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Highly pathogenic H5N1 avian influenza virus epidemic in Egypt: Detection and protection studies

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Dedication

To my homeland Egypt …
for better future …

To my mother, father, family and friends…
I hope I didn't disappoint them…

Contents

CHAPTER 1

General introduction

1. Introduction

Emergence of the highly pathogenic avian influenza virus (HPAIV) of the H5N1 subtype in Egypt in mid-February 2006 caused magnificent losses in the poultry industry and was considered a potential threat to public health. Since late 2007, there is increasing evidence that stable lineages of H5N1 viruses are being established in chickens and humans in Egypt despite the blanket vaccination policy in all poultry sectors. Presence of the virus under immune pressure in vaccinated birds accelerated the mutation rate of the virus to escape from the repertoire of immune response. Existence of deleterious mutations in the primers and probe specific sites resulted in loss of sensitivity of the currently recommended real time reverse transcription polymerase chain reaction (RT-qPCR) by the World Organization of Animal Health "OIE" (Slomka et al., 2007) targeting the H5 gene. Furthermore, and recently, there are two different H5N1 sublineages co-circulating in Egypt referred to as variant 2.2.1 strains isolated from vaccinated poultry and classic or proper 2.2.1 strains usually isolated from backyard birds and accidently transmitted to humans (WHO, 2011a). Development of sensitive and specific RT-qPCR to detect and differentiate between those sublineages is of epidemiological concern and will improve the early detection and subsequently the control of the disease in poultry.

Increase in incidence of H5N1 outbreaks in vaccinated poultry in Egypt criticized the effectiveness of the current used vaccines to protect birds in the face of antigenic variation of the virus and increased the demand for testing a new vaccination regime and the development of new vaccines (Peyre et al., 2009; Hafez et al., 2010). Moreover, transfer of maternal antibodies from vaccinated breeders to their offspring might impair the efficiency of the vaccines. Studies described in this dissertation were planned to improve the diagnosis of the virus, as a first step in the control of the disease, and to evaluate a commonly used commercial vaccine to protect birds against currently circulating field virus. To achieve this aim the following studies have been conducted:

1. Development of modified H5 RT-qPCR oligonucleotides for detection of divergent HPAIV H5N1 in Egypt.
2. Development of new multiplex RT-qPCR for detection and differentiation of HPAIV H5N1 classic and variant viruses currently co-circulating in Egypt.
3. Effect of multiple dose vaccination of broiler breeder chickens with an H5N2 vaccine against challenge with classic and variant HPAIV H5N1 viruses of clade 2.2.1.
4. Study the influence of maternal immunity on infection and/or vaccination of one day old chicks against infection with Egyptian HPAIV H5N1.

2. Review of literature

2.1. Avian influenza viruses

2.1.1. Taxonomy and structure

Influenzavirus type A is a member of the family Orthomyxoviridae, which is composed of five genera, influenzavirus A, B, C, Thogotovirus and Isavirus (Büchen-Osmond, 2003). Influenza A viruses, the only orthomyxoviruses known to infect birds are negative-sense; single-stranded, enveloped ribonucleic acid (RNA) viruses contain genomes composed of eight separate segments encoding 11 viral proteins divided into three main categories: A) surface proteins (hemagglutinin; HA, neuraminidase; NA and Matrix 2; M2), B) internal proteins (polymerase subunits; PB2, PB1, PA, nucleoprotein; NP, matrix 1; M1 and nuclear export protein; NEP) and C) non-structural proteins (NS1 and PB1-F2) (Webster et al., 1992; Brown, 2000; Tollis and Di Trani, 2002; Cheung and Poon, 2008).

Haemagglutinin (HA) is responsible for attachment and fusion of the virus with the host cell receptors. The HA is synthesized as a precursor polypeptide, HA0 then cleaved by ubiquitous proteolytic enzymes into HA1 and HA2 (Steinhauer, 1999). The HA monomer consists of a globular head region mainly of HA1 connected to a fibrous stalk domain formed by the two polypeptide segments of HA1 and HA2. Several structures are present on the HA1 protein namely receptor binding domain (RBD), proteolytic cleavage site (PCS), N-Linked glycosylated carbohydrate (GS), antigenic sites and immunogenic epitopes (Chen et al., 1998; Steinhauer, 1999; Brown, 2000), while the transmembrane domain and fusion peptide are associated with the HA2 protein (Steinhauer et al., 1995; Armstrong et al., 2000). The proximity of the globular head region harbours the receptor-binding pocket of the virus which usually binds to α2-3 linkage sialosides abundant in the intestinal tract of birds in case of avian influenza viruses (AIV) while human-adapted viruses are specific for the α2-6 linkage mainly in the respiratory tract (Parrish and Kawaoka, 2005). A switch from the α2-3 linkage to the α2-6 linkage receptor specificity is a prerequisite for emergence of avian viruses with pandemic potential (Stevens et al., 2006).

