intracellular (HPAIV) into HA1 and HA2 parts by cellular proteolytic enzymes, i.e. trypsin cleaves LPAIV and secreted by mucosal cells in the respiratory and intestinal tracts and furin-like proteases cleave HPAIV secreted by all types of cells. Thereafter, acidification of the endosome occurred with increasing the H+ ions transferred from the cell-cytoplasm to the endosome via M2 channels. The **Fusion** peptide in the HA2 C-terminal end, formed after cleavage of the HA, brings the viral and endosomal membranes in contact results in the release "**uncoating**" of the viral genome into the infected cell. The viral RNPs enter the nucleus where the replication occurs. The viral RNA-dependent RNA polymerase (i.e. PB2, PB1 and PA) steal "cap-snatching" a methylated cap structure "10-15 nucleotides" from the cellular messenger RNA "mRNA" acting as a primer for the **synthesis** of complementary RNAs (cRNAs) and viral mRNA. The cRNAs will be used for the synthesis of progeny virion RNAs. Translation of viral mRNA to the corresponding proteins occurs in the Endoplasmic Reticulum in the cytoplasm. The NS1 protein antagonizes the innate antiviral responses of the host enabling effective replication of the virus. The translated PB2, PB1, PA and NP are imported back to the nucleus to encapsidate the cRNAs and vRNAs, while the HA and NA are glycosylated in the Golgi apparatus before being transferred to the cell membrane beside the M2 protein. M1 and NEP facilitate the transport of RNPs from the nucleus and then to the cytoplasmic membrane. **Packaging** of the nascent virions occurs by a help of M1 protein where all viral structures are

packed together in a lipid bilayer envelop taken from the host-cell membrane during **budding**. Finally, release of the progeny virion occurs after removal of SA from the HA by the NA protein.

2.1.3 Virus evolution

Influenza virus continues to evolve in nature via two major genetic strategies: antigenic drift and antigenic shift. **Antigenic drift** is defined as small and gradual changes in the virus. These changes occur spontaneously and can be accelerated by pressure exerted by the host immune response or upon transmission to a new host. For example: (1) after infection or vaccination of a host with influenza, the immune system induces antibodies against that virus. Influenza virus responds to these antibodies by pin-point mutations in the major immunogenic or antigenic structures as a "camouflage" to escape from the host-defense system (49). This explains the re-occurrence of flu in people each season (56). However, this strategy was not common among AIV before mass-vaccination of poultry in some developing countries since the 1990s (14, 94, 134, 201). (2) After transmission of influenza virus to a new host, genetic changes occur to maximize the replication "adaptation" of the virus in that host. Transmission of AIV from wild birds to domestic poultry and/or to human is usually preceded with or accompanied by changes in one or more of the viral proteins particularly the HA, NA, PB2 and/or NP (88, 107, 189). **Antigenic shift** is defined exchange "reassortment" of gene segments between influenza viruses resulting in new gene constellations (50, 197). It occurs when two or more influenza viruses infect the same cell, their genome segments mixed then new viruses emerge that differ from the parent viruses in one or more segments (Figure 3). The emergence of novel viruses with HA and/or NA can lead to severe pandemic in human due to lack of immunity to this novel virus (197). This strategy resulted in at least four human pandemics in 1918-1919, 1957, 1968 and 2009 (210). The antigenic shift in human influenza viruses has occasionally reported but it is thought to be more frequent in animals (e.g. wild-birds, pigs, etc.) (57, 100).

2.1.4 Virus pathogenicity

According to their pathogenicity in chickens, AIV is divided into two pathotypes: HPAIV and LPAIV (24).

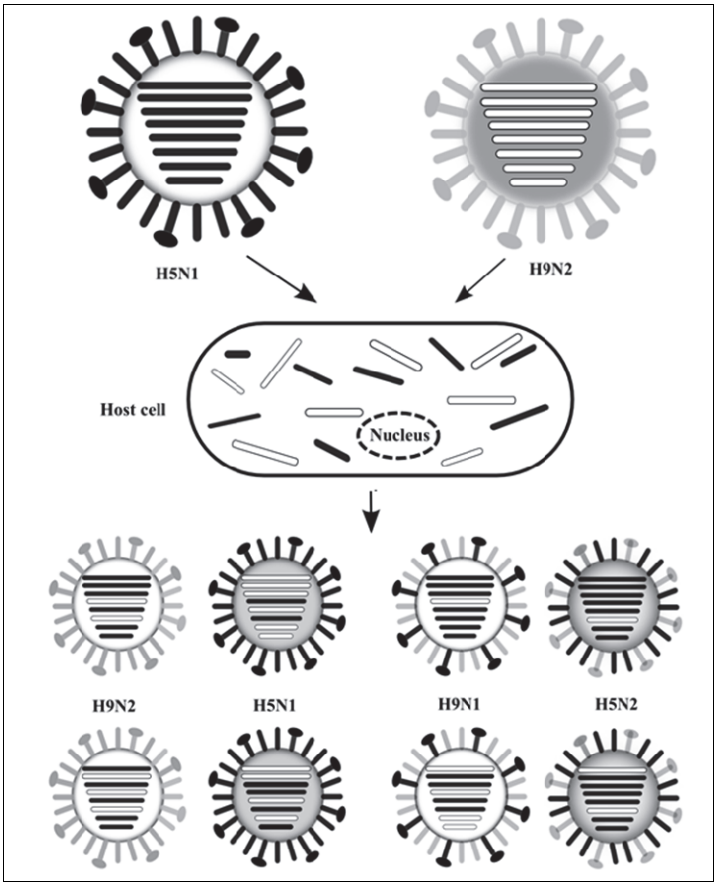


Figure 3: Reassortment of two influenza viruses. Shown are two different influenza A viruses (e.g. H5N1 and H9N2) co-infect the same cell. Theoretically, this could result in 2^8 different viral genotypes (i.e., 254 new genotypes in addition to the two parental viruses).

The HPAIV is also known as fowl plague was firstly described in 1878 in Italy then reported in several countries including Germany, Austria, Switzerland, France, Belgium, The Netherlands, England, Egypt, China, Japan, Argentina, Brazil, England and USA (205). All AIV are low pathogenic strains while some viruses of H5 and H7 subtypes can be highly pathogenic which evolve from LPAI ancestors after circulation in domesticated birds for a period of time (7). Therefore, infections by either LP or HP H5 and H7 viruses are notifiable to the OIE (161).

In human, to date there is no definition for high and low virulence of AIV. Infections with LPAIV in human have been reported by H6N1, H7N2, H7N3, H7N7, H7N9, H9N2 and H10N7 (91, 115, 184) as well as by HPAI viruses of subtypes H5N1, H7N3 and H7N7 (65, 115, 141).

2.1.5 Diagnosis

The vast majority of low pathogenic strains infect birds without showing any clinical signs or lesions. Nevertheless, some strains of LPAIV, particularly H9N2, induce respiratory illness, reduced body-weight gain and/or decreased egg production (97) especially when concomitant bacterial infections occur (126, 166). The HPAIV causes severe systemic fatal infection and

results in 100% mortality of chicken and turkey flocks. Cyanosis of comb and wattle, facial edema, hemorrhages on the shank, respiratory distress, diarrhea and complete cessation of egg production may indicate infection of poultry (mainly chickens and turkeys) with HPAIV (205). Lesions in chickens (or turkeys) infected with some strains of LPAIV can be restricted to nephritis, pneumonia and/ or enteritis. Lesions in HPAIV infected birds range from no-lesions (in peracute cases) to hemorrhages and focal necrosis in visceral organs. Hemorrhages on the epicardium, mucosa of the proventriculus and ventriculus, necrosis of the pancreas in addition to severe pneumonia, atrophy of cloacal bursa and thymus are frequently observed in HPAIV infections (205). The specific laboratory diagnosis of the disease is achieved by isolation and molecular techniques as well as using serological tests.

Virus isolation is the gold standard for diagnosis of AIV which is usually carried out by inoculation of tracheal and/or cloacal swabs medium in: (1) embryonated chicken eggs (ECE) obtained from specific pathogen free (SPF) or specific antibody negative (SAN) birds (161, 227) or (2) a variety of primary or continuous tissue culture obtained from different host species (128). ECE is typically inoculated through the chorioallantoic sac route at 9 -11 days old and seldom through yolk sac or the chorioallantoic membrane routes (227). Hemagglutinating activity after mixing with erythrocytes (e.g. from chickens) may indicate presence of AIV, paramyxoviruses and/or bacterial contamination (161). LPAIV may require than one passage, whereas HPAIV kills the embryo within 2-3 days of the first passage (227). Primary or continuous tissue culture is used for isolation, titration and pathogenicity studies with comparable sensitivity to ECE. The most common primary tissue cultures are chicken embryo kidney (CEK) and chicken embryo fibroblast (CEF) cells while the most cell lines used are Madin-Darby canine kidney (MDCK), African green monkey kidney (Vero), chicken fibroblast (DF-1), quail fibroblast (QT-35) and baby hamster kidney (BHK-21) cell lines (150, 149). It is worth mentioning that trypsin must be added to the cells to obtain LPAIV but HPAIV doesn't require trypsin for growth in vitro. In this regard, CEK was found to support replication of LPAIV without external trypsin because it secrets trypsin-like proteases (199).

Molecular diagnosis of AIV includes RNA detection tools, sequence of the viral genome and phylogenetic relatedness analysis. The most widely used tools for detection of influenza viral RNA are reverse-transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR (RT-qPCR). Several RT-PCR and RT-qPCR assays have been developed for specific detection, differentiation and/or subtyping of AIV. The RT-qPCR is more sensitive, more specific, safer and faster than the RT-PCR (192). Continuous mutation of the virus, the presence of inhibitors

particularly in cloacal swabs and carryover contamination are the most challenging for detection of AIV using PCRs (17, 63, 193). Sequence of viral genome can be done within 1 – 2 days due to the growing advancement in the modern sequencing technology. Sequence identification of the influenza virus genome is crucial to develop/update diagnostics, vaccines and therapies (167, 190). Phylogenetic analysis of the generated nucleotide sequences or the deduced amino acids is very useful for molecular epidemiological studies. Assignment of strains or new serotypes to taxonomic groups, inferring horizontal gene transfer from species to another (e.g.: from wild birds to domesticated poultry), or geographical spread (e.g. Eurasian vs. American lineages of AIV) could be well predicted by phylogenetic analysis of influenza gene sequences (194, 233).

Serological tests which are often used for detection of the anti-influenza antibodies or the virus are: enzyme linked immunosorbent assay (ELISA), haemagglutination inhibition test (HI) and agar gel precipitation test (AGPT). ELISA is mostly used for surveillance purposes either for detection of AIV (200) or the determination of HA and/or NA subtypes (101, 131, 179) as well as for detection of anti-AIV antibodies (235). Although it is rapid, sensitive and semi-automated test for screening of large number of samples, however false positive results are not uncommon (37, 36). The HI is commonly used for detection and subtyping of AIV (161, 168). It is also useful for monitoring of vaccination efficiency and protection (14, 129, 207). Nevertheless, HI is laborious, impractical for screening in case of surveillance and antigenic diversity within the same AIV subtype may lead to misinterpretation of the results (94, 95, 121, 183). AGPT is most widely used for detection of anti-AIV antibody in serum, plasma and egg yolk (47). However, it is time-consuming, has moderate sensitivity in comparison to other serological techniques and requires a large amount of both antigens and antibodies (205).

2.1.6 Prevention and control

Prevention and control of AIV in poultry are based mainly on enforcement of **biosecurity** to prevent introduction as well as further spread of the infection. This includes many measures to be applied, for example: (1) preventing the contact of domestic poultry with wild and feral birds (52) as well as rodents (20, 185) and insects (44, 158, 181). (2) Restriction of movement of infected and in-contact poultry flocks and poultry by-products. (3) Cleaning and disinfection of all poultry rearing and marketing facilities and equipment. (4) Continuous monitoring and surveillance. (5) And last but not least education and awareness programs for all personnel involved in the poultry production chain (54, 62, 160, 172, 195). **Stamping out** of infected flocks as well as the adjacent

flocks, including backyard birds is the most effective strategy to control AIV. However, costs of the mass culling of poultry are usually unbearable especially in under-resourced developing countries (97, 142). **Vaccination** of poultry against (HP)AIV decreases the morbidity, mortality, losses in production (egg, meat), viral excretion and horizontal transmission. It increases the host resistance to infection, but does not totally prevent virus replication and shedding (142, 206). A number of vaccines has been developed and used extensively in the field to prevent or eradicate AIV in poultry. The two major categories are inactivated and recombinant vaccines. Inactivated vaccines are homologous (have the same HA-NA genes of the field virus) or heterologous (have the same HA gene but different NA). In the field, the used recombinant vaccines are based mainly on fowl pox virus (FPV), Newcastle disease virus (NDV) or Turkey herpesvirus (HVT) and applied for dual protection against the vector virus and AIV (203). Under experimental condition, a number of recombinant viral vectored vaccines against AIV in poultry has been also developed (12).

2.2 The situation of AIV in Egypt

2.2.1 Poultry industry structure in Egypt

The available literatures described the early date of poultry production in Egypt in the recent history to early 1900s (27). This production was influenced by political and social circumstances. Annual eggs export was estimated to be over 200 million eggs in 1924, and increased to 1,053 million in 1944 then completely stopped at 1950. In 1985, production began to increase reaching 2,302 million (27). Early 1990s and 2000s, a total investment of LE18 billion and 2.5 million workers were estimated in the poultry industry (77) and in 2004-2005, poultry production was around 8.8% of the value of agricultural production in the country (77). Poultry production in Egypt has two main divisions; the commercial enterprises and backyard birds. In 2006, about 850 and 250 million birds, respectively in the commercial and backyard sectors were reported (8). The commercial enterprises are divided according to FAO into 4 sectors. Sector 1: is industrial integrated system with high level of biosecurity where birds/products marketed commercially (e.g. farms that are part of an integrated broiler production enterprise with clearly defined and implemented standard operating procedures for bio-security). Sector 2: Commercial poultry production system with moderate to high biosecurity and birds/products usually marketed commercially (e.g. farms with birds kept indoors continuously; preventing contact with other poultry or wildlife). Sector 3: Semi-commercial poultry production with low to minimal

biosecurity and birds/products usually entering live bird markets (e.g. a caged layer farm with birds in open sheds; a farm with poultry spending time outside the shed; a farm producing chickens and waterfowl) and sector 4: Village or backyard production with minimal biosecurity and birds/products consumed locally (8, 104). The backyard sector contains family poultry production which roaming in the streets, ponds and fields or on rooftops during the day and at night they stay in small cages or barns (8) and kept by about 4.5 – 7 million families (144). Backyard poultry is in-close contact to human particularly women and children as well as interwoven with the commercial farms in other sectors (8).

Marketing of poultry in Egypt relies on LBM in (1) retails shops: where birds are available all over the week, (2) traditional market: one-day a week for each or group of villages or (3) mobile vehicles (16, 104). In these markets, it is common to see different species and breeds of birds from several houses, farms or vendors are gathered mostly without any system for tracing the source of birds in each market. Also, the capacity of slaughterhouses in Egypt ranged from 18 to 30% of the total poultry production (8, 77). Therefore, reforming of marketing system in Egypt from selling live birds for frozen meat will require time and funds (8).

2.2.2 Epidemiology of AIV in Egypt

2.2.2.1 History and subtypes

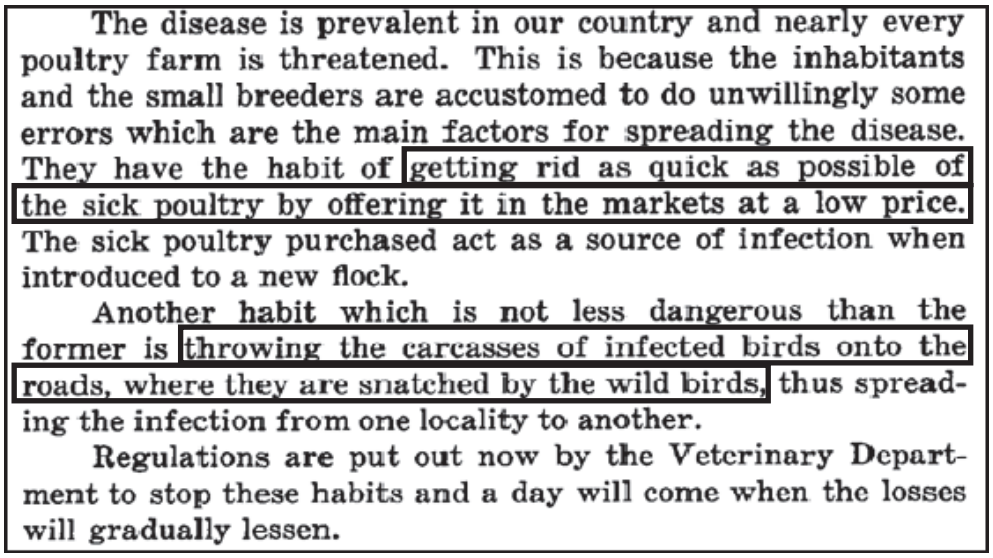
In Egypt the first recording of AIV was reported as a part of the worldwide fowl plague (H7N1) outbreak in 1923 (132, 133, 173). Nevertheless, the earliest quoted date for the beginning of the history of AI in Egypt, referring to the reports of the Local Veterinary Service, is as far back as 1912 (180). The disease was endemic for many decades in Egypt and nearly every poultry farm was threatened (70, 180). The virus was isolated from chickens, turkeys, waterfowls, peafowl, parrots and pheasants. Control of the disease had been mainly based on nationwide vaccination with whole-virus inactivated vaccines developed from circulating field strains (64, 145). By the 1960s, the disease was no longer mentioned and dramatically disappeared. Later the virus was classified as HPAIV H7N1 (A/fowl/Egypt/45 or A/FPV/Egypt/45) (39, 42, 226).

Egypt lies at the crossroads of two major spatially-overlapping migration flyways; the Black Sea-Mediterranean Flyway and the East Africa-West Asia Flyway linking Africa, Europe, and Asia with 34 wetlands and stopovers (8). Therefore, the role of wild birds in introduction of different pathogens in the last decade has been considered a risk factor (26, 28, 188). A considerable

number of AIV subtypes have been isolated from wild birds in Egypt through several collaborative research projects; nevertheless infections of domestic poultry and/or human with any of these viruses remain poorly defined (23, 28, 89, 92, 164, 188).

2.2.2.2 H5N1

Introduction of A/H5N1 into Egypt most probably occurred by migratory teal ducks in late 2005 (178). The secondary spread was due to rapid and random movement of poultry from place to another. Another important factor was human behaviors which were very similar to that observed in the 1940s (Figure 4) by marketing sick poultry at a low price and throwing the carcasses of dead birds onto the roads (8, 180). The virus has been rapidly and widely established in Egypt and became officially endemic since 2008. All domesticated birds have been infected with the virus including chickens, ducks, geese, turkeys, quails, ostrich and pigeons (8, 119), in addition to zoo birds, feral birds, donkeys and probably pigs (2, 71). To date, 63 human cases have died and 110 patients have recovered after infection with HPAIV H5N1. The majority of infected humans were women and children (223).



The disease is prevalent in our country and nearly every poultry farm is threatened. This is because the inhabitants and the small breeders are accustomed to do unwillingly some errors which are the main factors for spreading the disease. They have the habit of getting rid as quick as possible of the sick poultry by offering it in the markets at a low price. The sick poultry purchased act as a source of infection when introduced to a new flock.

Another habit which is not less dangerous than the former is throwing the carcasses of infected birds onto the roads, where they are snatched by the wild birds, thus spreading the infection from one locality to another.

Regulations are put out now by the Veterinary Department to stop these habits and a day will come when the losses will gradually lessen.

Figure 4: Common practices result in the spread of AIV in Egypt (180)

The main features of dynamic of A/H5N1 in poultry and human in Egypt where backyard birds play a significant role in human and poultry infections are summarized in Figure (5). To date, about 167 out of 173 human infections were linked to the close contact with backyard birds. Slaughtering, de-feathering and evisceration are the main pathways for women infections while playing with infected birds for children. Only two workers in the commercial sector were infected

with the virus and they were recovered. In addition, two poultry sellers acquired the infection from live bird markets. The source of infection of other two cases of human was not confirmed (116, 119, 231).

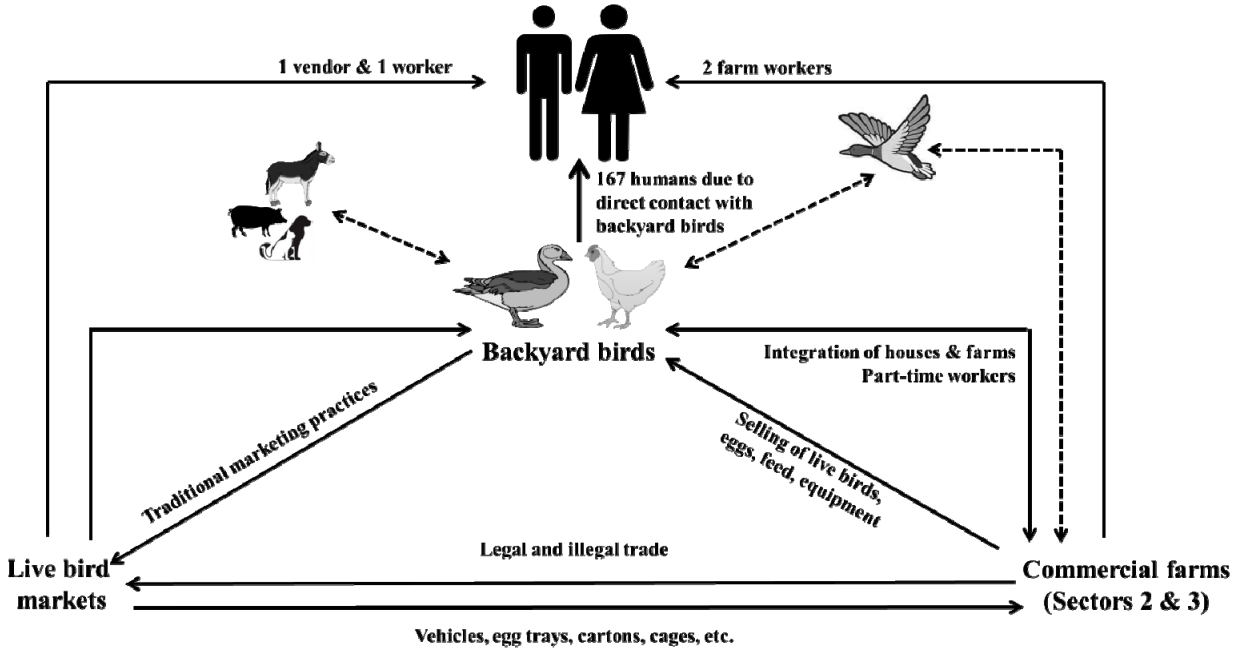


Figure 5: Transmission of A/H5N1 in different hosts/sectors in Egypt. The cycle of backyard birds-LBM-commercial farms in Egypt is very close where the backyards play a central role in the epidemiology of A/H5N1 in Egypt. As of October 06, 2013, 167 out of total 173 infected humans were due to close contact with backyard birds and only two patients were infected from commercial farms in addition to one worker in LBM and one poultry vendor and two other infections due to unknown sources. Also, backyard birds were thought to be the source of infection for donkeys and probably pigs, cats and dogs (2, 8, 71). A/H5N1 was isolated from wild ducks in 2006 in Egypt, but it is unknown whether the infection spread to backyard birds firstly or commercial poultry or both. This figure was modified from Abdelwhab and Hafez (10).

Backyard birds usually acquire the infection mainly through (1) commercial farms in the "sector 3" through the selling of unused equipment (i.e.: hoppers, drinkers, feeders, etc.), feed, selling of infected birds (e.g. apparently normal birds), and eggs or via (2) introduction of live birds from the markets which was a common history told by householders in many of surveyed backyards. Live bird markets play an important role in the epidemiology of the virus in Egypt. Infection is usually acquired via commercial birds or eggs in sectors 2 and 3. Commercial farms are usually being infected through a breach in the biosecurity via partial time or temporary farm workers particularly in the layer chickens farms, contact with feral and wild birds, inappropriate

disinfection, disposal of dead birds and/or contaminated litter (8, 95). The temporal pattern of the disease in winter was typical for outbreaks in 2006-2008 (25, 95); however in 2009-2012 the disease has been reported year-round particularly in the backyard sector (32). Geographically, the outbreaks concentrated mainly in the Nile Delta (Northern of Cairo), where dense poultry and human populations exist. Conversely, few cases, if any, have been reported in the Suez Canal region (Eastern of Cairo) or the Upper Egypt (Southern of Cairo) (8).

Genetic evolution studies of A/H5N1 in Egypt showed co-circulation of two major genotypes; designated variant and classic genotypes. The variant genotype has been recently classified to be clade 2.2.1.1 and the classic genotype as 2.2.1/C. The variant clade has been exclusively isolated from commercial poultry (two isolates were from humans) whereas the classic is broadly and frequently isolated from backyard birds, small-scale commercial poultry in sector 3, human after 2009 and donkeys. Extensive mutations in the immunogenic epitopes are typical to the variant strains, meanwhile deletion at the RBD and changes in the glycosylation pattern are characteristic of the classic viruses. It is thought that these mutations enable the variant strains to evade the host-immune system in vaccinated birds and increased of the adaptation of the classic strains on human (18, 32, 31, 170, 221).

2.2.2.3 H9N2

First isolation of H9N2 virus in Egypt was reported in December 2010 and May 2011 from chickens (5, 147) and quail (34) (see also publication No. 4 of the present dissertation) in the commercial sector. Retrospective serological investigation indicated widespread of the virus in the commercial farm settings between February 2009 and April 2012 (21). All serologically positive or infected chicken flocks suffered respiratory distress (21, 147) and decrease in egg production in breeder and layer flocks (21), nevertheless both broiler and quails were clinically healthy (34, 75). Infected flocks were located along main migration routes of birds in several provinces in Egypt assuming that the virus was probably introduced through wild birds (133). The Egyptian H9N2 clustered phylogenetically with viruses currently circulating in neighboring countries and in the Middle East region which may indicate epidemiological links through legal or illegal transportation of infected poultry (75). Although all cases were reported from the commercial sector, only 3 cases of backyards were recorded (33).

2.2.3 Major challenges for the control of AIV in poultry in Egypt

Surveillance, stamping out, vaccination, restriction of movement, reform of the poultry industry and awareness are the major lines for controlling AIV in Egypt (8). Monitoring of commercial poultry and wild birds was routinely conducted for three years before emergence of A/H5N1 in 2006. Therefore, diagnosis of the disease was successfully done within a few days; from 10th to 16th February 2006. The preparedness plan for control of AIV in Egypt depended mainly on culling of the primary cases and birds in 1-3-km surrounding zone. Compensation was provided during the first wave of the disease. Nevertheless, due to the widespread of the virus nationwide within few weeks, a mass vaccination strategy was introduced as an ancillary tool to the test-and-slaughter campaign. A number of inactivated homologous and heterologous H5 vaccines or recombinant NDV-H5, FPV-H5 or HVT-H5 vaccines are applied in the field (174, 203). To control H9N2, vaccination with inactivated local field strains has been recently implemented. Even more, bivalent or trivalent vaccines with H5, NDV and/or IBV have been also used particularly in the breeder and layer flocks (Abdelwhab and Abdel-Moneim, unpublished data).

2.2.3.1 Stamping-out

Egypt appears as a small village where more than 90% of the 85 million inhabitants are living on less than 10% of the country; around the Nile and the Nile Delta. Concentration of poultry industry within the Nile Delta and close contact between the commercial and backyard sectors, rendered depopulation of all poultry in 3-km zones around the infected spots is utmost impossible (8, 77, 104). Also, the majority of broiler and layer poultry farms in Egypt is not registered. At the first wave of the A/H5N1 outbreak in 2006, compensation was given, although at a low rate, only for the registered farms (22, 144). Then compensation was ceased because of the wide-spread of the virus and lack of funds. Thereafter, reporting and/or culling was done mostly by the owner, therefore passive or voluntary reporting of A/H5N1 was approximately nil (8).

2.2.3.2 Backyard birds

To combat the dilemma of the huge backyard sector in Egypt the authorities firstly banned keeping of live birds or trading them and enforced free-of-charge vaccination program twice a year until 2009. Vaccination of backyard birds resulted in masking of clinical signs in infected birds and increased the risk of infection of human (218). Moreover, positive (clinically healthy) birds discovered during the routine surveillance were culled mostly without compensation (8).

Subsequently, the farmers didn't cooperate to enforce the control strategy and the disease became endemic and deeply entrenched in backyards (112).

2.2.3.3 Vaccine and vaccination

Egypt embarked early on a nationwide blanket vaccination policy attempting to reach all poultry species to control the disease (25). Incidence of A/H5N1 outbreaks was decreased after the first wave of infections in 2006 due to use of several tens of millions of doses of inactivated H5 vaccines (98, 118, 183). However, since late 2007, 2.2.1.1 variants were widely entrenched in the vaccinated commercial poultry in Egypt (4, 18, 31, 40). In vivo experimental studies in chickens and meat turkeys indicated that the current vaccines (1) conferred clinical protection against 2.2.1/C as well as the original 2.2.1 genotype (14, 94), (2) partially protected vaccinees against early 2007-2008 antigenic drift variants (124, 212) and (3) no protection was afforded against variant strains of 2.2.1.1 (14, 15, 94, 98, 121). However; H5N1 vaccines developed from local field variant strains protected chickens (and turkeys) against morbidity, mortality, decreased virus shedding (15, 94, 98), reduced bird-to-bird transmission and virus RNA was not detected in vaccinated chicken meat and edible organs (98) after infections with variant 2.2.1.1 clade and/or 2.2.1/C group. Importantly, maternal immunity transmitted from vaccinated breeders to their offspring interfered with vaccination of chickens with the same H5N2 vaccine at 3 days old (15). Therefore, it was recommended that chickens had anti-H5 maternal antibodies should not be vaccinated immediately after hatch but after waning of the maternal immunity (15, 124).

In 2009, nationwide surveillance showed that viruses of 2.2.1/C group were isolated frequently from clinically-ill and/or asymptomatic vaccinated poultry (32, 73, 122) (see also publication No. 3 of the present dissertation). Since 2011, 2.2.1/C viruses are prevailing in the commercial poultry in Egypt (32) subsequently emergence of variant strains exhibiting antigenic drift from this 2.2.1/C group is plausible. It is worth mentioning that immune pressure induced by vaccination accelerated the evolution of the virus (the 2.2.1.1 variants) (18, 58) and fasten the escape of the virus from the vaccine strains. Therefore, continuous development of (bivalent) vaccines from classic and variant strains is required (94). On the other hand, vaccination coverage of backyards was low ranged from 1 to 50% while all commercial poultry farms applied their own vaccination programs which is not uniform (e.g. different vaccine strains, sources, vaccination scheme, doses, ages, etc.) (171). Also, vaccination of commercial poultry negatively influenced the passive reporting of infected flocks because infected birds may show no or mild clinical disease (218).

2.2.3.4 Infra-structure

Although industrialization of poultry production in Egypt has begun since many decades, however it has mostly occurred randomly with indefinite long-term planning (8). The huge number and capacity of small-scale commercial farms in sector 3, the social, economic and cultural aspects linked to the backyard production, preferences for consumption of live poultry, capacity of slaughterhouses and insufficient veterinary and hygiene inspection in LBM consider a real challenge for control AIV in Egypt. Reforming of sector 3, backyards and marketing-system has been planned for years but no significant improvement has been done so far. Establishment of satellite accredited laboratories; updating the diagnostics, continuous surveillance and development of vaccines from field viruses have been achieved. Compartmentalization (establishing small zones or compartments that free from H5N1) as a gradual eradication plan for the virus is in progress. Importantly, awareness programs about the disease are currently lacking (17, 8, 19).

2.2.4 Potential health hazards of AIV in Egypt

Continuous interspecies and intraspecies transmission of A/H5N1 in Egypt warrants being worried about a possible adaptation to human. The original "parent" H5N1 in 2006-2008 in Egypt killed 23 out of 51 infected human cases, with a 55% fatality rate. A dramatic change in the fatality rate was observed in 2009 where 4 out of 39 infected humans died due to the infection with the virus. Another observation in 2009 is the increased number of infected babies, toddlers and women (13). Thereafter, the fatality rate was 45% (n=13/29) in 2010, 38% (n=15/39) in 2011, 45% (n=5/11) in 2012 and 75% (n=3/4) in 2013 (as of October, 08) (224). Genetically, in comparison to the parent 2.2.1 virus, the classic 2.2.1/C group of viruses carries a handful of mutations in the HA protein. A deletion of the receptor binding site 129S lead to increased affinity of the virus to the human receptors and retained its specificity for avian receptors (221). Recently, air-born-transmission of A/H5N1 or reassortant H5N1-H1N1 viruses were known to be governed by about five mutations (103, 108); three of them exist in the Egyptian isolates until 2011 (156). In 2012, a suspicious fourth mutation has been observed in the recent human isolates which may probably enhance human-to-human transmission (Abdelwhab et al., unpublished). This also may support the possible subclinical infection with A/H5N1 in human in Egypt. Also, it is highly expected that co-circulation of diversified H5N1 and H9N2 virus will produce new reassortants with unpredictable genetic and phenotypic features either for poultry or human (33).

Transmission of H5N1, H9N2 or combinations thereof from Egypt, where two major migration flyways exist, to Africa, Europe or Asia is not impossible. Taken together, international collaboration to control the disease in Egypt is highly and urgently required.

3. Summary of publications in the current thesis

3.1 Publication 1:

El-Zoghby EF, Abdelwhab EM, Arafa A, Selim AA, Kholousy SG, Kilany WH, M. K. Hassan MK, El-Kanawati Z, Aly MM, Hafez HM. 2011. Active surveillance of avian influenza virus in backyard birds in Egypt. The Journal of Applied Poultry Research 20:584-588 (74).

In this publication we conducted surveillance on backyard birds at the border of an artificial migratory wetland in Egypt, El-Abassa fish Lake about 80 km east of Cairo, in 2007 after isolation of an H7 virus from migratory birds few weeks before this surveillance. Tracheal and cloacal swabs were collected from chickens, ducks, geese, pigeons and turkeys from 45 houses in 11 villages roaming in and around the Lake. Viral RNA was extracted from the swabs then subjected to RT-qPCR for detection of M and HA genes of AIV. A total of 207 serum samples was collected from chickens (n=73), ducks (n=67), geese (n=52), turkeys (n=9) and pigeons (n=6). Sera of chickens and turkeys were tested against anti-AIV NP antibody using a commercial ELISA kits and all serum samples were tested H5 and H7 subtypes using HI test. None of the swabs was positive for AIV in the RT-qPCR. Nonetheless, 4.9% of chickens and turkeys serum samples (n=4/82) were positive for AIV, 3.9% of all sera (n=8/207) were positive for H5 subtype and no antibody against H7 subtype was recorded. This study indicated that H7 virus probably was not transmitted to the backyard birds around this Lake. Vaccination of poultry and/or previous exposure to H5(N1) virus may explain the positive results of the H5-HI test. We concluded that the threat of wild birds for transmission of AIV to backyards should not be neglected and the role of backyards in perpetuation of A/H5N1 in Egypt should be "seriously considered".

3.2 Publication 2:

El-Zoghby EF, Aly MM, Nasef SA, Hassan MK, Arafa AS, Selim AA, Kholousy SG, Kilany WH, Safwat M, Abdelwhab EM, Hafez HM. 2013. Surveillance on A/H5N1 virus in domestic poultry and wild birds in Egypt. Virology Journal 10:203 (76).

This publication summarized the Egyptian official national surveillance on A/H5N1 in poultry in commercial poultry farms, backyards and LBM in 2009 as well as in wild birds sampled during the migration season 2009-2010. Tracheal and cloacal swabs were collected from chickens, ducks

and/or turkeys in 22024 commercial farms, 1435 backyards and 944 live bird markets (LBMs). Also, 1297 samples were collected from 28 different types of migratory birds. A/H5N1 was detected by RT-qPCR in 0.1% (n=23), 10.5% (n=151) 11.4% (n=108) of examined commercial poultry farms, backyards and LBMs, respectively. None of the wild birds was positive for A/H5N1. The highest incidence of the virus in summer and spring particularly in backyard birds, however it was also detected in winter and fall seasons. Positive cases were concentrated mainly in Lower Egypt where 95.7% (n=22) of commercial poultry farms, 68.9% (n=104) of backyard birds and 52.8% (n=57) of LBMs were detected positive. Higher prevalence (56%, n=85) was reported in backyards that had mixed chickens and waterfowl together in the same vicinity and LBMs that had waterfowl (76%, n = 82). The findings of this study indicated (1) broad circulation of the endemic A/H5N1 among poultry in 2009 in Egypt (2) the epidemiology of A/H5N1 has changed over time with outbreaks occurring in the warmer months of the year (3) backyard waterfowl may play a role as a reservoir and/or source of A/H5N1 particularly in LBMs (4) the virus has been established in poultry in the Nile Delta where major metropolitan areas, dense human population and poultry stocks are concentrated (5) in the context of this study, wild birds may not play a role in spread of A/H5N1 in Egypt. Based on our findings we recommended that the surveillance in poultry (particularly backyards) must continue, tracing the source of live birds in the markets is essential, and global collaboration is needed to control the spread of the virus in Egypt.

3.3 Publication 3:

El-Zoghby EF, Arafa AS, Kilany WH, Aly MM, Abdelwhab EM, Hafez HM. 2012. Isolation of avian influenza H5N1 virus from vaccinated commercial layer flock in Egypt. Virology Journal 9:294 (73).

This publication described in more details a table-egg layer-chicken flock that was infected with the A/H5N1 virus in May 2009. In this flock, 23 699 Hisex-brown chickens were vaccinated three times at 1, 7 and 16 weeks of age with an inactivated "homologous" H5N1 vaccine. Four weeks after the last vaccination some birds had cyanosed comb and wattles, hemorrhages in the shank and up to 27% mortality in addition to about 20% drop in egg production. One week after the onset of symptoms birds had a mean HI titer of 3.2 $\log_2$ against the vaccine antigen supplied by the vaccine-producing company. The virus was detected in tracheal and cloacal swabs using RT-qPCR and was successfully isolated in SPF-ECE with a titer estimated to be 7.2 median egg infectious dose (EID_{50}) pro 0.1mL. Sequence and phylogenetic analysis of the HA and NA genes

of this virus indicated high genetic identity to H5N1 viruses of clade 2.2.1/C that isolated from LBM and a 32-month-old boy from the same village. Also, it was closely related to viruses isolated from backyard birds. This study highlighted important aspects in the epidemiology of A/H5N1 in Egypt: (1) insufficient "clinical" protection afforded by the current used vaccines due to improper vaccination or antigenic differences (2) possible circulation of the classic 2.2.1/C in commercial layer flocks after multiple vaccination (3) the role of backyard birds as a common source of infection in the commercial sector as well as humans (4) the significant importance of tracing the source of birds in LBM (5) also this study raised a question about the possible biological role of subclinically infected human (poultry workers, traders, veterinarians, etc.) in transmission of A/H5N1 to poultry.

3.4 Publication 4:

El-Zoghby EF, Arafa AS, Hassan MK, Aly MM, Selim A, Kilany WH, Selim U, Nasef S, Aggor MG, Abdelwhab EM, Hafez HM. 2012. Isolation of H9N2 avian influenza virus from bobwhite quail (*Colinus virginianus*) in Egypt. Archives of Virology 157(6):1167-1172 (75).

In this research paper we recorded the first isolation of H9N2 virus in poultry in Egypt in 2011. Five-week old 5 000 bobwhite quails in a commercial farm were vaccinated twice using H5 commercial vaccines at 3 and 9 days of age. Tracheal and cloacal swabs were collected during a routine pre-slaughter surveillance and the birds were clinically healthy. RT-qPCR and subsequent virus isolation indicated positive results for the AIV of H9N2 subtype and no evidence for the H5N1 or H7 subtypes. Genetic analysis of the HA and NA genes showed that the virus belonged to G1-like H9N2 lineage and very closely related to other H9N2 viruses circulating in poultry in the Middle East region. Moreover, the HA protein carried L234 which is known to be a human-receptor specific marker and fortunately the NA protein had Q277 and R292 which are known to be associated with sensitivity to neuraminidase inhibitors. This study showed that H9N2, on the contrary to H5N1, (1) most probably was introduced from neighboring countries not from the far east (2) produced no clinical disease in quails which considered a mix-vessel host for AIV of zoonotic potential (3) existence of the human-receptor specific genetic marker increases the burden of human infection (4) possible reassortment of the virus with H5N1 in poultry (or any other host including human) should be carefully and continuously monitored (5) finally, this study raised another important question about the interaction between H9N2 and H5N1 viruses (i.e. cross protection).