All influenza A viruses have PCS of an arginine (R) residue adjacent to a conserved glycine (G) amino acid, the later becomes the N-terminus of HA2 protein (Garten and Klenk, 1983). Avian influenza of low pathogenicity phenotypes has monobasic amino acid, arginine or lysine (K) residues, in the cleavage site while the existence of multibasic amino acids with an R-X-K/R-R motif is a feature of high pathogenic subtypes (Klenk and Garten, 1994). Two different classes of proteases are responsible for cleavage-activation of influenza viruses, and the distribution of these proteases in the host appears to be the prime determinant of tropism and pathogenicity (Klenk and Garten, 1994; Steinhauer, 1999). The proteases that cleave non-pathogenic viruses are encountered in a limited number of cell or tissue types, so these viruses normally cause localized infections in, for example, the respiratory tract of mammals or the intestinal tract of wild birds. On the contrary, proteases that activate pathogenic influenza viruses are ubiquitously expressed, allowing for the systemic spread of the virus in infected hosts (Munch et al., 2001).

Five immunogenic epitopes (denoted A – E) of recent H5N1 hemagglutinin was mapped (Kaverin et al., 2007; Somvanshi et al., 2008; Duvvuri et al., 2009). The repertoire of immunocompetent antibody-producing cells is directed almost against the upper surface of the H5 HA molecule (Kaverin et al., 2007). Therefore, most of the positively selected sites were found to be within or adjacent to the immunogenic epitopes with a higher evolution rate which could help the virus to circumvent the host immune response (Lee et al., 2004; Duvvuri et al., 2009).

2.1.2. Subtypes

Based on the two surface proteins, HA and NA, there are 16 HA (H1 - H16) and 9 NA (N1 - N9) subtypes. An influenza virus must have one type of the HA and one type of the NA surface proteins resulting in 144 possible distinct HN combinations (Büchen-Osmond, 2003; Fouchier et al., 2005). All subtypes of influenza A virus are prevalent in wild and domesticated birds (Webster et al., 1992). Three HA subtypes (H1, H2 and H3) and two NA subtypes (N1 and N2) are usually infecting humans. However, and recently, human infections by the previously avian-restricted subtypes H5, H7 and H9 have been frequently reported (Perdue and Swayne, 2005). Likewise, swine and horses are infected with a much narrower range of AIV subtypes (Alexander, 2000).

2.1.3. Pathotypes

Avian influenza viruses are classified according to the pathogenicity for poultry into two main categories; low pathogenic strains (LPAIV) result in mild or asymptomatic infections and HPAIV causing up to 100% morbidity and mortality (Swayne, 2009). To date, only H5 or H7 subtypes fulfilled the defined criteria of high pathogenicity. Meanwhile, the existence of H5 and H7 viruses of low pathogenicity were also documented and these strains can potentially evolve into high path subtypes (Garcia et al., 1996; Senne et al., 1996; Perdue et al., 1997; Halvorson, 2002).

All H5 and H7 viruses have been listed as a “notifiable disease” by the OIE which mandates all member countries to report the OIE within 24 hours of confirming AIV infections (Pearson, 2003). Therefore, the OIE defined the HPAIV as: (1) viruses cause 75% mortality of 8 susceptible 4- to 8-week-old chickens within a 10 days observation period or (2) viruses have an intravenous pathogenicity index (IVPI) of greater than 1.2 upon inoculation of 10 susceptible 4- to 8-week-old chickens or (3) H5 or H7 AIV with PCS amino acid sequence similar to any of those that have been previously observed in HPAI viruses. Moreover, non-pathogenic H5 and H7 in chickens that do not posses PCS similar to any of those that have been observed in HPAI viruses are designated as notifiable LPAI viruses while other non-H5 or non-H7 AIV that are not virulent for chickens are identified as LPAI viruses (OIE, 2009).

In the European Union (EU), the Council directive 2005/94/EC defined HPAIV infection in poultry or other captive birds as: (1) infection with any influenza A virus of the subtypes H5 or H7; or with any influenza having an IVPI >1.2 in 6-week old chickens and/or (2) infection

with H5 or H7 AIV subtypes that have multiple basic amino acids at the PCS of the HA similar to that observed for HPAI viruses. AIV of subtypes that do not comply with the previously mentioned criteria were defined as LPAIV (EC, 2005).

2.1.4. Genetic and antigenic variation

Constant genetic and antigenic variation of AIV is an intriguing feature for continuous evolution of the virus in nature (Brown, 2000). Gradual antigenic variation via incremental acquisition of point mutations is defined as “antigenic drift” which is commonly regarded as the driving mechanism for influenza virus epidemics from one year to another. However, possible “antigenic shift” of influenza virus occurs by exchange genes from different subtypes of influenza “reassortment” leading to a complete alteration in the antigenic structure and emergence of new viruses with novel gene constellations (Brown, 2000). This unpredictable process is relatively infrequent, however it results in severe pandemics since the human population has no prior immunity to these de-novo surface proteins (Ferguson et al., 2003).

The H5N1 hemagglutinin gene has evolved into ten phylogenetically distinct clades (designated as clade 0 – 9) (WHO/OIE/FAO, 2009) as shown in figure (1). Two major phylogenetic clades are wide-spread: Clade 1 viruses in Cambodia, Thailand, and Vietnam and clade 2 viruses spread from China and Indonesia to the Europe, Middle East and Africa. To date, six distinct subclades of clade 2 have been identified (WHO/OIE/FAO, 2009). In a previous study, genome analysis of viruses collected from Europe, Northern Africa and the Middle East from late 2005 to 2006 in addition to Asian H5N1 revealed emergence of a new European-Middle Eastern-African (EMA) lineage which further was diversified into 3 distinct independently evolving clades, designated as EMA clade 1, EMA clade 2 and EMA clade 3. The early Egyptian strains in 2006 clustered within EMA clade 1 (Salzberg et al., 2007). In another study, African strains were classified into 3 sublineages denominated A – C, where the early Egyptian strains clustered within the sublineage B along with isolates from South-west Nigeria and Djibouti (Ducatez et al., 2007). Later on, H5N1 viruses isolated from Egypt, Israel, the Gaza Strip, Nigeria, and Europe in 2006 and 2007 were classified as clade 2.2.1, within this clade the Egyptian viruses further diversified to several subclades or groups (WHO/OIE/FAO, 2009). The most recent WHO classification allocated the Egyptian strains from human and backyard origin within the 2.2.1/C group, meanwhile viruses from vaccinated chickens belong to the 2.2.1/F group (WHO, 2011a).