Chapter 2

El-Zoghby EF
Abdelwhab EM
Arafa A
Selim AA
Kholousy SG
Kilany WH
Hassan MK
El-Kanawati Z
Aly MM
Hafez HM

Surveillance on backyard birds around artificial fish-lake

Pool in Nebamun's estate garden
Tomb-chapel of Nebamun
Thebes, Egypt
Late 18th Dynasty, around 1350 BC
British Museum

Active surveillance of avian influenza virus in backyard birds in Egypt

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Primary Audience: Flock Supervisors, Veterinarians, Researchers

SUMMARY

The lack of biosecurity measures for backyard birds in Egypt and their continuous contact with wild migratory birds make backyard birds much more vulnerable to avian influenza virus (AIV) than those on commercial farms. The wild bird-backyard flock pathway was claimed to be the source of the first intrusion of H5N1 virus in Egypt in February 2006. The Egyptian government reported the isolation of low-pathogenic AIV subtype H7 in migratory ducks from El-Abassa Lake, a fish-farming lake located 80 km east of Cairo. Fear of repeating the scenario of introducing H5N1 into the poultry sectors prompted us to survey backyard birds around the borders of the lake. Screening of 207 serum samples (73 from chickens, 67 from ducks, 52 from geese, 9 from turkeys, and 6 from pigeons) using a commercial generic anti-influenza A nucleoprotein antibody enzyme-linked immunosorbent assay revealed 4.9% positive chicken sera. No antibody against the AIV H7 subtype was recorded when a hemagglutination inhibition test was used, whereas 4.3% of the serum samples examined were positive for the H5 AIV subtype. Nevertheless, AIV was not detected in swabs examined by real-time reverse transcription-polymerase chain reaction. However, continuous surveillance of backyard birds should be emphasized.

Key words: avian influenza, backyard bird, Black Sea-Mediterranean flyway, Egypt, migratory bird

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Chapter 3

El-Zoghby EF
Aly MM
Nasef SA
Hassan MK
Arafa A
Selim AA
Kholousy SG
Kilany WH
Safwat MA
Abdelwhab EM
Hafez HM

National surveillance on poultry and wild birds

Flock of Geese
Tomb-chapel of Nebamun
Thebes, Egypt
Late 18th Dynasty, around 1350 BC
British Museum

Nebamun hunting birds
Tomb-chapel of Nebamun
Thebes, Egypt
Late 18th Dynasty, around 1350 BC
British Museum

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VIROLOGY JOURNAL

RESEARCH Open Access

Surveillance on A/H5N1 virus in domestic poultry and wild birds in Egypt

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Abstract

Background: The endemic H5N1 high pathogenicity avian influenza virus (A/H5N1) in poultry in Egypt continues to cause heavy losses in poultry and poses a significant threat to human health.

Methods: Here we describe results of A/H5N1 surveillance in domestic poultry in 2009 and wild birds in 2009–2010. Tracheal and cloacal swabs were collected from domestic poultry from 22024 commercial farms, 1435 backyards and 944 live bird markets (LBMs) as well as from 1297 wild birds representing 28 different types of migratory birds. Viral RNA was extracted from a mix of tracheal and cloacal swabs media. Matrix gene of avian influenza type A virus was detected using specific real-time reverse-transcription polymerase chain reaction (RT-qPCR) and positive samples were tested by RT-qPCR for simultaneous detection of the H5 and N1 genes.

Results: In this surveillance, A/H5N1 was detected from 0.1% (n = 23/) of examined commercial poultry farms, 10.5% (n = 151) of backyard birds and 11.4% (n = 108) of LBMs but no wild bird tested positive for A/H5N1. The virus was detected from domestic poultry year-round with higher incidence in the warmer months of summer and spring particularly in backyard birds. Outbreaks were recorded mostly in Lower Egypt where 95.7% (n = 22), 68.9% (n = 104) and 52.8% (n = 57) of positive commercial farms, backyards and LBMs were detected, respectively. Higher prevalence (56%, n = 85) was reported in backyards that had mixed chickens and waterfowl together in the same vicinity and LBMs that had waterfowl (76%, n = 82).

Conclusion: Our findings indicated broad circulation of the endemic A/H5N1 among poultry in 2009 in Egypt. In addition, the epidemiology of A/H5N1 has changed over time with outbreaks occurring in the warmer months of the year. Backyard waterfowl may play a role as a reservoir and/or source of A/H5N1 particularly in LBMs. The virus has been established in poultry in the Nile Delta where major metropolitan areas, dense human population and poultry stocks are concentrated. Continuous surveillance, tracing the source of live birds in the markets and integration of multifaceted strategies and global collaboration are needed to control the spread of the virus in Egypt.

Keywords: Egypt, Highly Pathogenic Avian Influenza H5N1, Epidemiology, Surveillance, Commercial Farms, Backyard Birds, Live Bird Markets, Wild Birds

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Background

The unprecedented spread of H5N1 high pathogenicity avian influenza virus (A/H5N1) from Asia to Africa in 2005 was considered as a global epidemiological twist [1] due to poor infrastructure of poultry industry and diagnostic laboratory and lack of "accredited" preparedness control plans. Infection of domestic poultry with A/H5N1 in Egypt since mid-February 2006 caused enormous losses in poultry industry and the slaughter-campaign has overwhelmed the resources of the Egyptian veterinary and public health authorities [2]. Thereafter, Egypt has adopted a strategy to combat the disease based mainly on mass vaccination of backyard birds by inactivated H5N1 vaccine provided by the government free of charge (no longer supplied) whereas the commercial sector applied vaccination programmes with widely varying standards. Several types of inactivated H5N1 and H5N2 vaccines with different seed viruses were supplied by a number of vaccine manufacturers and used in the field [2,3].

The capacity of the commercial poultry sector in Egypt was estimated to be 850 million birds in 2006, where the majority of farms are small-scale units (5000 – 20000 birds) with poor or no biosecurity and usually used for broiler and layer poultry production. Conversely, the breeders and grandparent farms have strict biosecurity measures with all-in all-out production systems. Backyard birds in Egypt are a major source for cheap animal protein and essential financial resources for the farmers and small enterprises in rural areas. The backyard sector estimated to have 250 million chickens, ducks, geese, turkeys and rarely pigeons which are usually kept together in the same house [2]. In addition, due to insufficient slaughterhouses, marketing facilities and cultural preference for consumption of freshly slaughtered poultry trading of poultry meat in Egypt depends mainly on live bird markets (LBMs) [4]. In LBMs, birds of different species with various ages from several locations and different sources (backyards/barnyards and commercial flocks) are usually mixed. Therefore, LBMs are an indicator for A/H5N1 infections in poultry. Previous surveillance in Egypt has highlighted continuous and wide circulation of the virus in vaccinated and non-vaccinated commercial farms, backyard birds and LBMs [3-10] and bird-to-human transmission has occurred due to contact and/or slaughtering and defeathering of infected backyard birds [11]. Genetic analysis indicated that the Egyptian A/H5N1 has diversified into multiple genotypes where at least two distinct genotypes are currently prevalent: the 2.2.1.1 clade isolated mainly from vaccinated commercial poultry (and rarely from backyard birds) and the 2.2.1/C viruses isolated from backyard birds, small-scale commercial poultry and human [12,13].

On the other hand, Egypt acts as a bridge between Europe, Asia and Africa and millions of migrating birds pass through Egypt on their flights annually particularly in winter seasons where the northern Nile Delta lakes act as a major refuge for a multitude of bird species. Lake El-Manzala in the Nile Delta (north-east of Egypt: 32.20 East, 31.27 North) is one of the largest wetland on the Egyptian Mediterranean Coast (about 77,000 ha) where four provinces share its borders (Figure 1). It is an important wetland for wild birds either migratory or winter visitors along the Black-Sea-Mediterranean migratory flyway [14]. Many low pathogenic avian influenza viruses have been isolated from several species of migratory birds in Egypt [15-18] and A/H5N1 virus was possibly introduced into domestic poultry in 2006 by an infected common teal duck near Lake El-Manzala [18].

In previous publications we described the surveillance conducted in domestic poultry in 2006 [3], and 2007–2008 [7]. Here we describe the results of our nationwide surveillance on A/H5N1 in commercial poultry farms, backyard birds and LBMs in Egypt in 2009 as well as in wild birds in Lake El-Manzala in 2009–2010.

Results

Field investigations

Clinical signs and lesions of birds in the surveillance was varied; from clinically healthy birds to cyanosis of comb (snout in turkey) and wattle, haemorrhages on the shank, respiratory and intestinal disorders. In layer and breeder flocks complete cessation to slight decrease in egg production was recorded. Congestions and haemorrhages, particularly in parenchymatous organs, were observed. All wild birds tested in this study were apparently healthy.

Results of virus detection by RT-qPCR

In this surveillance samples were obtained from poultry on 22024 commercial farms, 1435 backyard flocks and 944 LBMs from Lower and Upper Egypt in 2009. The detection rate of A/H5N1 was 0.1% (n = 23/22024) of the examined commercial poultry farms as shown in Table 1. There were 1, 2, 8 and 10 infected broiler breeder, layer breeder, layer and broiler farms, respectively and 2 infected duck farms recorded in 2009 (Table 2). Moreover, 60% (n = 14/23) and 8.7% (n = 2/23) of positive commercial poultry farms used H5N1 and H5N2 vaccines, respectively (Table 2). The virus was detected in commercial farms in each season where 3, 7, 7 and 6 infected farms were detected in winter, spring, summer and autumn, respectively (Table 1).

On the other hand, a total of 10.5% (n = 151/1435) of the examined backyard flocks were detected positive (Table 1). Incidence of the virus was higher in summer ($p < 0.05$) and spring in backyards where 15.3% (n = 25/163) and 11.9% (n = 48/403) of examined backyard flocks were positive, respectively in comparison to 10.6% (n = 51/481) in winter and 6.9% (n = 27/388) in autumn.

No	Province	Commercial poultry farms	Backyard flocks	Live bird markets
1	Alexandria	1	5	4
2	El-Buhaira	0	19	5
3	Kafr El Sheikh	1	5	4
4	El-Dakhalia	0	22	2
5	Dumietta	0	1	1
6	El-Gharbia	0	13	10
7	El-Menofia	0	16	9
8	El-Quliubia	17	1	10
9	El-Sharkia	1	7	6
10	Cairo	0	0	0
11	Giza	0	4	0
12	6th October	2	7	0
13	Helwan	0	4	6
14	Port-Said	0	3	0
15	El-Ismailia	0	0	4
16	El-Suez	0	5	1
17	El-Fayoum	1	18	14
18	Beni-Suif	0	3	6
19	El-Menia	0	6	3
20	Assuit	0	2	6
21	Sohag	0	4	15
22	Qena	0	0	1
23	Luxor	0	3	0
24	Aswan	0	3	1
Total		**23**	**151**	**108**

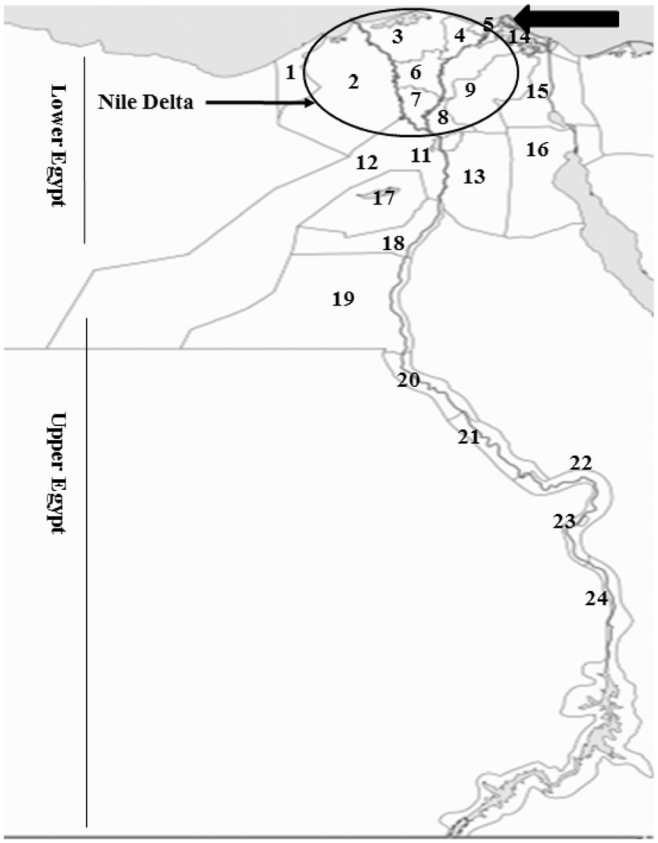

Figure 1 Geographical distribution of avian influenza H5N1 outbreaks in commercial farms, backyards and LBMs in Egypt in 2009. Provinces numbers 1 to 13 are located in Lower Egypt, numbers 14 to 16 in the Canal region and numbers 17 to 24 in Upper Egypt. Provinces numbers 1 to 9 is forming the Nile Delta region. Black arrow refers to the location of El-Manzala Lake where four provinces share borders "provinces 4, 5, 9 and 14".

Prevalence of A/H5N1 subtype virus in different backyard birds were 56% (n = 85/151), 24% (n = 36/151) or 13% (n = 19/151) of positive backyards had chicken-waterfowl, chicken or waterfowl, respectively (Figure 2).

Results of our surveillance revealed that 11.4% (n = 108/944) of the LBMs were positive for A/H5N1 (Table 1). The detection rate of A/H5N1 in winter, spring, summer and autumn was 14.3% (n = 36/252), 11.8% (n = 47/400), 9% (n = 22/245) and 6.4% (n = 3/47), respectively. The highest prevalence of H5N1 was in LBMs which had waterfowl (76%; n = 82/108), while LBMs that had waterfowl-chicken-turkey; waterfowl-chicken and waterfowl-turkey represented 9% (n = 10/108), 7% (n = 8/108) and 5.5% (n = 6/108), respectively (Figure 2). The detection rate of A/H5N1 in commercial poultry was significantly lower than that in backyards and LBMs ($p < 0.05$) whereas the detection rate in LBMs was not significantly different than that in backyards ($p = 0.39$). There was a medium positive correlation ($r = 0.31$) between seasonal incidence of the virus in poultry in backyards and LBMs whereas seasonal incidence of the virus in commercial poultry was negatively correlated with

Table 1 Seasonal distribution of A/H5N1 in commercial poultry farms, backyard birds and live bird markets in Egypt during 2009

Season	Commercial farms		Backyard flocks		Live bird markets	
	Examined	Positive	Examined	Positive	Examined	Positive
Winter	3665	3 (0.08%)	481	51 (10.6%)	252	36 (14.3%)
Spring	6086	7 (0.12%)	403	48 (11.9%)	400	47 (11.75%)
Summer	6710	7 (0.10%)	163	25 (15.3%)	245	22 (9%)
Autumn	5563	6 (0.11%)	388	27 (6.9%)	47	3 (6.4%)
Total	22024	23 (0.1%)	1435	151 (10.5%)	944	108 (11.4%)

Table 2 Type of birds and vaccines used in positive commercial poultry farms infected with A/H5N1 in Egypt in 2009

Type of birds/ vaccine used	Number of outbreaks in commercial chicken farms				Number of outbreaks in commercial duck farms	Total
	Broiler breeders	Layer breeders	Layers	Broilers		
H5N1	0	0	7	7	0	14 (60%)*
H5N2	1	0	0	0	1	2 (8.7%)
Unknown	0	2	1	2	1	6 (26%)
Unvaccinated	0	0	0	1	0	1 (4.3%)
Total	1	2	8	10	2	23 (100%)

* Percent refers to number of infected farms /total of 23 positive farms.

incidences in backyards and LBMs (r = – 0.1 and - 0.52, respectively).

Out of 1297 examined wild bird samples only one teal duck was found positive for the matrix gene of AIV but not for H5 and/or N1 genes (Table 3). Attempts to isolate this virus were unsuccessful (data not shown).

Spatial distribution of the virus

There were 95.7% (n = 22/239) positive commercial farms in Lower Egypt and only one farm in Upper Egypt. In addition, there were 68.9% (n = 104/151) positive backyard flocks in Lower Egypt and 25.8% (n = 39/151) in Upper Egypt while 5.3% (n = 8/151) were reported in the Canal region (Figure 1). Moreover, the incidence of the disease in LBMs was higher in Lower Egypt as 52.8% (n = 57/108) of surveyed markets were positive in comparison to 42.6% (n = 46/108) in Upper Egypt and 4.6% (n = 5/108) in the Canal region. Respectively, 87% (n = 20/23), 59% (n = 89/151) and 47% (n = 51/108) of positive commercial farms, backyards and LBMs were reported in the Nile Delta as shown in Figure (1).

Sequence and phylogenetic analyses

Sequence of the HA and/or NA genes of seven randomly selected viruses from chickens, ducks or turkeys in different poultry sectors from Upper and Lower Egypt was generated (Table 4). The topology of the phylogenetic trees was similar in all methods (data not shown). As shown in Figure 3, the phylogenetic analysis of the HA gene indicated that four viruses (Ck6-BY, Tk1-M, Ck534-BY and Dk224-F) are located within the 2.2.1.1 clade characterized by mutations in residues 74, 97, 110, 123, 140, 141, 144, 154, 156, 162, 165, 184, 226 and 238 (Figure S1) and shared 97.1 – 97.5% and 95.4 – 96.3% nucleotides and amino acids identity with the parent virus, respectively (Additional file 1: Table S1). Whereas two viruses (Dk71-M and Ck18-F) clustered within two different extinct groups [12] and shared more than 98% identity with the

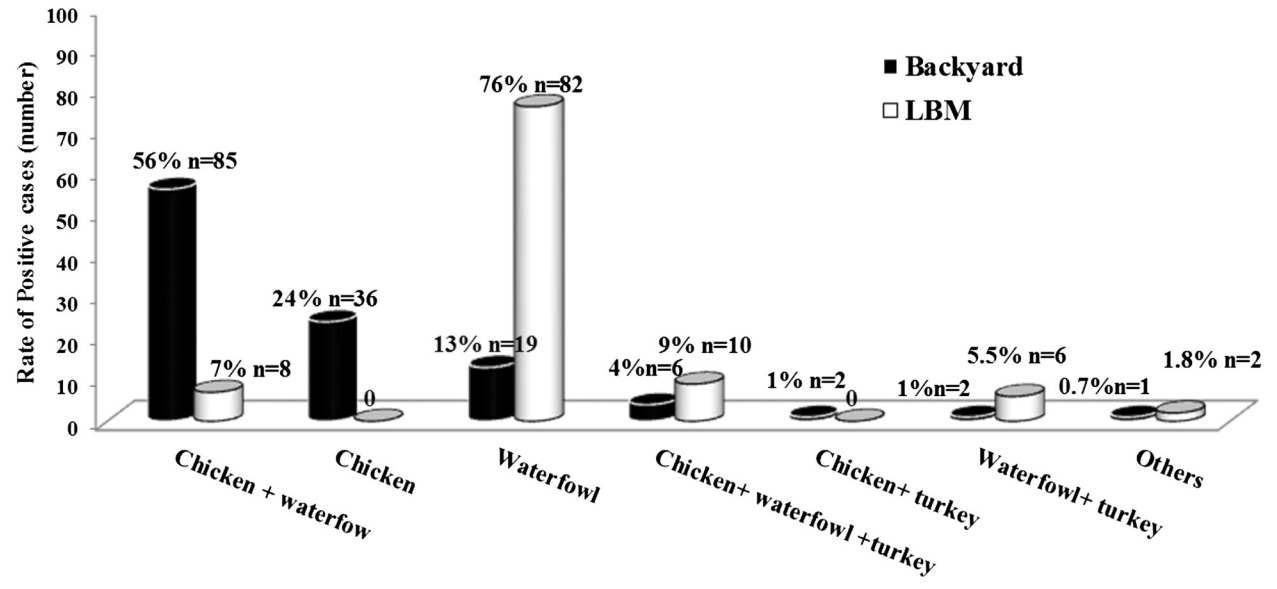

Figure 2 Poultry infection with A/H5N1 in backyards and live bird markets (LBM) in Egypt in 2009 detected by RT-qPCR. Species present in the examined backyards and LBMs. Percent refer to positive backyards or LBMs per species/total 151 positive backyards or 108 positive LBMs, respectively. Waterfowl represent duck and/or geese. Others in backyard refer to sample was taken from letter whereas in LBM refers to LBM sell only turkeys.