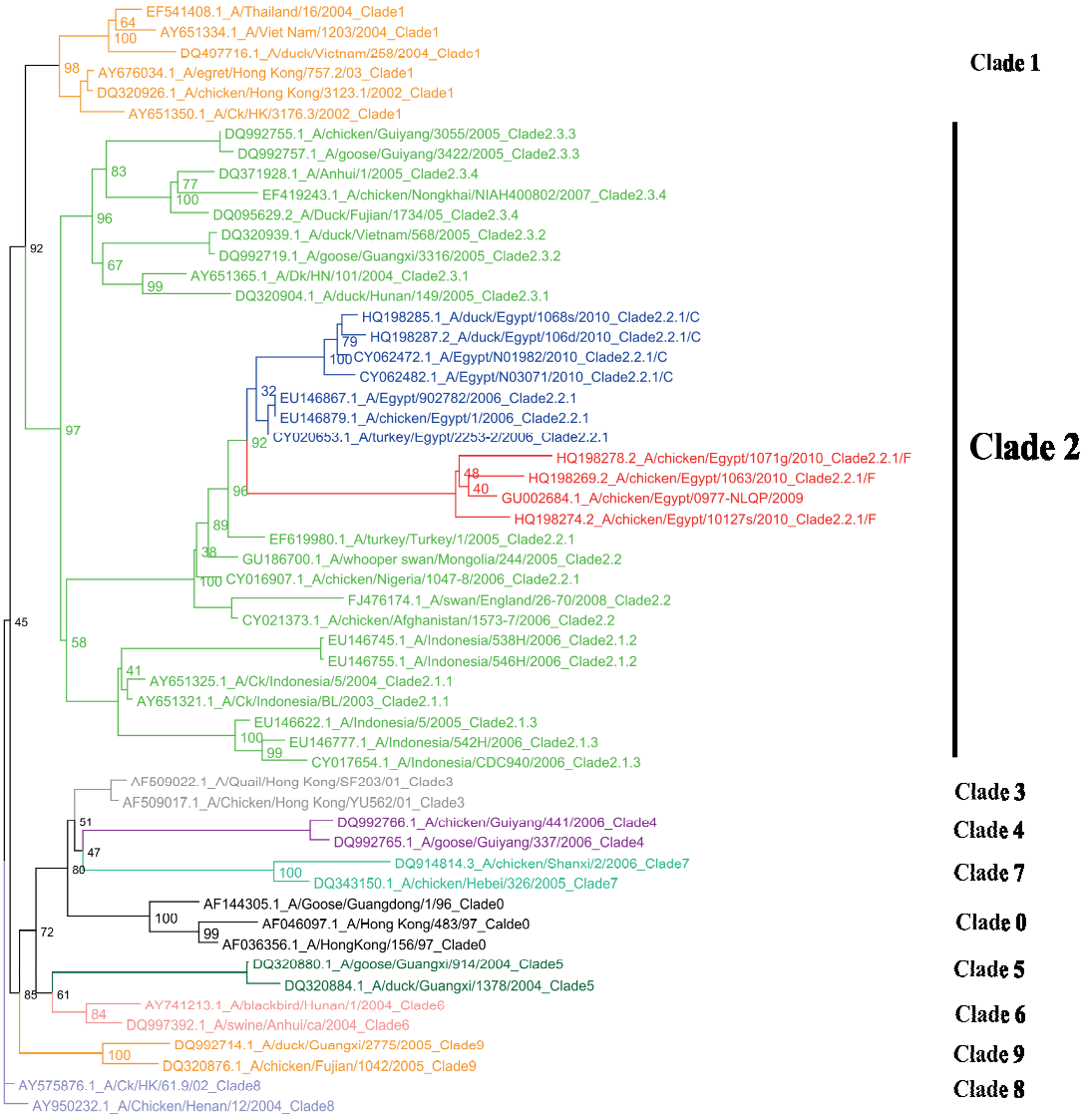

Fig. 1: Phylogenetic relatedness of H5N1 clades constructed by using Topali version 2 (Milne et al., 2008) and further edited by Dendroscope (Huson et al., 2007). Viruses were assigned to each clade after WHO/OIE/FAO (2008).

2.1.5. Global spread of AIV

Several studies have been conducted to determine the pathways of intercontinental or country-to-country spread of AIV based on the phylogenetic relatedness of isolated viruses, flyways of migratory birds, trade in poultry and wild birds and geospatial risk analysis (Kilpatrick et al., 2006; Lebarbenchon et al., 2010; Takekawa et al., 2010; Li et al., 2011; Williams et al., 2011; Gilbert et al., 2011). Migratory birds were claimed to be the main source for introduction (and re-introduction) of AIV into many countries in Asia, Europe, and Africa (Liu et al., 2005; Saad et al., 2007; Gall-Reculé et al., 2008; Starick et al. 2008; Fusaro et al., 2010; Prosser et al., 2011). Nevertheless, smuggling of infected wild birds and/or illegal trade of infected domesticated poultry have been frequently reported as a source of virus introduction (Van

Borm et al., 2005; Li et al., 2006; Lee et al., 2007; van den Berg, 2009; Fusaro et al., 2010). Moreover, introduction of H5N1 virus via legal trade routes through shipment of live chickens (Normile, 2005) or importation of duck meat (Tumpey et al. 2002; Mase et al. 2005) was also reported in several countries which highlights the need to re-assess the international trade regulations (Beato and Capua, 2011).

2.1.6. Diagnosis of avian influenza viruses

In the European Union, "EU", diagnosis of the primary infection of birds with HPAIV must be carried out in compliance with the "Council directive 2005/94/EC of 20 December 2005 on Community measures for the control of avian influenza and repealing Directive 92/40/EEC" (EC, 2005). The confirmation of AIV should be carried out with appropriate laboratory tests following the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2009). This includes samples collection, and in the primary outbreak in a given country virus isolation and identification and assessment of the pathogenicity.

2.1.6.1. Virus isolation

Embryonated chicken eggs (ECE) obtained from specific pathogen free (SPF) or specific antibody negative (SAN) fowls are the method of choice for isolation and propagation of AIV (Woolcock, 2008; OIE, 2009). Inoculation of 9 -11 days old ECE via the chorioallantoic sac has been used for decades as a superior route for growing of AIV. Occasionally, yolk sac or the chorioallantoic membrane routes might be useful in isolation of non-chicken originated AIV (Woolcock, 2008). Allantoic fluid collected from inoculated ECE which have haemagglutinating activity when mixed with chicken erythrocytes could indicate presence of an AIV; however other hemagglutinating viruses (e.g. paramyxoviruses) and contaminating bacteria should be ruled out (Woolcock, 2008; OIE, 2009). Typically, an HPAIV kills the embryo within 24-48 hours after inoculation of ECE but further passages are required to propagate viruses of low pathogenicity (Woolcock, 2008; OIE, 2009). High cost, availability, less specificity and sensitivity are the main disadvantages of ECE for AIV isolation (Suarez, 2008; Woolcock, 2008). On the other hand, cell cultures and cell lines were found to be as sensitive as egg inoculation in terms of virus isolation, titration, selection and pathogenicity. Madin-Darby canine kidney (MDCK), primary chicken embryo kidney (CEK), primary chicken embryo fibroblast (CEF) cell cultures, chicken bone marrow macrophage (HD11), chicken fibroblast (DF-1), mink lung epithelial (Mv1Lu) cells, quail fibroblast (QT-35) and baby hamster kidney (BHK-21) cell lines are efficient systems for growth of AIV. However, MDCK, CEK, and CEF were found useful and cost-effective to process a higher volume of samples (Moresco et al., 2010). In case of LPAI virus propagation in tissue cultures, trypsin must be added. Nevertheless, chicken kidney cells produce trypsin-like proteases which could allow replication of LPAI virus without prior addition of trypsin (Suarez, 2008). General speaking, virus isolation remains the only tool for providing a live virus for further investigation (Charlton et al., 2009). Yet, confirmation and subtyping of AIV after primary isolation is usually done by HI, agar gel immunodiffusion assay (AGID), commercial immunoassay kits or RT-PCR (Spackman et al., 2008; Woolcock, 2008; OIE, 2009).

2.1.6.2. Virus detection by RT-PCR

Several types of RT-PCR methods have been developed since the early 2000s for diagnosis and differentiation of AIV which are widely employed in surveillance, monitoring of outbreaks, and research activities. Among those methods, the RT-qPCR was described to be of high sensitivity, high specificity, rapid, cheap, quantitative and cost-effective method (Fouchier, et al., 2000; Suarez et al., 2007; Spackman and Suarez, 2008). A number of RT-qPCR assays for diagnosis and characterization of AIV have been published. These assays target the matrix gene (Spackman et al., 2002; Di Trani et al., 2006), the nucleoprotein gene (Muradrasoli et al., 2010), the neuraminidase or the hemagglutinin gene (Chen et al., 2007; Hoffman et al., 2001; Ng et al., 2006; Payungporn et al., 2006; Slomka et al., 2007; Li et al., 2008; Lu et al., 2008; Monne et al., 2008). Using specific primers and probes, amplification of a conserved region within the matrix gene among all AIV subtypes followed by or simultaneously with HA and NA subtype-specific RT-qPCR is the common used approach (Spackman et al., 2002).

2.1.6.3. Sequence

Identification of AIV genome sequence data is very important to develop novel influenza vaccines, therapies and diagnostics and increase our understanding for molecular evolution, virulence-associated genetic markers and host-pathogen interaction (Pasick, 2008; Spackman et al., 2008). Genome sequence of AIV has become relatively less expensive due to the recent advancement in the field of automated sequencing technology (Spackman et al., 2008). In contrast to the standard tests for assessment of AIV pathogenicity which is time-consuming, laborious and logistically complex, sequencing of the PCS motif of the HA for rapid assessment of the virulence potential of AIV could be generated easily within 24 hours and has been considered by the OIE as a criteria for notifiable HPAIV (Horimoto and Kawaoka, 1995; Senne et al., 1996; Pasick, 2008; Spackman et al., 2008; OIE, 2009). Furthermore, subtyping of AIV is achievable by direct sequencing of whole or partially amplified HA and NA gene segments (Spackman et al., 2008). In addition to rapid pathotyping and subtyping of the AIV, sequence analysis was applied successfully in molecular epidemiology to likely identify the possible source of infection, spectrum of susceptible species, ecological niche and geographic range (Guan et al., 2009; van den Berg, 2009; Shi et al., 2010).

2.1.6.4. Antibody detection

Serodiagnosis is an appropriate and broadly used tool for the detection and differentiation of anti-AIV elicited antibody during routine surveillance or monitoring of post-vaccination immune response in poultry. Enzyme-linked immunosorbent assay (ELISA) and haemagglutination inhibition (HI) are commonly used laboratory tests which have variable specificity and sensitivity. The ELISA is a rapid, semi-quantitative, semi-automated and sensitive tool in screening of AIV wherein large numbers of serum samples had to be examined (Snyder et al., 1985; Abraham et al., 1988; Davison et al., 1998; Naeem et al.,

2003, Jin et al., 2004; Al-Natour and Abo-Shehada, 2005). Mostly, anti-NP protein antibodies are the primary target of AIV-ELISA; however subtype-specific ELISA for detection of antibodies to different AIV subtypes (mostly against H5 and H7 subtypes) has been reported (Dlugolenski et al., 2010; Postel et al., 2011). Moreover, new approaches use ELISA targeting antibodies elicited against different viral genes; NS1, M2e or heterologous NA proteins were used to discriminate between naturally infected and vaccinated-only birds (DIVA) (Tumpey et al., 2005; Zhao et al., 2005; Lambrecht et al., 2007; Kwon et al., 2009; Moreno et al., 2009; Upadhyay et al., 2009; Liu et al., 2010; Avellaneda et al., 2010; Kim et al., 2010; Takeyama et al., 2011). Overall, generation of false positive results using ELISA is frequently seen, consequently confirmation of the obtained results is usually required (Avellaneda et al., 2010; Postel et al., 2011).

The HI is used for a subtype-specific serodiagnosis especially in monitoring of LPAIV H5 or H7 subtypes. However, HI is laborious, time-consuming and impractical for high-throughput screening in case of surveillance; in addition unspecific reactions could be obtained due to existence of other non specific proteins (Postel et al., 2011). Still, HI is the recommended test in the evaluation of post-vaccination immune response to assess the efficiency of a vaccination campaign or as indicator for protection (Brugh and Stone, 1986; Tian et al., 2005; Kumar et al., 2007; OIE, 2009; Swayne, 2009). However, antigenic diversity and emergence of drift variants within the same AIV subtype could limit such application. To overcome this limitation and for better evaluation of vaccination efficacy, antigens from circulating field viruses should be used (EFSA, 2008; Hafez et al., 2010; Grund et al., 2011; Kilany et al., 2011; Shany et al., 2011).