Table 3 Number of samples collected from different wild birds in Lake El-Manzala

Number of collected swabs	Type of wild bird
331	Coot
195	Teal duck
166	Cormorant
120	Quail
95	Shoveler
60	Purple swamp-hen
58	Moorhen gallinula
53	Stock dove
41	Pintail
32	Great egret
25	Common moorhen
19	Ferruginous duck/ Mahogany/ White-eyed pochard
16	Little crake
16	Squacco heron
12	Mallard/wild or Mammon duck
10	African sacred ibis
10	Egyptian vulture
10	Wigeon duck
8	Rose-ringed parakeet
5	Slender billed gull
4	Kestrel
2	Black headed gull
2	Dalmatian pelica
2	Gerfalcon
2	Tufted duck
1	Common pochard
1	House sparrow
1	Lanner falcon
1297	Total

parent virus. Except for the Ck18-F (isolated from poultry in a commercial farm in El-Qaluibia province) which clustered within the variant 2.2.1.1 clade characterized by T168I substitution (Additional file 2: Figure S2), the topology of NA was similar to the HA and shared 98.2 – 98.5% and 99.1 – 99.7% nucleotide and amino acid identity with the parent virus, respectively (Additional file 3: Table S2). The NA sequence of Dk184-BY virus isolated from backyard ducks clustered with the classic 2.2.1/C group characterized by A46D, L204M, S319F and S430G substitutions (Additional file 2: Figure S2) and respectively shared 98.9 and 99.3% nucleotide and amino acid identity with the parent virus (Additional file 3: Table S2).

Discussion

Active surveillance on avian influenza virus in Egypt has been extensively performed on a regular basis since February 2006. For instance, our surveillance in 2009 confirmed continuous A/H5N1 infections in commercial poultry farms, backyard birds and LBMs in Egypt. Although, outbreaks in commercial poultry farms (0.1%, n = 23/22024) could be neglected as a great risk for the commercial poultry sectors in contrast to the backyards (10.5%, n = 151/1435). However, lack of compensation hampers the reporting of outbreaks and might result in a skewed/blurred picture of the actual field situation. It is worth mentioning that the current capacity of slaughterhouses in Egypt was estimated roughly to be 30 – 60% of the national meat poultry production and veterinary inspection is insufficient [2]. Moreover, illegal trading of unexamined commercial poultry and backyard birds into LBMs is not uncommon; therefore this might explain the higher incidence of the virus in LBMs (11.4%, n = 108/944). These results are in accordance with our surveillance conducted in cooperation with the Food and Agriculture Organization of the United Nations (FAO) in 2006 [3] and 2007 – 2008 [7]. In contrast to our results, Kayali et al. [19] reported 6.8% (n = 192/2827) positivity rate in commercial farms, 3.3% (n = 34/1024) in LBMs and only 0.9% (n = 12/1381) in the backyard flocks. However, the latter group conducted a targeted surveillance from August 2009 to July 2010 in six governorates only where 53%

Table 4 Viruses subjected to sequence analysis in this study and their GenBank accession numbers

No.	Virus	Abbreviation	HA*	NA*	Sector	Province	Date of isolation
1	A/duck/Egypt/0971SM-NLQP/2009	Dk71-M	GU002697	HQ908471	LBM	El-Monofia	February-2009
2	A/chicken/Egypt/0918Q-NLQP/2009	Ck18-F	GU002687	HQ908466	Farm	El-Qaluibia	March-2009
3	A/chicken/Egypt/096 L-NLQP/2009	Ck6-BY	GU811726	HQ908463	Backyard	Luxor	May-2009
4	A/turkey/Egypt/091Q-NLQP/2009	Tk1-M	GU002702	HQ908465	LBM**	El-Sharkia	January-2009
5	A/chicken/Egypt/09534S-NLQP/2009	Ck534-BY	GU002694	HQ908470	Backyard	6th October	May-2009
6	A/duck/Egypt/09224 F-NLQP/2009	Dk224-F	GU002686	HQ908464	Farm	El-Qaluibia	May-2009
7	A/duck/Egypt/09184SM-NLQP/2009	Dk184-BY	Not done	HQ908472	Backyard	Assuit	March-2009

* accession numbers, **LBM = live bird market.

Figure 3 Phylogenetic relatedness of the HA and NA genes generated in this study with other relevant H5N1 viruses isolated from poultry and human in Egypt. Phylogenetic relatedness of the H5 (left) and N1 (right) sequences generated in this study and relevant genes were done using the Bayesian (MrBayes algorithm) method launched from TOPALi v2 with general time-reversible (GTR) likelihood model and 10 independent runs with the initial 25% of each run discarded as burn-in. Each run consisted of two independent tree files with each 100,000 Markov Chain Monte Carlo-sampled trees. Viruses obtained from this study are written in bold and the two co-circulating genotypes are highlighted in pink (variant clade 2.2.1.1) and yellow (2.2.1/c subclade). Both trees were rooted to the corresponding sequences of A/goose/Guangdong/1/96. Final trees were further edited by Inkscape 0.48 software.

(n = 2959/5562) of their collected samples were from poultry from two governorates.

The viral circulation in vaccinated and non vaccinated birds was previously reported; particularly during the winter seasons of 2006 – 2008 [3,7]. However, the results obtained herein showed that the epidemiology of A/H5N1 in Egypt in 2009 has changed over time with outbreaks, especially in backyards, occurred in the warmer

months of the year; spring and summer which may indicate establishment and adaptation of the virus to the environmental conditions. This observation is in accordance with findings of Kayali et al. [19] in Egypt in 2009–2010 and was also found in Vietnam [20] and in opposition to a winter-associated pattern of AIV in other countries [21-23]. In the current study, A/H5N1 was more prevalent in LBMs that had waterfowls (and/or turkeys) but not chickens alone. Unfortunately, paucity of epidemiological data is an obstacle for identification of the source of birds, particularly waterfowl, in the markets and curbs trace back of infection. Nevertheless, due to cultural factors the source of ducks in LBMs is usually the backyards while chickens are usually come from commercial farms [24]. Also, in contrast to chickens, waterfowl can be silently infected with A/H5N1 [25-29] which may maintain the virus in the LBMs for longer periods. It has been previously described that A/H5N1 infections are high in Upper Egypt particularly in the Nile Delta which could be considered as the influenza epicentre in Egypt where major metropolitan areas with dense human populations are concentrated and a lot of poultry are likely to be traded and consumed [3,7,10,13,19,26].

Previous comprehensive phylogenetic analyses described temporal pattern of A/H5N1 in Egypt and neither geographical nor species-linked pattern were observed [12,13]. Interestingly, Ck18-F and Dk224-F were isolated from two different poultry farms at the same governorate (El-Qaluibia) but they belonged to two different genetic lineages (Figure 3) whereas Ck6-BY and Tk1-M isolated from LBM and commercial farms, respectively from two different provinces (about 800 km apart) clustered together within the 2.2.1.1 clade. This could be explained by the rapid and random movement of poultry nationwide and mix of different poultry and marketing sectors. The topology of the HA gene of Ck18-F isolated from commercial poultry is different from the topology of the NA gene (Figure 3) which possibly is due to reassortment. However, the full genome sequence is required to confirm this notion.

Although isolation of many AIV subtypes from wild birds in Egypt has been previously reported, we failed to identify any A/H5N1 from wild birds which may indicate, in the context of this surveillance, no role of wild birds for spread of the virus in domesticated poultry. This is also in accordance with previous negative A/H5N1 results in samples collected from wild birds in Lake El-Manzala in 2006 [30] and 2009 [16]. Indeed, commercial poultry-LBMs-backyards cycle in Egypt is closely integrated and any breach will eminently affect poultry and endanger public health. Therefore we suggest that enforcement of biosecurity measures should be the first line of defence while vaccination acts only as an ancillary tool for control of A/H5N1. Depopulation of infected holdings requires prompt and fair compensation. Lack thereof will severely hamper effective eradication of the disease.

Conclusion

Our findings indicated broad circulation of the endemic A/H5N1 among poultry in 2009 in Egypt. In addition, the epidemiology of A/H5N1 has changed over time with outbreaks occurring in the warmer months of the year. Backyard waterfowl may play a role as a reservoir and/or source of A/H5N1 particularly in LBMs. Continuous surveillance, tracing the source of live birds in the markets and integration of multifaceted strategies and global collaboration are needed to control the disease in poultry in Egypt.

Materials and methods

Surveillance

Samples were collected during the routine nationwide avian influenza surveillance program after the ministerial decree number 221/2006 in charge the National Laboratory for Veterinary Quality Control on Poultry Production (NLQP) for official diagnosis and surveillance for avian influenza virus (AIV) in Egypt. The surveillance in domestic poultry was conducted in 2009 in 24 out of 29 provinces in Egypt which allocated in Lower Egypt (13 provinces), Upper Egypt (8 provinces) and Canal region (3 provinces) as shown in Figure (1). Nile Delta (9 provinces) is located in Lower Egypt between Damietta and the Rosetta branches of river Nile and represents approximately 4% of Egypt area where 95% of human population and poultry are living together. Surveillance was carried out in commercial poultry farms, backyard birds and LBMs. Samples were collected from some commercial farms on more than one occasion whereas each backyard and LBM was visited only once. In this study, a commercial farm was considered as an epidemiological unit regardless of the number of houses or flocks in the farm. Likewise, the house was considered as an epidemiological unit regardless of the type of backyard birds or species. Therefore, the number of positive backyard holdings is the number of positive backyard flocks. Also, the positive LBMs refer to the market rather than the species.

Up to 20 cloacal and tracheal swabs were collected from 22024 commercial poultry farms, 1435 backyards and 944 LBMs (Table 1). A maximum of ten tracheal or cloacal swabs collected from commercial poultry were pooled separately and the examined sample was a mix of tracheal and cloacal swabs. Swabs from each species in each of surveyed backyards or LBMs were pooled together; however for economical reasons examined samples in the laboratory represent the house or the LBM as a whole; if multiple species were present. The available

history of vaccination of poultry in positive farms is summarized in Table 2.

The surveillance in wild birds was conducted during January 2009 and January, September and October 2010 in Lake El-Manzala. Wild birds were captured by hands or using mist nets, traps or shot by professional hunters. Cloacal and/or tracheal swabs were collected from 1297 wild birds representing 28 different types where 907 (69.9%) samples were obtained from Coot, Teal, Cormorant, Quail and Shoveler (Table 3). All swab samples were collected on viral transport medium containing antibiotics, transported to NLQP without breaking the cold chain and then stored at −80°C until used [31].

Real-time reverse-transcription polymerase chain reaction (RT-qPCR)

RNA was extracted from a mix of cloacal and tracheal swabs by using a MagNA Pure LC Total Nucleic Acid Extraction kit according to the manufacturer's instructions using a MagNA Pure LC instrument (Roche, Mannheim, Germany). Samples were amplified using One-step Real Time RT-PCR Kit (Qiagen, Valencia, Calif.) for detection of type A avian influenza viruses targeting the matrix gene using primers and probe described by Spackman et al. [32]; forward primer 5′-AGA TGA GTC TTC TAA CCG AGG TCG-3′, reverse primer 5′-TGC AAA AAC ATC TTC AAG TCT CTG-3′and probe 5′ FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA-3′. The test was conducted in a Stratagene MX3005P real time PCR machine as mentioned before [3]. Positive AIV samples were used for further H5N1 subtype identification using H5N1 Real Time RT-PCR Kit (Roche Diagnostics Ltd) following the instructions of the manufacturer using LightCycler® 2.0 machine (Roche, Mannheim, Germany).

Sequence and phylogenetic analyses

Seven viruses were randomly selected from chickens (n = 3 viruses), ducks (n = 3) and turkeys (n = 1) from commercial farms (n = 2), backyards (n = 3) and LBMs (n = 2) in Lower (n = 5) and Upper Egypt (n = 2) as shown in Table 4. The nucleotide sequence of a total of 6 HA and 7 NA genes was generated as previously described [5] using BigDye Terminator v3.1 Cycle Sequencing Kit on an automatic sequencer (ABI-3130; Applied Biosystems, Foster City, CA). The obtained sequences were assembled and aligned to the related A/H5N1 viruses using MAFFT [33], BioEdit version 7.0.9.0 [34] and the results were further enhanced by manual editing. The generated sequences were deposited in the GenBank database and their accession numbers are listed in Table 4. Nucleotide and amino acid identity matrices of sequences generated from this study and the putative parent virus (A/chicken/Egypt/06207-NLQP/2006) isolated during the first outbreak in February 2006 were calculated. Amino acids of H5 and N1 proteins were numbered according to the mature protein of the putative parent virus. Phylogenetic relatedness of the H5 and N1 sequences conducted in this study and relevant genes retrieved from the GenBank were done using the Bayesian (MrBayes algorithm) method launched from TOPALi v2 [35] with general time-reversible (GTR) likelihood model and 10 independent runs with the initial 25% of each run discarded as burn-in. Each run consisted of two independent tree files with each 100,000 Markov Chain Monte Carlo-sampled trees. The resultant trees were compared with the consensus trees of 1000 bootstrap replicates produced by neighbor-joining, maximum-likelihood and maximum-parsimony implemented in MEGA5 [36]. All trees were rooted to the corresponding sequences of A/goose/Guangdong/1/96. Final trees were further edited by Inkscape 0.48 software (Figure 3).

Statistics

Chi-square and Pearson product moment correlation coefficient were used to analyse difference and correlation of A/H5N1 infections in poultry sectors in different seasons.

Availability of supporting data

The data sets supporting the results of this article are included within the article (and its additional files; Additional file 4: Figure S1, Additional file 2: Figure S2, Additional file 1: Table S1 and Additional file 3: Table S2.

Additional files

Additional file 1: Table S1. Sequence identity matrix of the HA of viral sequences generated in this study compared to corresponding sequence of the putative parent virus ($^{\text{nucleotides}}$ identical $_{\text{amino acids}}$).

Additional file 2: Figure S2. Alignment of amino acid sequences of the NA protein generated compared to the corresponding sequence of the putative parent virus (A/chicken/Egypt/06207-NLQP/2006).

Additional file 3: Table S2. Sequence identity matrix of the NA of viral sequences generated in this study compared to corresponding sequence of the putative parent virus ($^{\text{nucleotides}}$ identical $_{\text{amino acids}}$).

Additional file 4: Figure S1. Alignment of amino acid sequences of the HA protein generated compared to the corresponding sequence of the putative parent virus (A/chicken/Egypt/06207-NLQP/2006).

Abbreviations

A/H5N1: H5N1 high pathogenicity avian influenza virus; AIV: Avian influenza viruses; FAO: Food and agriculture organization of the united nations; HA: Hemagglutinin; NA: Neuraminidase; LBMs: Live bird markets; NLQP: National laboratory for veterinary quality control on poultry production; OIE: World organization for animal health; RT-qPCR: Real-time reverse transcription polymerase chain reaction.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EFE, AS, SGK, WHK, MS, carried out sample collection and examination. AA carried out the sequence of the selected viruses. EMA did sequence and phylogenetic analyses and prepared the manuscript, MMA, SN, MKH and

HMH conceived and coordinated the study and revised the manuscript. All authors read and approved the final manuscript.

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Table S1. Sequence identity matrix of the HA of viral sequences generated in this study compared to corresponding sequence of the putative parent virus ($^{\text{nucleotides}}$ **identical** $_{\text{amino acids}}$)

No.	Virus	Abbreviation	Genotype	1	2	3	4	5	6	7
1	EU372943_A/chicken/Egypt/06207-NLQP/2006	Parent virus	2.2.1	**ID**	99.2	98.3	97.2	97.1	97.5	97.5
2	A/chicken/Egypt/0918Q-NLQP/2009	Ck18-F	Extinct	99.0	**ID**	97.6	97.4	97.3	97.4	97.5
3	A/duck/Egypt/0971SM-NLQP/2009	Dk71-M	Extinct	98.1	97.2	**ID**	95.9	95.8	96.3	96.1
4	A/duck/Egypt/09224F-NLQP/2009	Dk224-F	2.2.1.1	96.0	96.5	95.1	**ID**	99.4	98.2	97.8
5	A/chicken/Egypt/09534S-NLQP/2009	Ck534-BY	2.2.1.1	95.8	96.3	94.7	99.6	**ID**	98.1	97.8
6	A/chicken/Egypt/096L-NLQP/2009	Ck6-BY	2.2.1.1	96.3	96.5	95.6	97.8	97.4	**ID**	98.0
7	A/turkey/Egypt/091Q-NLQP/2009	Tk1-M	2.2.1.1	95.4	96.0	94.7	96.9	96.5	96.7	**ID**

Table S2. Sequence identity matrix of the NA of viral sequences generated in this study compared to corresponding sequence of the putative parent virus ($^{\text{nucleotides}}$ **identical** $_{\text{amino acids}}$)

No.	Virus	Abbreviation	Genotype	1	2	3	4	5	6	7	8
1	GQ184251_A/chicken/Egypt/06207-NLQP/2006	Parent virus	2.2.1	**ID**	98.4	98.4	98.5	98.5	98.5	98.2	98.9
2	A/chicken/Egypt/0918Q-NLQP/2009	Ck18-F	2.2.1.1	99.1	**ID**	97.2	99.9	99.8	98.8	98.5	97.4
3	A/duck/Egypt/0971SM-NLQP/2009	Dk71-M	Extinct	98.6	97.7	**ID**	97.3	97.4	97.2	97.0	97.4
4	A/duck/Egypt/09224F-NLQP/2009	Dk224-F	2.2.1.1	99.7	98.8	98.4	**ID**	99.7	98.7	98.5	97.5
5	A/chicken/Egypt/09534S-NLQP/2009	Ck534-BY	2.2.1.1	99.5	98.6	98.2	99.7	**ID**	98.9	98.7	97.6
6	A/chicken/Egypt/096L-NLQP/2009	Ck6-BY	2.2.1.1	99.1	98.2	97.7	99.3	99.1	**ID**	98.6	97.7
7	A/turkey/Egypt/091Q-NLQP/2009	Tk1-M	2.2.1.1	99.7	98.8	98.4	100.0	99.7	99.3	**ID**	97.4
8	A/duck/Egypt/09184SM-NLQP/2009	Dk184-BY	2.2.1/C	99.3	98.4	97.9	99.5	99.3	98.8	99.5	**ID**

Figure S1. Alignment of amino acid sequences of the HA protein generated compared to the corresponding sequence of the putative parent virus (A/chicken/Egypt/06207-NLQP/2006)

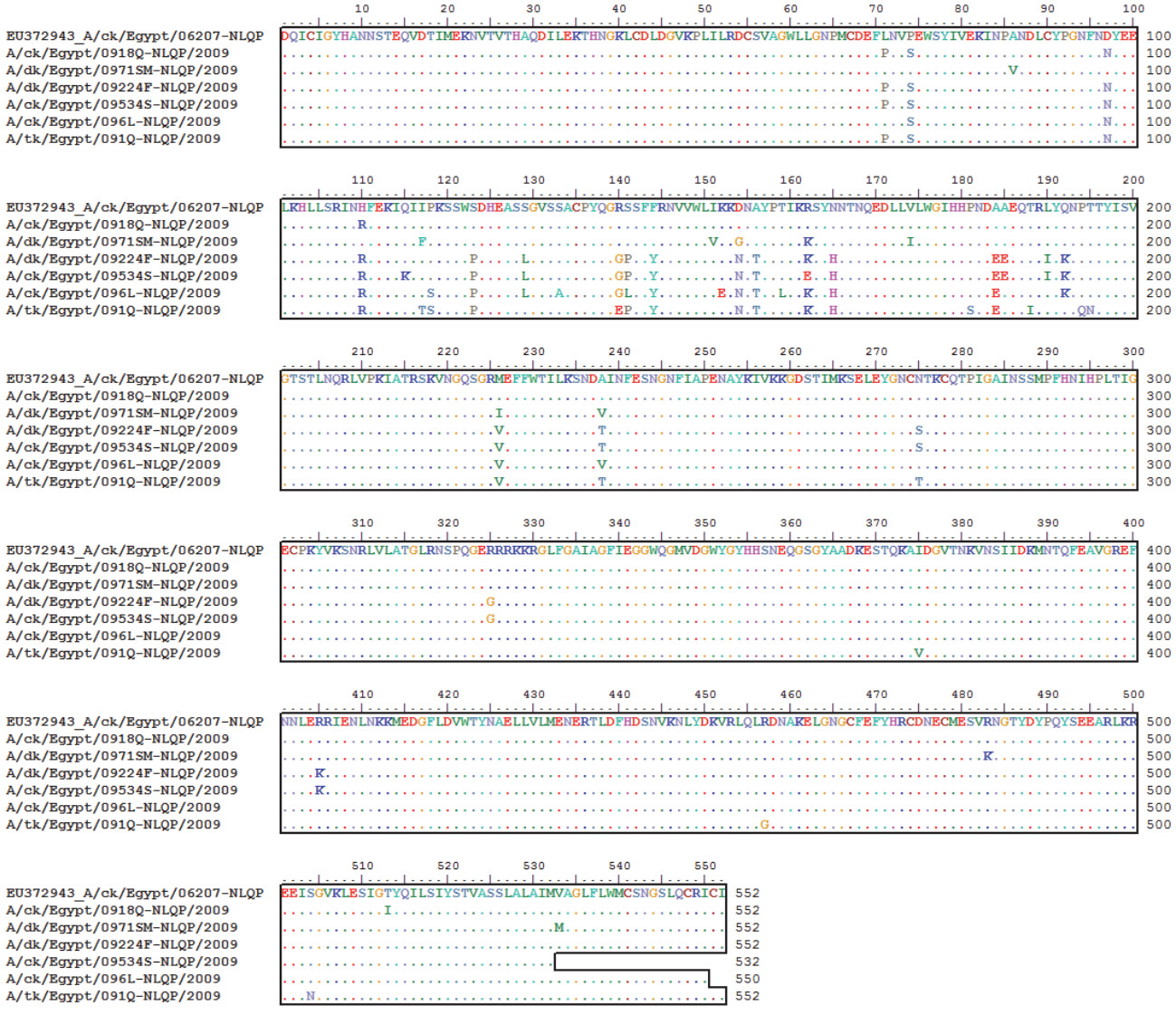
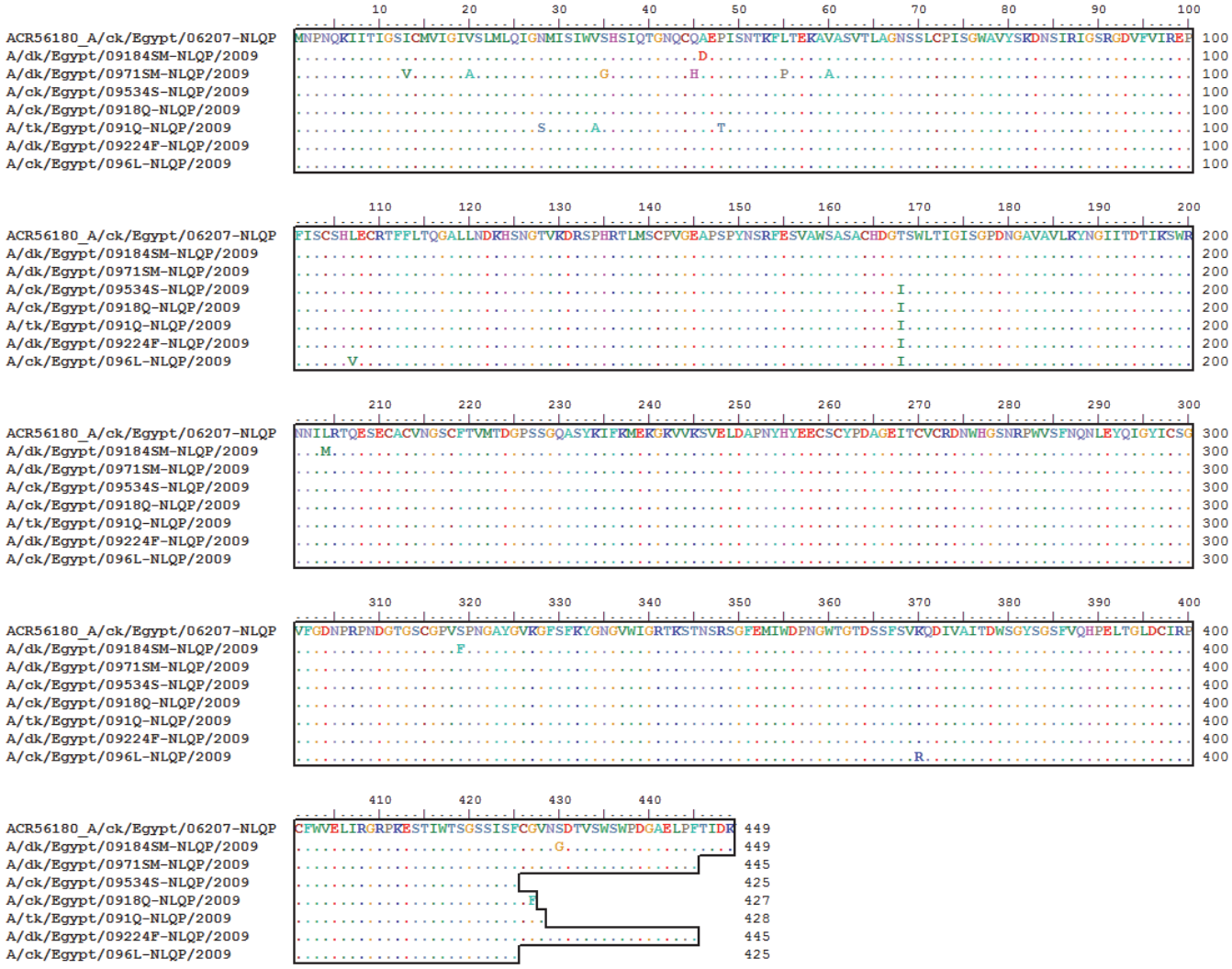


Figure S2. Alignment of amino acid sequences of the NA protein generated compared to the corresponding sequence of the putative parent virus (A/chicken/Egypt/06207-NLQP/2006)



Chapter 4

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Arafa A
Kilany WH
Aly MM
Abdelwhab EM
Hafez HM

A closer look on an infected commercial table-egg layer chickens flock

Bearers bring geese to an offering table
The tomb of Nakht
Thebes, Egypt
The 18th dynasty c.1421-1413 BC

El-Zoghby et al. Virology Journal 2012, 9:294
http://www.virologyj.com/content/9/1/294

VIROLOGY JOURNAL

SHORT REPORT Open Access

Isolation of avian influenza H5N1 virus from vaccinated commercial layer flock in Egypt

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Abstract

Background: Uninterrupted transmission of highly pathogenic avian influenza virus (HPAIV) H5N1 of clade 2.2.1 in Egypt since 2006 resulted in establishment of two main genetic clusters. The 2.2.1/C group where all recent human and majority of backyard origin viruses clustered together, meanwhile the majority of viruses derived from vaccinated poultry in commercial farms grouped in 2.2.1.1 clade.

Findings: In the present investigation, an HPAIV H5N1 was isolated from twenty weeks old layers chickens that were vaccinated with a homologous H5N1 vaccine at 1, 7 and 16 weeks old. At twenty weeks of age, birds showed cyanosis of comb and wattle, decrease in egg production and up to 27% mortality. Examined serum samples showed low antibody titer in HI test (Log_2 3.2± 4.2). The hemagglutinin (HA) and neuraminidase (NA) genes of the isolated virus were closely related to viruses in 2.2.1/C group isolated from poultry in live bird market (LBM) and backyards or from infected people. Conspicuous mutations in the HA and NA genes including a deletion within the receptor binding domain in the HA globular head region were observed.

Conclusions: Despite repeated vaccination of layer chickens using a homologous H5N1 vaccine, infection with HPAIV H5N1 resulted in significant morbidity and mortality. In endemic countries like Egypt, rigorous control measures including enforcement of biosecurity, culling of infected birds and constant update of vaccine virus strains are highly required to prevent circulation of HPAIV H5N1 between backyard birds, commercial poultry, LBM and humans.

Keywords: Highly pathogenic avian influenza, H5N1, Egypt, Vaccination failure, Backyards, Live bird markets

Findings

Background

A devastating highly pathogenic avian influenza virus (HPAIV) of H5N1 subtype first emerged in poultry and further transmitted to human in Hong Kong in 1997 [1]. The spread of the H5N1 virus to Europe and Africa was due to a large-scale outbreak of H5N1 infection that occurred in migratory waterfowl in Qinghai Lake (China) in 2005 [2]. To date, ten different genetic clades (0 – 9) of H5N1 virus have been distinguished which further diversified into subclades [3]. The HPAIV H5N1 of clade 2.2.1 has been introduced into Egypt in early 2006 [4] probably via infected wild ducks [5]. Despite control efforts, the virus had become endemic in poultry in Egypt since 2008 [6]. Egypt embarked mainly on inactivated H5N1 and H5N2 vaccines to limit the spread of H5N1 virus and minimize its socioeconomic impacts [7]. However, circulation of the HPAIV H5N1 in different poultry species (chickens, ducks, turkeys, etc.), in addition to donkeys and possibly pigs was reported [8-10]. Moreover, out of 168 infected human in Egypt, 60 cases were fatal until July 27, 2012 [11].

In Egypt, H5N1 isolated from humans and a large group of viruses isolated from backyard ducks and chickens clustered in a distinct genetic group designated "2.2.1/C" [12], meanwhile, majority of viruses derived from vaccinated poultry in commercial farms were found in newly named 2.2.1.1 clade [3,13]. A virus belonging to 2.2.1/C group has been also isolated from donkeys in Egypt [8]. Those 2.2.1/C viruses harbor conspicuous mutations in the HA and NA proteins, were thought to be responsible for decreasing virulence in

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mammals as a step towards adaptation to the human population [14,15]. On the contrary, viruses cluster in clade 2.2.1.1, also known as variant 2.2.1 viruses, had extensive amino acids substitutions in or adjacent to the immunogenic epitopes at the proximal globular head region of the HA protein which could enable continuous circulation of the virus in and among commercial poultry despite large scale vaccination campaigns [16,17]. Regular nationwide active, passive and targeted surveillance revealed that HPAV H5N1 is perpetuated in many commercial farms, backyards and live bird markets (LBM) [4,7,9,16,18]. Culling of infected birds occurs infrequently in Egypt [6].

Backyard birds and commercial poultry are kept in very close contact with humans due to integration of both farms and houses in the same buildings. Employees in commercial farms usually maintain their own household birds. Furthermore, selling of remaining feed, utensils and equipment from commercial farms to the rural family poultry often occurs in Egypt. Backyard chickens, ducks and geese are mostly reared together and roam freely in the vicinity of the house in close contact with human, particularly children. More than 70% of the Egyptian poultry production from commercial or backyard sectors is marketed through LBM [6]. All H5N1 infected human cases, except three cases, were linked to direct contact of human with sick or apparently healthy birds in backyards and/or LBM [19].

Here we describe the isolation and molecular characterization of an HPAI H5N1 virus isolated from twenty weeks old chicken layer flock, which was vaccinated three times with a commercial inactivated H5N1 vaccine.

Methodology

A commercial chicken farm with 23,699 Hisex-brown layers kept in cages; chickens were vaccinated three times at weeks 1, 7, and 16 with a commercial inactivated H5N1 vaccine seeded by A/Goose/Guangdong/1/1996/H5N1 (Re-1 YEBIO, Harbin, China). Vaccination of birds was conducted by the owner as part of usual practice. Ten tracheal swabs and ten cloacal swabs were collected randomly at day 7 after onset of clinical signs. At the same time, ten serum samples were collected for further laboratory investigation. Ten swabs were pooled according to Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [20]. No experimental research was conducted in this study and the birds were handled according to the standard guidelines [20]. Samples were collected during the routine nationwide influenza surveillance program after the ministerial decree number 221/2006 in charge the National Laboratory for Veterinary Quality Control on Poultry Production (NLQP) for official diagnosis and surveillance of AIV in Egypt.

Virus isolation trials were carried out in 10-day-old SPF hatching eggs via allantoic sac inoculation [20]. The virus titer was estimated by mean egg-infective dose (EID_{50}/0.1mL) according to Reed and Muench [21]. For detection of avian influenza H5–specific antibodies in the serum samples; hemagglutination inhibition (HI) test was performed in V-bottom; 96-well microtiter plates with four hemagglutinating units (4HAU) of the indicated homologous A/Goose/Guangdong/1/1996 H5N1 antigen, supplied by the vaccine-producing company (YEBIO, Harbin, China) and 1% of chicken erythrocytes according to the standard protocol [20]. Results were interpreted as the reciprocal of the last well that showed complete inhibition of the hemagglutination activity of the used H5 antigen.

Viral RNA from fluid of pooled swabs was extracted using a MagNA Pure LC Total Nucleic Acid Extraction kit following manufacturer's instructions and MagNA Pure LC instrument (Roche, Mannheim, Germany). The RT-qPCR reaction was done using one step Real-Time PCR Kit (Qiagen, Valencia, CA.) as recommended by the manufacturer. Partial matrix (M) gene segment of AIV from viral RNA was amplified according to Spackman et al. [22] using forward primer 5′-AGA TGA GTC TTC TAA CCG AGG TCG-3′, reverse primer 5′-TGC AAA AAC ATC TTC AAG TCT CTG-3′and probe 5′ FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA-3′. The RT-qPCR reaction was done in Stratagene MX3005P real time PCR machine (Stratagene, Agilent Technologies, Santa Clara, CA). Thereafter, H5 and N1 genes were amplified using generic avian influenza virus H5N1 Real Time RT-PCR RT3 Kits (Roche, Mannheim, Germany) according to the manufacturer guidelines in LightCycler® 2.0 machine.

Amplification of the open reading frame of the HA and NA gene segments was conducted as previously described [16]. The full coding sequences of the HA and NA genes of the isolated virus were conducted using Big-Dye Terminator v3.1 Cycle Sequencing Kit on an automatic sequencer (ABI-3130; Applied Biosystems, Foster City, CA). The produced sequences were aligned with BioEdit version 7.0.9.0 [23]. Amino acid sequence was deduced and a BLASTN search was performed to identify the query sequence and to find similar sequences. Phylogenetic trees of the obtained H5 and N1 genes and other relevant H5N1 genes retrieved from the GenBank data base were generated using the neighbor-joining method with 1000 bootstrap replicates and the evolutionary distances were computed using the Maximum Composite Likelihood method implemented in MEGA5 [24]. Trees were edited for publication using Inkscape software 0.48.1 as shown in Figure 1. Prediction of N-linked glycosylation sites was done by the NetNGlyc 1.0 Server that examines the sequence context of N–X–S/T sequons [25].

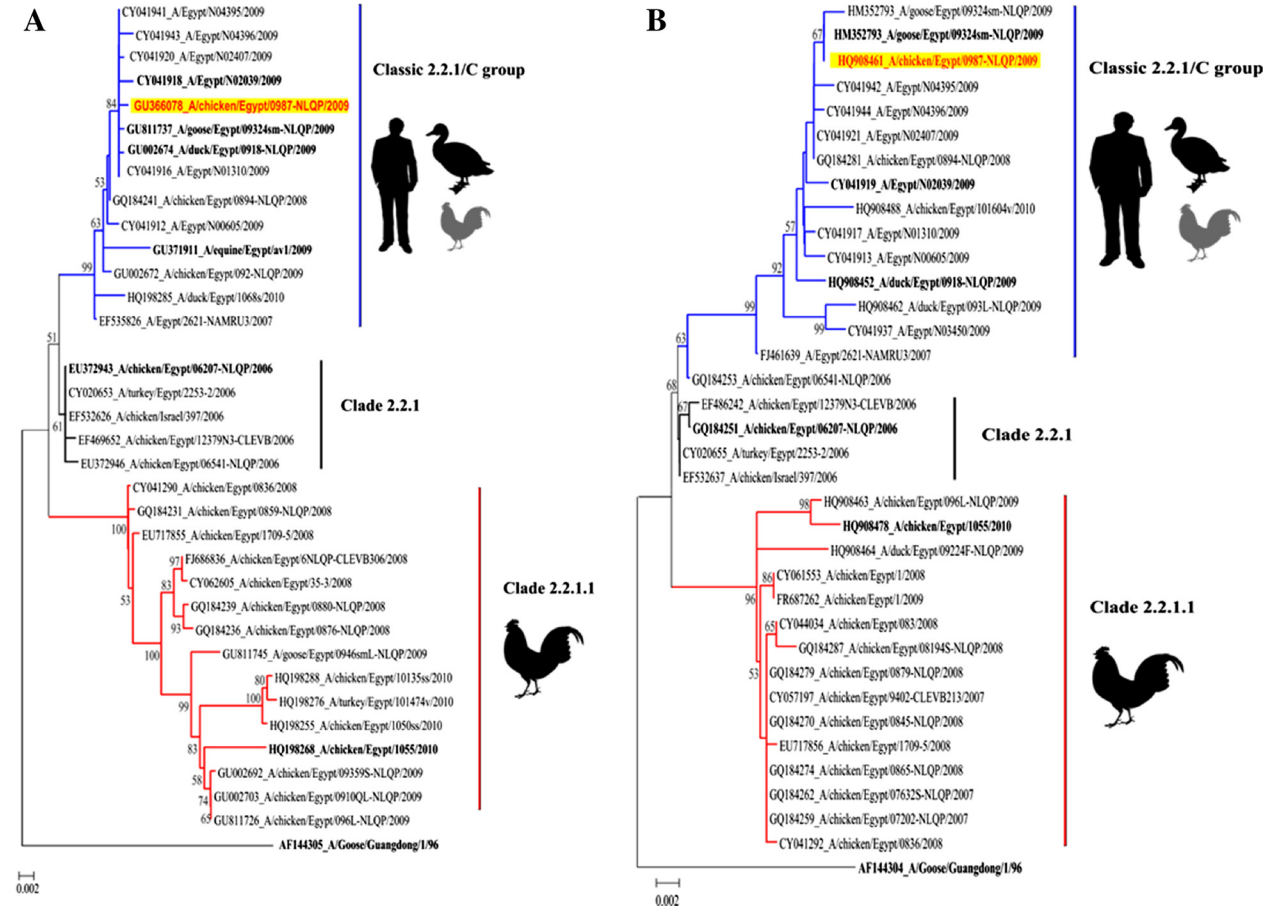

Figure 1 Phylogenetic trees of H5 (A) and N1 (B) genes of A/chicken /Egypt/0987-NLQP/2009 virus with other H5N1 viruses currently circulating in poultry and human in Egypt. The evolutionary history of the HA and NA was inferred using the Neighbor-Joining method with 1000 bootstrap replicates and the evolutionary distances were computed using the Maximum Composite Likelihood method implemented in MEGA5. The bootstrap values are shown next to the branches. Trees were edited for publication using Inkscape software. Virus obtained from this study is highlighted in yellow while other related viruses are written in bold. The virus clustered with H5N1 viruses isolated from poultry in backyards, live bird market and confirmed human infections. Two major H5N1 genetic clusters are currently co-circulating in Egypt: newly designated 2.2.1.1 clade (depicted in red) representing variant viruses isolated exclusively from vaccinated poultry in commercial farms and group C of 2.2.1 clade (blue) denoted 2.2.1/C group that mainly isolated from human and backyard birds according to the recent classification of WHO/OIE/FAO Evolution Working Group. The 2.2.1 index virus introduced into 2006 (black) was no longer isolated and was denoted as 2.2.1 clade.