2.1.7. Immunity against HPAI H5N1 virus

Both humoral HA-specific antibodies as well as cellular immune response likely provide homo- and heterotypic immunity following infection and/or vaccination (Gao et al., 2006). Antibodies specific to the HA protein are the main determinant for protection against AIV infection in poultry, therefore vaccination for AIV is mainly based on the HA subtype. Quantification of anti-H5 antibody titers is commonly measured by the HI test (Suarez and Schultz-Cherry, 2000). Several studies correlated the level of HI titer of the same HA subtype with protection against HPAI H5N1 virus infection in poultry (Tian et al., 2005; Kumar et al., 2007).

On the other hand, neutralizing antibodies elicited against the NA protein in chickens could be protective against an HPAIV infection (McNulty et al., 1986), although less important than the HA (Sylte et al., 2007). Antibody immune response against other AIV proteins as well as mucosal and cell-mediated immunity play a less important role in protection of chicken against an active infection but could be a useful tool for the diagnosis and differentiation of different AIV subtypes (Suarez and Schultz-Cherry, 2000; Seo et al., 2002).

2.1.8. Vaccination against HPAI H5N1 virus

Although enforcement of biosecurity measures and an eradication strategy of an infected flock should be the basic line in any control against H5N1 virus infections (Capua and Marangon 2007); Vaccination as a "tailored synergy" has been implemented as a main tool to confront the disease in many of developing countries and to mitigate the impact of the unbearable pre-emptive culling of infected birds (van den Berg et al., 2008; Swayne, 2009).

Several types of H5 vaccines are available to protect birds against H5N1 virus infection. Conventional inactivated heterologous LPAIV (H5N2, H5N3, H5N9) or homologous whole HPAIV H5N1 virus after removal of the PCS by means of reverse genetics are commonly used vaccines in the field (Swayne, 2009; van den Berg et al., 2008). Furthermore, vaccines include recombinant viral vectors (e.g.: adenovirus, fowl poxvirus, Newcastle disease virus, baculovirus, turkey herpes virus and infectious laryngotracheitis virus) with an inserted AI H5 gene are a recently developed promising approach (Beard et al., 1991; Webster et al., 1991; Crawford et al., 1999; Swayne et al., 2000; Lüschow et al., 2001; Bublot et al., 2006; Gao et al., 2006; Veits et al., 2006; Toro et al., 2008; van den Berg et al., 2008; Soto, 2011). Prevention of the clinical signs, mortality, reduced shedding of the virus in the environment, increased the resistance of birds to an infection, decreased bird-to-bird transmission and limited decrease in the egg production are the main advantages of the AI vaccines (Capua and Marangon 2007; van den Berg et al., 2008; Swayne, 2009).

Yet, the virus is still able to infect vaccinated birds and subsequent silent spread usually occurs (Savill et al., 2006; van der Goot et al., 2007). It is worth pointing out that continuous circulation of AIV under immune pressure in vaccinated populations for extended period favour the antigenic drift of the field virus away from the vaccine strain as reported in the H5N2 epidemic in Mexico (Lee et al 2004) and the endemic H5N1 in China (Smith et al., 2006; Tian et al., 2010) as well as in Egypt (Peyre et al., 2009; Hafez et al., 2010; Kilany et al., 2011; Abdel-Moneim et al., 2011; Eladl et al., 2011).

Generally, the immunity induced by vaccination is of short duration and it is necessary to apply the vaccine several times during one rearing period. There are little or no data available about the frequency of vaccinations required to keep the breeder and layer flocks protected during the entire production period (Hafez, 2008). Furthermore, there are several factors which could affect the vaccine and vaccination against HPAI such as: subtype of the vaccinal strain, heterogeneity of the vaccine and circulating virus, potency of the vaccine, dose, antigen mass, adjuvant, surfactant, age of birds, species and the breed of birds (Wood et al., 1985; Stone, 1988; Beard et al., 1991; Tripathy and Schnitzlien, 1991; Swayne et al., 1999; Capua and Marangon 2000; Swayne et al., 2000; Qiao et al., 2003; Suarez, 2005; Lavoie et al., 2006; Gardin, 2007; Philippa et al., 2007). Inappropriate storage, handling and improper administration are further factors for vaccination failure. The quality of the vaccine application is crucial since all non injected chickens are not protected, and improperly injected chicks will be poorly protected. Using post-vaccination necropsy (residue of oil at the

site of injection) or serological testing demonstrated that it is not uncommon to see as much as 20 – 30% or even more of chickens that were not injected (Gardin, 2007).

Finally, continuous antigenic and genetic drift of AIV, differentiating vaccinated from field-exposed birds and inevitable circulation of the virus in vaccinated birds "silent infection" are considered major challenges of any AIV vaccine (Capua and Marangon, 2007). Therefore, vaccination alone is inadequate to eliminate H5N1 virus in endemic countries. Thus, it is essential to incorporate a sustainable awareness campaign and education programs about the virus and modes of transmission for veterinarians and para-veterinarians involved in the poultry production chain (Hafez, 2008).