The virus isolated in this study was designated as A/chicken/Egypt/0987-NLQP/2009(H5N1) and referred to as "Layer/0987". Identity matrix of the isolated virus with other six H5N1 viruses was done: (1) A/goose/Egypt/09324sm-NLQP/2009 [GenBank: GU811737 and HM352793 for the HA and NA genes, respectively] isolated from geese in live bird market (LBM) in the same region and referred to as LBM/Gs. (2) A/duck/Egypt/0918-NLQP/2009 [GenBank: GU002674 and HQ908452] isolated from backyard birds referred to as BY/Dk. (3) A/Egypt/N02039/2009 [GenBank: CY041918 and CY041919] was detected from 32 months old boy few days before the incidence of the current outbreak in commercial-layer flock from the same village (case number 57 in the WHO [26]) and referred to as Human/N02039. Those three viruses belong to the 2.2.1/C group. (4) A/chicken/Egypt/06207-NLQP/2006 [GenBank: EU372943 and GQ184251] was isolated in February 2006 and referred to as Index/2006. (5) A/chicken/Egypt/1055/2010 [GenBank: HQ198268 and HQ908478] a representative virus of the 2.2.1.1 variant clade that was isolated from vaccinated chickens and referred to as Variant/1055. (6) Layer chickens in this study were vaccinated by a commercial vaccine modified by reverse genetic where monobasic-HA and NA genes originated from H5N1 A/Goose/Guangdong/1/96 [GenBank: AF144305 and AF144304] referred to as GsGd/96. The latter was considered to be the parent virus of clade 2.2 [27]. Numbering of amino acid residues of the HA (H5 numbering after removal of the signal peptide) and NA proteins were done in comparison to GsGd/96 as standard (N1 numbering).

Tertiary structures of H5 and N1 glycoprotein monomers were generated as PDB using 3D-JIGSAW from Index/2006 virus [28]. Location of amino acid substitutions was imposed on the HA and NA proteins using RasTop software version 2.7.1 [29] and further edited by Inkscape.

Results

At twenty weeks of age, some birds showed cyanosis of comb and wattles, hemorrhages on the shank and a total mortality estimated to be 27%. In addition, there was a drop in egg production (20%) and an increase of the number of misshaped eggs (depigmentation, soft eggshell, shell-less and rough eggs). The course of the disease took more than 21 days from onset of clinical illness until depopulation of the flock.

After inoculation of embryonated chicken eggs with tracheal and cloacal swabs media, all embryos died within 48 hours post inoculation. Hemagglutination activity in the harvested allantoin fluid was determined. The isolated virus was confirmed to be an H5N1 virus by partial amplifications of the M, HA and NA genes of avian influenza viruses using specific real-time reverse transcription– polymerase chain reaction (RT-qPCR). The median egg infectious dose was 7.2 EID_{50}/0.1mL. Serum samples collected at 7 days after the onset of clinical signs had mean HI titer of 3.2 log_2 (standard deviation ± 4.3 log_2) using homologous A/Goose/Guangdong/1/1996 H5N1 antigen, supplied by the vaccine-producing company (YEBIO, Harbin, China).

The full coding H5 and N1 gene sequences generated in this study were submitted to GenBank under accession numbers GU366078 and HQ908461 for the HA and NA genes, respectively. A/chicken/Egypt/0987-NLQP/2009(H5N1) isolated in this study had 99.76% and 99.65% HA and 99.85% and 99.78% NA nucleotides and amino acids identity, respectively with an H5N1 virus isolated from LBM from the same village as shown in Table 1. A/chicken/Egypt/0987-NLQP/2009 had 99.82% and 99.65% HA and 99.64% and 99.49% NA nucleotides and amino acids identity, respectively with an H5N1 virus isolated from backyard birds in addition to 99.77% and 99.47% HA and 99.55% and 99.55% NA nucleotides and amino acids identity, respectively with an H5N1 virus isolated from 32 months old boy from the same village. However, the virus isolated in this study had nucleotides and amino acids identities of 94.28% and 94.53% for the HA and 93.1% and 92.04% for the NA with the vaccine strain (GsGd/96), respectively as shown in (Table 1). Both HA and NA genes

Table 1 Percentage identity of the hemagglutinin and neuraminidase sequences of A/chicken/Egypt/0987-NLQP/2009 in relation to other Egyptian H5N1 viruses and vaccine strain

Hemagglutinin	[1]Layer/0987	[2]LBM/Gs	[3]BY/Dk	[4]Human/N02039	[5] Index/2006	[6]Variant/1055	[7]GsGd/96
Layer/0987		99.77	99.83	99.77	98.90	96.19	94.28
LBM/Gs	99.65		99.94	99.88	98.96	96.19	94.46
BY/Dk	99.65	100.00		99.94	99.02	96.13	94.52
Human/N02039	99.47	99.82	99.82		98.96	96.25	94.46
Index/2006	98.94	99.29	99.29	99.12		96.88	96.02
Variant/1055	94.89	95.24	95.24	95.06	95.59		92.96
GsGd/96	94.53	93.47	94.71	94.53	95.41	91.71	
Neuraminidase	**Layer/0987**	**LBM/Gs**	**BY/Dk**	**Human/N02039**	**Index/2006**	**Variant/1055**	**GsGd/96**
Layer/0987		99.85	99.64	99.55	98.58	97.30	93.01
LBM/Gs	99.78		99.75	99.78	98.73	97.43	91.54
BY/Dk	99.49	99.58		99.49	98.56	97.20	90.59
Human/N02039	99.55	99.70	99.41		98.74	97.43	91.63
Index/2006	98.88	99.10	98.98	98.89		98.49	92.62
Variant/1055	97.96	98.19	97.96	97.96	99.09		91.76
GsGd/96	92.04	92.26	91.35	92.04	93.18	92.19	

Percentage identity of nucleotide (above the diagonal) and amino acid (below the diagonal).

[1] The virus isolated in this study was designated as A/chicken/Egypt/0987-NLQP/2009 under GenBank accession numbers GU366078 and HQ908461 for the HA and NA genes, respectively.

[2] A/goose/Egypt/09324sm-NLQP/2009 [GenBank: GU811737 and HM352793] isolated from geese in live bird market (LBM) in the same region.

[3] A/duck/Egypt/0918-NLQP/2009 [GenBank: GU002674 and HQ908452] isolated from backyard birds.

[4] A/Egypt/N02039/2009 [GenBank: CY041918 and CY041919] isolated from 32 months old boy from the same village.

[5] A/chicken/Egypt/06207-NLQP/2006 [GenBank: EU372943 and GQ184251] was isolated in February 2006 and represents the index 2.2.1 Egyptian viruses.

[6] A/chicken/Egypt/1055/2010 [GenBank: HQ198268 and HQ908478] variant 2.2.1.1 virus isolated from vaccinated chickens in a commercial farm.

[7] A/Goose/Guangdong/1/96 [GenBank: AF144305 and AF144304] vaccine strain used in vaccination of the current commercial layer flock.

clustered with 2.2.1/C group but not with viruses isolated from vaccinated commercial poultry in 2.2.1.1 clade (Figure 1).

The proteolytic cleavage site of the HA had polybasic amino acid motif "ERRRKKR/GLF", typical for HPAIV of clade 2.2. A deletion within the receptor binding site at position 129S (H5 numbering) and additional four amino acid substitutions namely; D43N, S120N, I151T and S320G of viral H5 protein in comparison to H5N1 virus introduced into Egypt in 2006 (Index/2006) were found. All mutations located in the HA1 on the globular head domain (Figure 2). In addition, 14 synonymous mutations were also found. Seven potential glycosylation sites (residues 11, 23, 165, 193, 286, 484 and 543) in the HA protein were predicted. De-glycosylation (loss of asparagine and existence of aspartic acid instead) of amino acid residue 154 was observed.

The NA of A/chicken/Egypt/0987-NLQP/2009 has a deletion of 20 amino acids in the stalk region (49 – 68; N1 numbering) in comparison to GsGd/96. Furthermore, in comparison to H5N1 virus introduced into Egypt in 2006, A/chicken/Egypt/0987-NLQP/2009 had five amino acid substitutions in the NA protein namely; A46D, N200D, L224M, S339V, and S450G and 14 synonymous mutations in the NA gene. There was no molecular marker of neuraminidase inhibitor resistance. The NA protein had three predicted potential glycosylation sites; residues 68, 126 and 215. On the contrary, GsGd/96 virus had additional four potential glycosylation sites that located in the deleted stalk region of A/chicken/Egypt/0987-NLQP/2009.

Discussion

Twenty weeks old layer chickens flock vaccinated three times with inactivated H5N1 vaccine suffered from respiratory distress and some birds had signs commonly seen in poultry infected with HPAIV. HPAIV H5N1 was isolated from tracheal and cloacal swabs media collected after onset of clinical signs during regular national surveillance. Full H5 and N1 coding gene sequences showed a close genetic relationship between the obtained virus and viruses of LBM, backyards and human origins. Due to insufficiency of epidemiological data, the source of this infection through either LBM, backyard birds or from humans cannot be deduced.

Despite repeated vaccination using a homologous H5N1 vaccine, the chickens exhibited observable distress and mortality. This suggests updated poultry vaccine may be necessary in endemic areas like Egypt. Tian et al., [30] found that the HI titer of > $4\log_2$ indicated sufficient protection of vaccinated chickens against HPAIV H5N1 infection. In this study, anti-H5 antibody titer was low with variable individual titers (3.2 ± 4.3 $\log_2$) after three successive vaccinations of Hisex brown-layers. Recent

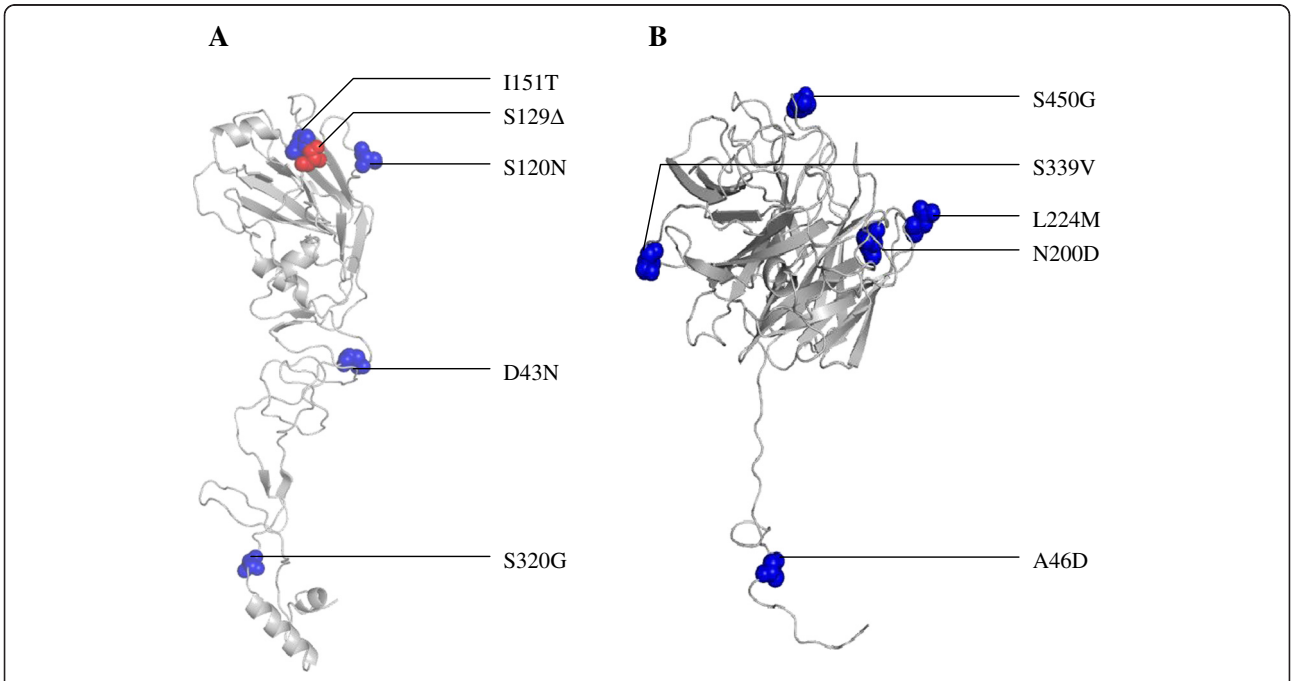

Figure 2 Predicted location of amino acid substitutions found in the isolated virus on the tertiary structure of HA H5 (A) and N1 NA (B) monomers. Shown are the substitutions in D43N, S120N, I151T and S320G in the HA and A46D, N200D, L224M, S339V and S450G in the NA proteins. Substitutions have been imposed upon the tertiary structure of A/chicken/Egypt/06207-NLQP/2006(H5N1) (index virus of clade 2.2.1 introduced into Egypt in early 2006). Protein modeling was generated by JIGSAW [28] and edited by RasTop version 2.7.1 and further edited by Inkscape.

experimental studies showed that specific-pathogen-free (SPF) chickens vaccinated with this inactivated H5N1 vaccine evoked high HI titer (7.0 ± 0.8 $\log_2$) and were protected against challenge with an Egyptian H5N1 virus belonged to the 2.2.1/C group [31]. Therefore, we assume that improper administration, mishandling and inappropriate storage of the vaccine [7] or suppression of the immune system (i.e.: due to chicken anemia virus infection or ingestion of mycotoxins) [32] could be responsible for such weak immune response and subsequently lack of protection. Moreover, the ability of inactivated H5N1 vaccine to evoke effective immune response in Hi-Sex brown layers remains to be investigated. Taken together, vaccination with regularly updated H5N1 vaccines to protect poultry against the evolving H5N1 virus in Egypt is highly recommended.

The high level of genetic identity of A/chicken/Egypt/0987-NLQP/2009 to viruses in LBM markets and backyard birds suggests a direct transmission link which is not uncommon scenario in Egypt. On the other hand, close genetic relationship between A/chicken/Egypt/0987-NLQP/2009 and H5N1 virus isolated from 32 months old boy from the same village could be explained by existence of a common source of infection; most probably backyard birds and/or LBM [7,9]. However, another possible source of infection could be human-to-chicken transmission which instantly could neither be excluded nor confirmed. Subclinical infections of human with H5N1 virus probably due to poultry-to-human, limited human-to-human transmission or environmental source (s) have been reported in China, Cambodia, Vietnam, Thailand and Turkey [33-37]. It is worth pointing out that subclinical infections of apparently healthy pigs and donkeys with HPAIV H5N1 have been reported in Egypt [8,10]. Unfortunately there is a paucity of information on subclinical spread among people in Egypt, particularly those in close contact to infected backyard birds or shared in culling of infected commercial farms. However, since late 2008, symptomless cases infected with mild virulent H5N1 in Egypt have raised concern that the virus might be adapted to Egyptians without getting sick [38].

It is well known that affinity of H5N1 virus to avian-type α2-3 and mammalian-like α2-6 linked sialic acid receptors seems to be governed by a number of residues in the HA protein including serine at position 129 which is a part of the receptor binding domain (RBD) [39]. It has been found that the Egyptian H5N1 viruses had a potential to use mammalian receptors resembling seasonal H1N1 virus [40]. Deletion of RBD 129S found in A/chicken/Egypt/0987-NLQP/2009 (Figure 2) existed also in all recent isolates of human and backyard origins in Egypt but had neither been reported from the parent GsGd/96 virus [27] nor from Index/2006, the virus originally introduced into Egypt in 2006 . Intriguingly, virus isolated from donkeys belonged to the same 2.2.1/C sublineage (Figure 1). Recently, deletion of RBD 129S combined with I151T in this unique Egyptian genetic group increased affinity of the Egyptian viruses to mammalian receptors and retained its avian receptor specificity [15]. Mutation in this residue was associated with a less virulent H5N1 phenotype causing milder or asymptomatic courses of infection and increased transmissibility in mice [41]. Interestingly, other significant substitutions such as Q192R, G222L and Q224S associated with adjustment of the virus from avian to mammalian receptors [42] were not observed in any Egyptian H5 gene sequences, including A/chicken/Egypt/0987-NLQP/2009 [13].

It is well known that N-linked glycosylation of the HA of influenza A viruses can affect receptor binding preferences or mask antigenic regions [41]. A/chicken/Egypt/0987-NLQP/2009 like other recent Egyptian H5N1 viruses of human and backyard origin, lost the potential glycosylation site at residue 154 (near the RBD) [13]. This phenotype was associated with (1) increase the affinity of H5 viruses to α2-6 residues [43], (2) increased transmissibility in guinea pigs and decreased fatality and systemic spread in mice [44]. Taken together, these clues could support the possible scenario of human-to-poultry transmission in Egypt. Therefore, infected human (farm workers, dealers, visitors, etc.) in Egypt should be considered a possible source of infection not only mechanical but also as a biological vector. Moreover, targeted surveillance to identify subclinical infection of human in Egypt should be taken in consideration to avoid a sudden emergence of pandemic virus. On the other hand, residue A46 is located in the NA stalk region while the others are positioned at the surface of the NA monomer (Figure 2). None of NA mutations has a known biological function except 319S which is a part of an immunogenic epitope (C) (56). A similar deletion in human H1N1 virus brought with it compensatory changes in the NA to facilitate viral entry and release and a similar mechanism may be acting on the 2.2.1/C viruses of human origin in Egypt [45]. Recently, isolation of H9N2 has been isolated from poultry in Egypt and possible reassortment is expected [46-48].

In conclusion, circulation of HPAIV H5N1 in vaccinated birds continues to devastate the poultry industry in Egypt. Birds in backyards and LBM remain the main potential source of H5N1 infection to both commercial poultry and humans in Egypt. Targeted surveillance to elucidate the spread of HPAIV H5N1 among commercial poultry workers and/or householders should be considered.

Abbreviations

AIV: Avian influenza virus; D: Aspartic acid; EID: Egg-infective dose; HA: Hemagglutinin; HI: Hemagglutinin inhibition; HPAI: Highly pathogenic avian influenza; I: Isoleucine; G: Glycine; LBM: Live birds markets;

N: Asparagine; NLQP: National laboratory for quality control on poultry production; OIE: World organization of animal health; RBD: Receptor binding domain; RT-qPCR: Real-time reverse transcription polymerase chain reaction; SPF: Specific pathogen free; S: Serine; T: Threonine.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
EFE carried out sample collection and examination and helped to draft the manuscript. AA carried out the sequence of the isolated virus. WHK participated in virus isolation and/or serological examination. EMA did sequence and phylogenetic analyses and helped to draft the manuscript. MMA and HMH conceived and coordinated the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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Chapter 5

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Emergence of H9N2 virus in commercial bobwhite quails

Marshing bird
Tomb-chapel of Nebamun
Thebes, Egypt
Late 18th Dynasty, around 1350 BC
British Museum

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BRIEF REPORT

Isolation of H9N2 avian influenza virus from bobwhite quail (*Colinus virginianus*) in Egypt

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Abstract This study describes the first isolation of H9N2 avian influenza virus (AIV) from commercial bobwhite quail (*Colinus virginianus*) in Egypt. Infected birds showed neither clinical signs nor mortality. Virus isolation and real-time reverse transcription polymerase chain reaction confirmed the presence of the H9N2 virus in cloacal swab samples collected at 35 days of age and the absence of other AIV subtypes, including H5 and H7. The hemagglutinin and neuraminidase genes of the isolated virus showed 99.1% and 98.2% nucleotide identity and 97.3% and 100% amino acid identity, respectively, to those of H9N2 viruses currently circulating in poultry in the Middle East. Phylogenetically, the Egyptian H9N2 virus was closely related to viruses of the G1-like lineage isolated from neighbouring countries, indicating possible epidemiological links.

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Keywords H9N2 · Egypt · Bobwhite quail · RT-qPCR · Virus isolation

Abbreviations

AAF	Amnio-allantoic fluid
AIV	Avian influenza virus
HA	Hemagglutinin
HI	Hemagglutination inhibition
L	Leucine
NA	Neuraminidase
OIE	World Organisation for Animal Health
Q	Glutamine
RT-qPCR	Real-time reverse transcription polymerase chain reaction

It is accepted dogma for avian influenza virus (AIV) that aquatic birds are the natural reservoir for all 16 hemagglutinin (HA) and 9 neuraminidase (NA) AIV subtypes [16]. Infection of land-based poultry with H9N2 viruses has become ubiquitous and endemic in several countries, wherein several bird species are commonly affected and potentially transmit virus to humans and pigs [4]. Usually, poultry infected with H9N2 AIV, unless complicated with other pathogens, show no clinical illness or suffer mild respiratory signs and a drop in egg production [38]. Experimental infection of SPF chickens has shown that H9N2 avian influenza virus is low-pathogenic, but in the last decade, Asian and Middle Eastern countries have faced frequent outbreaks of H9N2 infection with high mortality [10, 21, 38]. On the other hand, co-infection with other pathogens such as *Staphylococcus aureus*, *Haemophilus paragallinarum*, *E. coli* or infectious bronchitis virus can aggravate H9N2 infections, resulting in high mortality rates [10, 23, 31].

Quail have been found to be highly susceptible to H9N2 viruses, with few changes in the HA gene required for efficient replication and transmission in quail [40]. Bobwhite quail express both sialic acid-α2, 3-galactose linked, avian-type receptors, and sialic acid-α2, 6-galactose linked, human-type receptors [21] and thus may be considered another "mixing vessel", like pigs, for the generation of reassortant viruses from mammalian and avian sources with potentially novel antigenic and genetic features [27]. Furthermore, A/Quail/Hong Kong/G1/97-like H9N2 virus has been assumed to be a donor of the internal gene segments of the lethal H5N1 virus that emerged in Hong Kong in 1997 [19].

Based on phylogenetic analysis of the HA gene of H9N2 viruses, there are, so far, two major genetic lineages: the North American and Eurasian lineages [9]. In the latter, several sublineages have been distinguished: The G1-like sublineage was established in the Middle East and on the Indian subcontinent in the 1990s while other sublineages (Y280 and Ck/bei-like) circulate mainly in countries of the Far East [44]. Extensive evolutionary genetic analysis has indicated that East Asia has been the major source of H9N2 in the Middle East area [17]. Circulation of H9N2 viruses in the Middle East and Northern Africa since the year 2000 has been frequently reported in Israel, Jordan, Lebanon, Saudi Arabia, the United Arab Emirates, Kuwait, Iraq and Libya, where inactivated vaccines have been implemented in some countries to combat H9N2-associated disease of economical importance in poultry [5, 8, 17, 36]. Nevertheless, isolation of H9N2 virus from poultry has not yet been reported from Egypt, whereas the endemic status of H5N1 virus in poultry in Egypt with frequent, and occasionally fatal, transmissions to the human population since 2006 is well known [3].

Here, we describe the first isolation and genetic characterization of H9N2 virus from commercial bobwhite quail in Egypt.

A commercial broiler bobwhite quail (*Colinus virginianus*) farm with 5000 birds, 35 days old, was routinely surveyed for H5N1 infections during pre-slaughter active surveillance. The flock was vaccinated against H5 with recombinant fowl pox-H5 (Trovac AIV-H5, Merial Select, Inc. Gainesville, USA) and inactivated H5N2 (Volvac AI KV, Boehringer Ingelheim, Mexico) commercial vaccines at 3 and 9 days of age, respectively. At the time of sampling, the examined flock showed neither clinical illness nor unusual mortality rates.

Swab sampling of birds in this study was conducted according to published guidelines of the OIE [39]. Samples were collected by professional veterinarians at the National Laboratory for Quality Control on Poultry Production (NLQP) and General Organization of Veterinary Services (GOVs), Egypt, as a part of the routine nationwide pre-slaughter surveillance programs according to ministerial decree number 221/2006, in which NLQP is responsible for official diagnosis and surveillance of AIV in Egypt. Ten tracheal and cloacal swabs were collected separately on May 28, 2011. The samples were immersed in cooled viral transport medium containing antibiotics and transported immediately to NLQP without interrupting the cold chain [39]. Pools of swab samples were used for virus isolation and/or RNA detection as described below.

Embryonated chicken eggs obtained from a national specific-pathogen-free chicken farm were incubated at 37°C. Fertile 9- to 11-day-old embryonated eggs were inoculated via the allantoic sac route with 0.1 ml of pooled swab materials as recommended [39]. Inoculated eggs were examined daily for embryo mortality. Amnio-allantoic fluid (AAF) was harvested after five days and tested for hemagglutination activity and bacterial contaminants and subsequent passaging.

The infected AAF collected from inoculated eggs was tested for hemagglutination activity according to the recommended protocol [39]. Hemagglutination-positive allantoic fluids were examined using the HI test as described [39], using reference antisera specific for H5N1, H5N2, H6N7, H7N7, H9N2 and H9N7 viruses provided by Istituto Zooprofilattico delle Venezie (IZVSe), Padova, Italy, through FAO Egypt (ECTAD unit). Both tests were carried out using 1% chicken erythrocytes in 96-well V-shape plates (Nunc, Wiesbaden, Germany) [39].

RNA was extracted from a pool of five cloacal and five tracheal swabs by using a QiaAmp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. RNA extracts, 5 µl per reaction, were reverse transcribed and amplified using a One Step Real-Time RT PCR Kit (QIAGEN, Hilden, Germany) according to the manufacturer's guidelines for detection of AIV using generic primers and probes targeting the matrix gene [46], H5 gene [2], H7 gene [45] and H9 gene [37]. Each gene was detected in a separate reaction using a Stratagene MX3005P real-time PCR machine (Stratagene, Amsterdam, The Netherlands). For the identification of the N2 gene, a conventional reverse transcription PCR assay described by Fereidouni et al. [14] was used (Biometra Thermocycler machine).

Viral RNA was extracted from the allantoic fluid of H9N2 AIV-infected eggs using a QiaAmp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and was reverse transcribed using a Super-Script III reverse transcriptase kit (Invitrogen, Carlsbad, CA). Amplification of 225 nucleotides (encoding 75 amino acids) of the HA gene and 334 nucleotides (encoding 110 amino acids) from the NA gene was done using specific primers [14, 25]. Gene sequencing was carried out using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) in an

ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) as described elsewhere [7].

BioEdit software version 7.0.9.0 [24] was used to analyze and edit the generated sequences of the HA and NA genes. The GenBank database was screened (BLAST) for closely related sequences. A phylogenetic analysis of the newly obtained HA and NA nucleotide sequences using the most homologous H9N2 gene sequences obtained from the BLAST search was carried out using the PhyML maximum-likelihood algorithms with 100 bootstrap replicates using TOPALI v2.5 [35]. A GTR+G model of nucleotide substitutions was chosen according to results obtained with the ModelTest software implemented in the Topali suite [35]. Unrooted HA and NA trees were drawn with Dendroscope [28] and further viewed and edited using Inkscape software version 0.48. Gene sequences of the virus isolated in this study were submitted to the GISAID database, and the accession numbers were WSS72090 and WSS72089 for the HA and NA gene, respectively.

In the present investigation, a hemagglutinating virus was isolated from swab samples obtained from 35-day-old commercial bobwhite quail in embryonated chicken eggs after two passages. The isolated virus was found to be H9N2 using an HI test and RT-qPCR. Tests for other AIV subtypes, namely H5 and H7, gave negative results. The virus isolated in this study had an average nucleotide identity of 99.1% for the HA gene and 98.2% for the NA gene, and 97.3% and 100% identity for the HA and NA proteins, respectively, in comparison to other viruses isolated from the Middle East region. Both the HA and NA genes clustered with those of the G1-like H9N2 virus currently circulating in the region in Gaza, Israel, Lebanon, Iran, the United Arab Emirates and Saudi Arabia (Figure 1). The presence of the amino acid leucine (L) at position 234 (H3 numbering: 226), a part of the receptor-binding domain, in the HA protein of the isolated virus was observed. The NA gene sequence had glutamic acid at position 277 and arginine at position 292, which are known to be associated with sensitivity to neuraminidase inhibitors.

Infection of different poultry species, including quail, with H9N2 in the Middle East has been reported frequently since the early 2000s [1, 5, 18, 36, 41, 42]. Here, for the first time, the isolation of H9N2 virus in Egypt is described. Despite previous intense surveillance activities conducted by our team and others in the context of H5N1 endemicity in Egypt, H9 viruses had not been detected before [6, 7, 13, 22]. In the present investigation, close sequence and phylogenetic identities with the G1-like H9N2 virus currently circulating in the neighbouring countries could indicate further spread of the virus into Egypt as a part of its extensive circulation in the nearby countries rather than a separate introduction from the Eastern Asian AIV epicentre. Similar observations have been noted with the H5N1 virus where it is endemic in Egypt and occasional sporadic outbreaks of phylogenetically closely related strains have been recorded in neighbouring countries [18, 32]. Possible pathways of introduction, in particular, illegally or legally transported poultry or poultry products, should be investigated. Migratory birds as a source of incursion cannot be ruled out presently, but it seems unlikely. Fereidouni et al. [15] have shown that the H9 lineages circulating in wild birds in Iran are distinguishable from those in poultry.

It is noteworthy that in Eastern Asia, quail were the first land-based birds from which H9N2 viruses were isolated; they still claim endemic status to date [33]. In addition, Guan et al. [20] reported that 17% of quail in live retail poultry markets in Hong Kong were positive for H9N2 AIV. Birds examined in this study were clinically healthy, and mortality was neither reported by the owner nor observed by the sampling team of veterinarians. This is in accordance with results obtained after experimental H9N2 infection of 4-week-old Japanese quail, resulting in efficient virus replication and transmission between quails without inducing any clinical signs [40]. Moreover, quails in the current commercial flock were vaccinated twice with H5 vaccines (as a part of the nationwide vaccination campaign to confront the endemic H5N1 virus infection). Although cross-protection, e.g., through the elicited cellular immune response [43] induced by these H5 vaccines or the anti-NA (N2 subtype) antibodies induced by the H5N2 vaccine [47] against an H9N2 infection, seems unlikely, experimental infections in naïve quail are required to confirm or exclude this cross-protection effect. Khalenkov et al. [30] reported that 90%-100% of chickens previously infected with a recent H9N2 Israeli virus survived an experimental infection with a lethal H5N1 virus. Likewise, results obtained by Imai et al. [29] indicated that chickens vaccinated with H9N2 vaccine were partially protected against challenge with highly pathogenic H5N1 virus. According to these results, we assume that vaccination of birds using H5N2 vaccine can probably modulate infections with the low-pathogenic H9N2 viruses.

Since the first emergence of H9N2 AIV in poultry in 1966 [26], the virus continues to be a surprisingly devastating disease of birds. Due to its zoonotic potential and spread in swine in China, it is also shortlisted as a candidate for a future influenza pandemic [12, 33]. Quail have become another focus of attention as mixing vessels of influenza A viruses from different sources [27, 40, 48].

Since 1998, human infections with H9N2 virus have been reported frequently in Asia [11, 34]. It has been observed that avian H9N2 viruses carry glutamine (Q) at position 234, a part of the receptor-binding domain of the HA protein, which increases the binding affinity of the

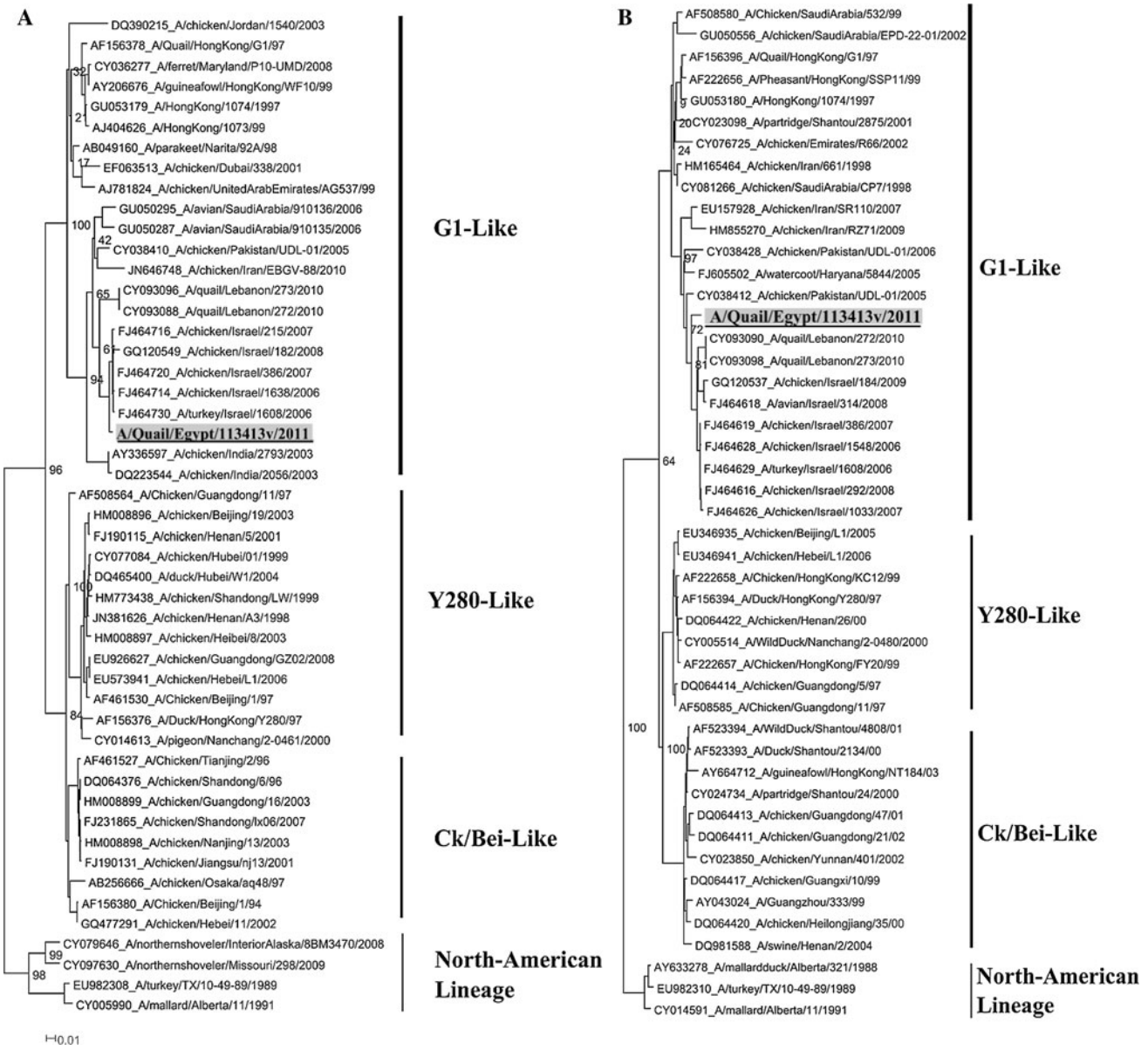

Fig. 1 Phylogenetic relation of H9 and N2 genes of A/quail/Egypt/113413v/2011 (H9N2) virus. Phylogenetic trees of the H9 hemagglutinin gene (**A**) and the neuraminidase (N2) gene (**B**) of A/quail/Egypt/113413v/2011(H9N2) isolated from 35 days old commercial bobwhite quail in Egypt. Bootstrap replicates (N=100) were calculated using maximum likelihood and a GTR+G model for nucleotide substitution as implemented in software Topali v2.5 [31]. The unrooted trees were visualized with Dendroscope [27] then edited by Inkscape 0.48 software. The virus isolated in this study is highlighted in grey; common H9N2 sublineages were depicted to the right of each tree

virus to the avian 2,3-linked sialic acid receptor. Human H9N2 viruses instead carry leucine (L) at this position, similar to human H2 and H3 viruses, which enhances the binding affinity to human 2,6-linked sialic acid receptors and improves direct-contact transmission in ferrets [11, 34, 49]. It is worth pointing out that highly pathogenic AIV H5N1 has established an endemic status in Egypt, even in vaccinated poultry [3]. Human cases of H5N1 infection continue to be reported from the country. Introduction of yet another AIV, which harbours a typical human virus L226 signature, calls for intensified nationwide surveillance, continuous monitoring, prompt control and retrospective studies to elucidate the spread of the virus in all Egyptian poultry sectors. Potential reassortment of the co-circulating H9N2 and HPAIV H5N1 in Egypt poses a major risk not only for poultry but also from a zoonotic point of view. Therefore, the complete genome sequence of the virus isolated in this study should be determined.

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Chapter 6

General Discussion and Recommendation

Defeathering and preparation of birds
The tomb of Nakht
Thebes, Egypt.
The 18th dynasty c.1421-1413 BC

7.1 General Discussion

In 2003, the HPAI H5N1 virus re-emerged and spread from the Southeast Asia to many countries in Eurasian. From 2003 to 2006, two institutions in Egypt started a nationwide surveillance in domestic poultry (NLQP; Ministry of Agriculture) and wild birds (NAMRU3; Ministry of Environment). A contingency plan was developed aiming at the rapid and effective containment of the disease in case of the emergence in domestic birds through stamping out of infected poultry at the index farm. It mandated the culling of poultry in a surrounding zone of 3 km which further surrounded by a buffer zone of 10 km diameter where movement of live poultry and eggs is tightly restricted (8).

Before February, 2006, none of the examined samples from domestic poultry was positive for AIV. Shortly, a few weeks before the end of the project, dead chickens, waterfowls and turkeys from seven backyards and LBMs in three different provinces around Cairo were submitted to the NLQP. After isolation and genotyping of HPAIV H5N1, the disease was officially announced to the public on February 16, 2006 (178). Although depopulation of poultry in the first reported outbreaks was done according to the prepared contingency plan; the infection, however, was reported nationwide within few weeks. Therefore, inactivated vaccines were introduced in a trial to mitigate the severe socioeconomic impact of the disease (8). Due to the blanket vaccination policy of birds all over the country and increase the temperature in summer season (from June to September), the incidence of the disease was dramatically decreased (25). After that, the outbreaks were frequently reported (4, 6, 32, 95, 119).

The first introduction of HPAIV H5N1 by legal or illegal trade of live poultry or imported feed components has been firstly suggested; however, it was never confirmed. It is now conceivable that the virus was introduced by wild birds because an HPAIV H5N1 with over 99% genetic identity was isolated from a common teal in the Nile Delta about two months before the emergence of the virus in the domestic poultry (178).

Also, the first isolation of the HPAIV H5N1 in February 2006 from seven outbreaks in three different provinces in the Nile Delta may support the hypothesis of the wild birds. In addition, it is widely accepted dogma in the epidemiology of avian influenza that wild birds are the natural reservoirs of the virus. All subtypes of AIV; mainly LPAIV and very rarely HPAIV H5N1, have been isolated from different species of wild birds. Some viruses transmit easily to

land-based poultry and/or human without prior adaptation. Nevertheless, the majority of these viruses must be adapted to domestic poultry (115, 191). Thus, there is a special concern on the wild-birds-backyards pathway for introduction of AIV into the domestic poultry (62, 110, 230).Then, the infection was probably acquired by the domestic ducks particularly in the backyards which roams freely around the lakes in the Nile Delta. Outdoor backyard birds are in frequent contact with wild and feral birds and bbackyard birds were claimed for the introduction of AIV in several countries (211, 216).

In the first publication of the present work, surveillance was conducted in 2007 in eleven villages encircle of EL-Abassa fish-lake, after isolation of an H7 virus from wild ducks few weeks before this surveillance, by colleagues in the Ministry of Environment and NAMRU3 (Unpublished data). None of the collected tracheal and cloacal swabs was positive for AIV in the RT-qPCR. Nonetheless, 4.9% of chickens and turkeys serum samples (n=4/82) were serologically positive for AIV, 3.9% of all sera (n=8/207) were positive for H5 subtype and no antibody against H7 subtype was recorded. This study indicated that H7 virus probably was not transmitted to the backyard birds around this Lake. Vaccination of poultry and/or previous exposure to H5 (N1) virus may explain the positive results of the H5-HI test.