2.2. Poultry industry in Egypt

Egypt is considered the largest poultry producer in the Arab world and roughly produces 23% of the total poultry production (Freiji, 2008). Since 1964, poultry production in Egypt has grown substantially with a growth rate of 301.2% in the 1990s worth L.E 17-18 billion (US$ 3 - 3.2 billion) (Hosny, 2006). Local poultry meat production was sufficient to satisfy the local consumption and up to 2 million birds were exported to the Arab countries annually (Taha, 2004). In 2005, it has been roughly estimated that the Egyptian national production was 240000 layer chickens producing around 3.5 – 5 billion eggs (Hosny, 2006). The size of the labour force involved in poultry production was about 1.5 million permanent workers and 1 million temporary workers, representing approximately 6% of Egypt's 23.7 million labour force and more than 15% of the agricultural work force (Hosny, 2006; El Nagar and Ibrahim, 2007). The structure of the poultry industry in Egypt consists of two main divisions: commercial enterprises and household poultry.

2.2.1. Commercial poultry

Commercial enterprises estimated in 2006 to cover 850 million birds and was estimated to be 1.444 billion birds by 2010 (Taha, 2004; Abdelwhab et al., 2009). The increase in the number of poultry houses and other associated establishments has occurred haphazardly and irrationally, without definite long term planning.

2.2.2. Backyard birds

Household poultry in Egypt is estimated to be 250 million birds kept by 8.1 million householders representing 4 – 5 million families out of the total Egyptian population of 82 million (Meleigy, 2007; Abdelwhab and Hafez, 2011). Few years ago, the government encouraged the house-poultry production sector by small loans and marketing facilities. Up to the end of the seventies, rural poultry production was an important source of Egypt's poultry meat and eggs. Although the majority of householders keep mainly ducks and chickens together, nevertheless rearing of geese, turkeys and pigeons in close contact with other

animals and humans in the same house is a common practice in Egypt (El Nagar and Ibrahim, 2007). Rural poultry production prior to the HPAI crisis was estimated to be approximately 10% of the market share of the meat production sector and 30% of the egg market. Backyard birds produce 22% of chicken meat, 64% of ducks, 34% of turkeys, and approximately all geese and pigeons (Taha, 2003). Flock size can range from 10-20 birds up to a few hundred (Hosny, 2006). Backyard birds are mostly reared in primitive cages, rooftops, or as scavengers with virtually no biosecurity. They are moving or grazing through streets, roads or fields. These birds are in close contact with either local feral birds and/or wild migratory birds (El Nagar and Ibrahim, 2007). The attitude of the backyard birds' householders hinders cooperation with vaccination committees. In some cases they are refusing the vaccine and hiding their birds without vaccination or they may vaccinate some birds and leave others without vaccination. Moreover, backyard waterfowl in Egypt are considered a potential reservoir of the virus and a mixing vessel for selection of variants to infect human (Abdelwhab et al., 2010b) or break through the immune system, and cause infection in vaccinated birds (Hafez et al., 2010; Kilany et al., 2010). But under village conditions it is not practical to separate the different species and such suggestion complicates the control efforts (Aly et al., 2008).

Based on implementation of the biosecurity measures, the Food and Agriculture Organization (FAO) classified the poultry production into 4 sectors. Hosny (2006) described the 4 sectors in Egypt and management related problems (Table 1). Sectors 1 and 2 include the integrated commercial companies, grandparent, parent and layer farms where biosecurity measures are usually enforced. Sector 3 includes non-regulated, non-registered small to medium-scale commercial activities while sector 4 contains backyard rural, in-house, and rooftop-raised poultry. In Egypt, household poultry production is in close contact with commercial farms of sectors 3 and 4, due to the presence of poultry in the same buildings with people, or by temporary workers in commercial farms keeping their own household birds at home and/or selling of unused feed, feeders and hoppers from commercial farms to the rural family poultry producers (Hosny, 2006; Aly et al., 2008).

Table (1) Classification of poultry production sectors in Egypt (Hosny, 2006)**.**

Sectors (FAO definition)	Poultry production systems			
	Industrial and integrated	Commercial		Village or backyard
		Biosecurity		
		High	Low	
	Sector 1	Sector 2	Sector 3	Sector 4
Biosecurity	High	Mod-High	Low	Low
Market outputs	Export and urban	Urban/rural	Live urban/rural	Rural/urban
Dependence on market for inputs	High	High	High	Low
Dependence on goods roads	High	High	High	Low
Location	Near capital and major cities	Near capital and major cities	Smaller towns and rural areas	Everywhere. Dominates in remote areas
Birds kept	Indoors	Indoors	Indoors/Part-time outdoors	Out most of the day
Shed	Closed	Closed	Closed/open	Open
Contact with other chickens	None	None	Yes	Yes
Contact with ducks	None	None	Yes	Yes
Contact with other domestic birds	None	None	Yes	Yes
Contact with wildlife	None	None	Yes	Yes
Veterinary service	Own Veterinarian	Pays for veterinary service	Pays for veterinary service	Irregular
Source of medicine and vaccine	Market or private Veterinarian	Market or private Veterinarian	Market	Government and market
Source of technical information	Company and associates	Sellers of input	Sellers of input	Government extension service
Source of finance	Banks and own	Banks and own	Banks and private	Private and banks
Breed of poultry	Commercial	Commercial	Commercial	Native
Food security of owner	High	Ok	Ok	From ok to bad