In the 2nd publication, described the results of a national surveillance on H5N1 in domestic poultry (backyards, commercial farms and LBMs) and wild birds. A total of 1297 cloacal and tracheal swabs were collected from 28 different types of migratory birds. Of them, only in one sample AIV - RNA for non-H5 could be detected. Unfortunately, few materials were available which hindered the full classification of this virus. This surveillance and others may indicate that the wild birds don't play any role in the secondary spread as well as re-emergence of HPAIV H5N1 viruses in Egypt. However, a number of surveillance in wild birds were conducted until 2010 in Egypt and a considerable number of AIV could be isolated, but none of them was an HPAIV H5 (28, 76, 92, 178). In the literature, there are also plenty of examples for the responsibility of wild birds for the introduction of H5N1 virus into the land based poultry (79, 83, 163). The prevalence of infection, however, was very high in the backyard birds (10.5%; n=151/1435) and LBMs (11.4%; n=108/944). The virus could be detected year round particularly in the backyard sector. Astonishing was the high incidence in summer and fall seasons, which is untypical for AIV to exist in a high temperature climate. Moreover, we observed also that a high incidence was reported in backyards that had mixed chickens and waterfowl together in the same vicinity (56%, n=85/151) and LBMs that had waterfowl (76%, n = 82/108). To elucidate possibility of the persistence of the infection in

backyard birds during the summer, a targeted surveillance was carried out in ducks in 111 backyards and 71 commercial farms in the mid-summer 2011 in five provinces (99). While chickens and imported duck breeds were negative, subclinical infection of native ducks was reported in three different backyards in only one province. Because ducks are usually infected with AIV without showing any clinical disease, they play a significant role in the spread of HPAIV H5N1 as reported previously in Asia (123). Therefore, it remains to be clarified, if backyard ducks are the "Trojan horse" of HPAIV H5N1 in Egypt and serving as a reservoir or they are victims being infected from other sources. The government and allies (FAO, OIE, WHO) have settled a control plan for the disease in backyards including new legislations, vaccination and awareness campaigns. Prohibition of keeping live birds on markets, houses or roof-tops in the major cities has been legalized since 2006. Nevertheless, such decision was not enforced in the reality because of many reasons including cultural correctitude, economic status and consumption preferences. As a part of mass vaccination of poultry in Egypt, backyard poultry was provided inactivated H5N1 vaccines without charge. The plan was to vaccinate all backyard birds twice a year. Nevertheless, this strategy failed and the vaccine is no longer provided. Vaccinated birds can be infected without showing any clinical signs "silent infection" and spread of infection to other poultry and humans may be increased. Vaccination coverage of backyard birds was very low; ranged from 1 to 50% in most of surveyed villages. Furthermore, the existence of vaccinated birds with unvaccinated birds (particularly waterfowl) may be an additional factor for increased evolution and subsequent adaptation of the virus. Awareness and education campaigns including media, religious men, leader villager women "Raeda Refiya" (29) targeted mainly women (as a main involved person in the household sector) was conducted. Unfortunately, these programs are not sustainable. Nevertheless, virus-induced mortality particularly in the winter encouraged the householder to keep lower numbers of birds, if at all, in winter. Similar practice has been also described in the commercial sector (30). Such strategy may be the key to control the disease in villages and thus should be encouraged. Simultaneously it is possible to provide cheap frozen birds in winter to gradually change the preferences of consumption of fresh-slaughtered birds. Another critical reservoir of HPAIV H5N1 is the LBMs. In the USA, H7N2 is endemic since the early 1990s and the virus spread from the LBMs to the commercial poultry and human (182, 216). A similar situation has been described in China, Vietnam and Indonesia where several AIV subtypes including HPAIV H5N1 were isolated year round (109, 113, 114, 169). Also, the recent LPAIV H7N9 in 2013 which caused mild or no disease in infected birds but killed a considerable number of humans in China and Thailand has been

linked to poultry in the LBMs (43, 137). In Egypt, marketing of poultry depends mainly on the trading of live birds where commercial poultry from several provinces is usually gathered in collection points then redistributed through the country. Meanwhile, backyard birds are usually sold in local or traditional LBMs. Over 16000 LBMs and 4300 small slaughtering and de-feathering points sell live birds, freshly slaughtered or chilled birds (105). The virus can perpetuate in the LBMs or is introduced with each new batch. Hence, surveillance indicated that LBMs are considered a hotspot in the epidemiology of HPAIV H5N1 in Egypt (16, 73, 95). AIV is poorly transmitted by air for long distances; therefore the virus spreads rapidly by moving objects (53). During outbreaks of AIV in The Netherlands, Pakistan and Canada movements of bird (e.g. during thinning, restocking, slaughtering, etc.) and human (e.g. workers, veterinarians, vaccination crews, etc.) were the most risk factors for the spread of the virus between farms (1, 51, 195). Also, smuggling of infected birds can spread the virus not only from region to another within a country but also between countries in different continents (217). In Egypt, the rapid and random movement of infected poultry between regions and mix of poultry in rearing and marketing sectors is a very common practice particularly in the small-scale commercial poultry and backyards (155). Although transport of live poultry or eggs without prior laboratory testing to confirm H5N1-free status is prohibited, nonetheless, smuggling of poultry is not uncommon (8). In the present investigation, two viruses from two adjacent poultry farms belonged to two different genetic lineages whereas two viruses from LBM and commercial farms from two different provinces (about 800 km apart) clustered together. A similar observation was reported by Abdel-Moneim and co-workers (6) who mentioned that two H5N1 viruses obtained on the same day from poultry in two neighbouring backyards were genetically distinct.

On the other hand, the disease has been rarely detected in the commercial sector (0.1%; n=23/22024). It is worth mentioning that the surveillance in the commercial sector is mostly passive surveillance. The owner must send samples for the laboratory investigation prior the movement of poultry flocks from province to another as well as before slaughtering of the birds at the end of fattening (broilers) or production periods (layers and breeders). In this passive surveillance, collection of samples from sick or dead birds is not expected. Moreover, all commercial birds are vaccinated and it is expected that the virus shedding in vaccinated birds will be very low. Furthermore, vaccination of the commercial flocks resulted in underreporting of the suspected HPAIV H5N1 infected birds as recently published (218).

In the 3rd publication, a closer look on a vaccinated commercial layer-chickens flock in sectors 3 with low biosecurity measures revealed that the virus can induce high losses in egg production, morbidity and mortality, despite of the vaccination. The course of the disease was approximately three weeks from the onset of clinical signs until total depopulation of the flock. This period is relatively long period for a typical HPAIV H5N1; where 100% mortality could be reached within a couple of days. Nevertheless, partially effective vaccination can delay onset of the disease, but not totally prevent it (102). Moreover, the chickens were kept in cages, where mostly infection spread slowly in comparison to the floor, outdoor or free-range production system as previously described during the HPAIV H7N7 in The Netherlands in 2003 (78). Introduction of the virus was probably via part-time workers, who keep infected backyard birds at home, which is very common in Egypt (81). Also, close genetic relatedness of the isolated virus to those that have been identified in birds in LBMs and backyards as well as in humans reflect the complicated situation of close integration of all poultry sectors in Egypt.

It is well known that vaccination can prevent the clinical disease but cannot prevent the infection. Vaccinal breaks have been previously reported in vaccinated flocks against H7N1 in Italy (55), H7N3 in Pakistan (152) and H5N1 in China (59), Vietnam (66) and Indonesia (220). Outbreaks of H5N1 virus in vaccinated poultry flocks is a hallmark in the epidemiology of H5N1 in Egypt. It emerged at the end of 2007 and increased thereafter due to the evolution of antigenic-escape 2.2.1.1 variants in the commercial vaccinated poultry in Egypt (4, 18, 40). However, in the present investigation, isolation and characterization of a virus from 2.2.1/C group in vaccinated poultry was carried out. These 2.2.1/C viruses are prevailing in the commercial poultry in Egypt in 2011 and subsequent emergence of variant strains exhibiting antigenic drift from this 2.2.1/C group is plausible (32). Viruses isolated from vaccinated poultry acquired stepwise mutations in the immunogenic epitopes of the HA and had diverged at an increased evolution rate to escape from the vaccine strains causing severe outbreaks, despite vaccination (18). This evolution rate is extremely high in comparison to the circulating H5N1 viruses in other countries (e.g. Indonesia, Turkey, Nigeria, Thailand, and China) (58, 202). Meanwhile, viruses isolated from non-vaccinated poultry in the backyard sectors and humans had diverged at a lower evolution rate (18, 58) but probably developed a unique strategy to evade the innate immune response and replicate at higher levels in the infected cells (Ahmed, A. S. et al., unpublished). These viruses acquired specific mutations with high probability to tune the receptor-binding affinity and/or increase the replication efficiency of the virus in a broad-range of hosts. This has been recently

confirmed in mice as a model for human infection (221) and there are speculations about the acquisition of species-specific genetic signatures of the Egyptian H5N1 viruses in donkeys and ducks (3, 106). Also, genetic markers in the recent Egyptian HPAI H5N1 viruses that might enable a sustainable transmission from human to human or by airborne droplets have been described (156, 170).

Potential reservoirs of the virus in Egypt have not been fully elucidated. Surveillance indicated infections of chickens, ducks, geese, turkeys, quails, ostrich and pigeons as well as zoo birds (8, 119). The virus has been recorded from the most common feral birds in Egypt: egrets, crows and herons (8, 69, 188). Intriguingly, the viral RNA has been recently detected in fish which was probably due to contaminated poultry manure used as a fertilizer or fish feed and/or via infected domestic and feral birds around the aquatic habitats (69). Also, HPAIV H5N1 was isolated for the first time from donkeys, one week after the infection of backyard birds at the same village (2). Moreover, antibodies against H5N1 virus were detected in humans, horses, pigs, cats and dogs (8, 72) but their role as a reservoir or vector of the virus remains to be investigated.

During the last decade, H9N2 viruses has been frequently reported in many countries in the in the Middle East and Northern Africa. Two main lineages of H9N2 viruses have been genetically identified: the North American and Eurasian lineages. Since the 1990s, H9N2 virus circulates in the Far East countries and has evolved into several sublineages. The Y280 and Ck/bei-like infect poultry in central and Far East Asia and the G1-like sublineage was established in the Middle East (87).

In the 4th paper, we described the first isolation of H9N2 in Egypt in a commercial quails flock in 2011. The flock was vaccinated with H5 vaccines and birds showed neither clinical signs nor mortality. The isolated Egyptian H9N2 virus belonged to the G1-like lineage, which is endemic in the Middle East region. Retrospective study was recently published by Afifi and colleagues (21), who found that 50% of serum samples, which were collected from February 2009 to April 2012 from 39 chicken flocks, were tested positive for antibody against H9 subtype in HI test . In 2011-2012, there were 165 outbreaks in the commercial broiler and layer poultry flocks and only 3 outbreaks in the backyard birds in 2011-2012 (33). From 2011-2013, another study described H9N2 in 24 out 60 broiler, one out of five layer and one out of five breeder flocks (38). The previous surveillance indicated wide circulation of H9N2 in the commercial poultry, but not in the backyard birds and to our knowledge there is no

infection in ducks to date. An alarm for reassortment of H5N1 and H9N2 has been raised after the co-infection of some commercial poultry flocks with the two viruses (33, 147). The reassortment between H5N1 and H9N2 viruses has been previously reported in China (228), Pakistan (111) and Bangladesh (148). The recent H7N9 also acquired the internal gene segments from two H9N2 viruses (140).

The regulations of the OIE do not mandate a test-and-slaughter campaign in case of infections with LPAIV. Therefore, vaccination against H9N2 is the most common tool to combat the disease and has been applied in several countries like China (59), Korea (135), Pakistan (151), Iran (159) and Israel (41). Also, amantadine has been illegally used by the Chinese farmers to limit the spread of the disease (9). Nevertheless emergence of vaccine-escape variants (232) and amantadine resistance strains (45, 136) have been also described. In Egypt, a local vaccination program has been implemented. The vaccines contain local field strains and sometimes bivalent or trivalent vaccines with H5N1, Newcastle disease virus and/or infectious bronchitis virus have been also used. Thus, it is expected from our experience from the H5N1 epidemic (14, 94, 174) and from other countries that antigenic drift of H9N2 virus will probably increase in Egypt due to vaccination pressure. To date, there are no confirmed data on the use of amantadine against AIV in poultry in Egypt. On the other hand, it has been published that vaccination or infection with H9N2 can protect or modulate virulence of H5N1 in chickens (120, 153) which remains to be studied in Egypt.

7.2 Recommendations and Conclusions

In the foreseeable future the elimination of the endemic H5N1 AIV in poultry in Egypt is unlikely. Therefore, more efforts are required to better understand the epidemiology and evolution of the virus. Multiple introductions of different AIV subtypes into Egypt by wild birds are expected any time and monitoring the migration spots and wetlands should be done all over the year. Biological and molecular characterization of these viruses should be examined.

Rigorous evaluation of the efficacy of the used vaccines is very essential to effectively control the disease in the commercial poultry that must be conducted annually or biennially under representative field conditions (e.g. against new emerging viruses and/or in the presence of maternal immunity). The current vaccines are highly diverse from the field viruses. There is

only one vaccine from a field variant strain, development of a bivalent vaccine from the classic strain or selection of a virus can protect against both strains is highly recommended (46, 118). Moreover, establishment of AIV-free zones or compartments should be conducted simultaneously with regional vaccination to eliminate the disease in the commercial poultry sector. This must be accompanied with restriction of movement of live poultry.

Control of the disease in the backyard sector should take into account the social and economic needs of the householders. Steady awareness programmes are helpful to prevent acquiring and spread of the infection, particularly in the rural areas and suburbs.

Surveillance should continue and should cover all sectors and LBMs and it is important to understand the ecological and biological reservoirs of the virus.

Tracking the infection in LBMs, establishment of new slaughterhouses and changing the consumer preferences are a real challenge for the control of AIV in Egypt, which must be considered by the policymakers. There are many suggestions to control the disease in LBMs including changing the consumer preferences by selling frozen birds at lower prices, which can be supported by the government or the poultry industry. The establishment of more slaughterhouses by investors can be encouraged through reduction of customs fees or taxes. A one-day rest each week or month for cleaning and disinfection of LBMs has been previously found to be helpful to interrupt AIV circulation in the LBMs in Asia and USA (86, 130). Fair compensation of the owners for the depopulation of infected birds is also important.

Emergence of LPAIV H9N2 in poultry should not be neglected. Reassortment between H5N1 and H9N2 is highly expected. In this regard, co-infection of quails and other poultry with H9N2 and H5N1 is of great concern for the public health because pandemic strains could suddenly emerge. Cross-neutralization induced by the H9N2 to H5N1 or vice versa and the impact on vaccination of poultry should be studied. Last but not least, due to their zoonotic nature, there is an urgent need to study the virulence and transmissibility of H5N1 and H9N2 in mammals (e.g. ferrets, mice, guinea pigs).

8. Summary

Avian influenza virus of H5N1 subtype, clade 2.2.1, was introduced into Egypt by wild birds In February 2006. Since then, these viruses caused significant losses in the poultry industry and posed a public health threat. Surveillance and monitoring of wild birds and domestic poultry described in this dissertation revealed important aspects in the epidemiology of AIV, particularly H5N1, in Egypt. In 2007, isolation of H7 from wild birds in an artificial wetland in Egypt incited us to carry out surveillance on household poultry on the border of the lake. None of the surveyed birds was positive for H7 viruses but few birds had antibodies against H5 (chapter 2).

In chapter 3, we conducted a national surveillance in domestic poultry in 2009 and wild birds in 2009-2010. In domestic poultry, the prevalence of the virus was very high in birds in the backyards and LBMs compared to the commercial enterprises. Prevalence of the virus was significantly high in households and LBMs that kept ducks. Infections of H5-vaccinated birds were not uncommon. Also, the virus was detected all over the year and from different provinces particularly in the Nile Delta region. We showed that the Egyptian H5N1 viruses are genetically diverse where two major groups are co-circulating since 2008: an antigenic-drift variant group in the vaccinated commercial poultry and a classic group in the backyard birds and human. Both genotypes developed powerful strategies to continue to evolve in different evolutionary pathways. Meanwhile, the viruses accumulated several point mutations in the HA immunogenic epitopes resulting in antigenic drift and the establishment of infections in vaccinated poultry. The classic viruses acquired mutations thought to improve the dual receptor binding affinity in avians and mammals. None of the samples tested from wild birds was positive for H5N1 virus.

In chapter 4, we described in details the vaccination failure of a layer chickens flock infected with a classic 2.2.1/C virus. The flock was vaccinated three times with a homologous H5N1 vaccine but morbidity, mortality and reduced egg production were reported. Breach in the biosecurity and/or poor vaccination was probably the main reasons for the infection. In chapter 5, we recorded for the first time the isolation of H9N2 from asymptomatic H5-vaccinated commercial bobwhite quails. The Egyptian G1-like virus was closely related to the viruses circulating in the neighbouring countries which may indicate a possible epidemiological link.

Based on the findings of this study we provided several recommendations to control the disease in poultry in Egypt. Although there are accumulating data from surveillance on the Egyptian H5N1 and H9N2 viruses in the last few years, many key knowledge gaps regarding the epidemiology and evolution of these viruses in poultry should be investigated.

9. Zusammenfassung

Epidemiologische Untersuchungen und molekulare Charakterisierung des aviäre Influenza Virus bei Geflügel in Ägypten

Im Februar 2006 wurde Aviäre Influenzavirus (AIV) des Subtyps H5N1, Clade 2.2.1, von Wildvögeln in Ägypten isoliert. Seitdem verursachen diese Viren erhebliche Verluste in der Geflügelindustrie und stellen eine Bedrohung der öffentlichen Gesundheit dar. In dieser Dissertation wird die Überwachung und Kontrolle von Influenzavirusinfektionen in Wildvögeln und Hausgeflügel in Ägypten beschrieben, welche wichtige epidemiologische Aspekte, insbesondere über das H5N1 AIV, aufzeigen konnte.

Im Jahr 2007 wurde in Ägypten AIV vom Subtyp H7 von Wildvögeln nahe einer Fischhälterung isoliert, weshalb eine Überwachung des Hinterhofgeflügels in der Umgebung dieser Hälterung durchgeführt wurde. Von keinem der untersuchten Vögel konnten H7-Viren isoliert werden, serologisch zeigten einige Tiere Antikörper gegen H5 (Publikation 1).

Die Publikation 2 fasst die nationale Überwachung des domestizierten Geflügels in kommerziellen Betrieben, Hinterhöfen/Haushalten und Geflügelmärkten (LBMs) im Jahr 2009 und Wildvögel in den Jahren 2009-2010 zusammen. Im domestizierten Geflügel war die Prävalenz in den Hinterhöfen und LBMs im Vergleich zu den kommerziellen Betrieben sehr hoch. Eine signifikant höhere Verbreitung von H5N1 AIV fand sich in Haushalten und LBMs, in denen auch Enten gehalten wurden. Infektionen in geimpften Beständen waren nicht ungewöhnlich. Auch konnte das Virus über das ganze Jahr und aus verschiedenen Provinzen in der Nil-Delta-Region detektiert werden. Es konnte gezeigt werden, dass die ägyptischen H5N1-Viren genetisch vielfältig sind. Seit 2008 zirkulieren zwei Genotypen: eine antigene Driftvariante (auch Clade 2.2.1.1 genannt) in geimpftem kommerziellen Geflügel und eine klassische Gruppe (auch Clade 2.2.1/C genannt) in Hinterhofgeflügel und Menschen. Beide Genotypen zeigen eine unabhängige evolutionäre Entwicklung. Die aus geimpftem Geflügel isolierten Virusvarianten 2.2.1.1 weisen mehrere Punktmutationen in den immunogenen Epitopen des HA auf. Die klassischen 2.2.1/C Virusvarianten erwarben dagegen Mutationen, die möglicherweise die duale Rezeptorbindungsaffinität dieser Viren zu Vogel- und Säugetierzellen verbessern konnte. In keinem der untersuchten Proben von Wildvögeln konnte H5N1-Virus nachgewiesen werden.

In der 3. Publikation wurde die Infektion einer Legehennenherde mit einer klassischen 2.2.1/C–Virusvariante beschrieben, obwohl die Herde dreimal mit einer homologen H5N1-Vakzine geimpft wurde. Die Infektion führte zu reduzierter Legeleistung und Morbidität sowie Mortalität. Als Ursache wurden eine ineffektive Impfung und/oder ungenügende hygienische Maßnahmen diskutiert.

Die 4. Publikation beschreibt die Erstisolierung von AIV vom Subtyp H9N2 aus asymptomatischen, H5-geimpften Wachteln. Die enge Verwandtschaft des ägyptischen H9N2 G1-ähnlichen Virus mit den zirkulierenden Viren in den Nachbarländern weißt auf eine mögliche epidemiologische Verbindung hin.

Basierend auf den Ergebnissen dieser Studien ergaben sich mehrere Empfehlungen für die Kontrolle der Aviären Influenza in Ägypten. Obwohl es eine Fülle von Daten aus der Überwachung der ägyptischen H5N1 und H9N2 Viren in den letzten Jahren gibt, sollten weiter Untersuchungen durchgeführt werden, um wichtige Wissenslücken in Bezug auf die Epidemiologie und Evolution dieser Viren im Geflügel zu schließen.

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12. 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 05.05.2014 Elham Elzoghby