2.2.3. Marketing system

Due to insufficient capacity of slaughterhouses (current capacity less than 30% of poultry production), lack of marketing infrastructure and cultural preference for consumption of freshly slaughtered poultry, poultry meat trade in Egypt depends mainly on live bird markets (LBM). Two types of LBM in Egypt exist: retail shops and traditional LBM where minimal, if any, veterinary control or inspection toward food safety are implemented. Moreover, slaughtering, defeathering and evisceration of birds are usually conducted manually in the markets which increase the risk of human infections. Multiple bird species of several ages with variant ecological niches and from different localities are usually present inside one market and even within one shop. Therefore, surveillances of LBM indicated broad circulation of the virus in poultry populations nationwide. Changing the consumer preference from live birds to frozen meat will need great efforts and time. Under the new legislation, only licensed slaughterhouses with a resident veterinarian are allowed to handle live poultry. Vigorous control efforts to stop smuggling of live poultry, increase the capacity of slaughterhouses, tracing the source of existing birds in the markets and financial support for regular monitoring of LBM will remain a significant challenge to the control of H5N1 in Egypt (Abdelwhab et al., 2010a).

2.3. Situation of HPAIV H5N1 in Egypt

Egypt was the second African country, after Nigeria, to declare infection of poultry with HPAIV H5N1 on 16 February 2006 (Aly et al., 2008). More than 30 million birds were culled in the first wave of the outbreak in 2006 (Meleigy, 2007; Aly et al., 2008) and 52 human fatalities out of 150 infected persons have been reported until 6, July 2011 (WHO, 2011b). The firstly stated control strategy was based on: stamping out of infected birds, implementation of quarantine measures and restriction of movement. However, the disease was spread rapidly and widely all over the country within short period. Therefore, Egypt has updated a control strategy based mainly on mass vaccination, surveillances and depopulation of infected birds to combat the endemic disease (Abdelwhab and Hafez, 2011).

Vaccination of backyard birds using inactivated H5 vaccines was provided by the government free of charge while the commercial sector adopted their pertained vaccination practices with widely varying standards (Hafez et al., 2010). However, vaccination coverage was 1-50% and increase risk of human infection due to silent circulation of the virus in vaccinated backyard incited the government to cesses vaccination of birds in the backyard sector (Peyre et al., 2009; Abdelwhab and Hafez, 2011). On the contrary, several types of inactivated vaccines based on H5N1 and H5N2 strains are supplied by a number of vaccine manufacturers and are permanently applied in the commercial sector (Abdelwhab et al., 2009). Active, passive and targeted surveillances were established to elucidate the spread of H5N1 usually in poultry sectors and rarely in other feral birds or farm animals. Surveillance highlighted the continuous and extensive circulation of the virus in different poultry despite ongoing control efforts (Aly et al., 2008; Abdel-Moneim et al., 2010; Abdelwhab et al., 2010a; Arafa et al., 2010a; Hafez et al., 2010; Kilany et al., 2010). Almost, culling of infected birds, if done, is selective and the

post culling procedures are implemented slowly in suboptimal conditions which increase the chances of virus spread to nearby birds and human without actual control of the disease. More information on surveillance, diagnosis and control activities mobilized to confront H5N1 virus in Egypt and the major challenges hampering the containment of the disease has been reviewed in details by Abdelwhab and Hafez (2011).

2.4. Major challenges to control HPAI H5N1 in Egypt

2.4.1 Genetic drift of the virus

It is widely accepted that the highly error prone replication of influenza viruses and viral genome reassortment facilitate the fitness of the virus to be one step ahead of its host (Suarez, 2010). Increased evolutionary rate of AIV might be accelerated either by the immune pressure (due to prior immunization or natural infection) exerted on the replicating viruses in different hosts and/or jumping of the virus from one species to another (Yassine et al., 2010). In Egypt, both immune pressure exerted by the extensive vaccination and/or continuous interspecies and intraspecies transmission of the virus are driving factors for genetic and antigenic drift of H5N1 which constitutes a major challenge for control of the disease. Evolution of the HPAIV H5N1 in Egypt since 2006 generated two major diversified sublineages; the first sublineage contains all immune escape mutants from vaccinated birds and the second sublineage contains the recent human isolates from 2009 – 2010 and most of viruses detected in backyard birds (Arafa et al., 2010; Kilany et al., 2011; Abdelwhab et al., 2010b).

In general, it was concluded that significant mutations in the virus hemagglutinin were eventually established (1) in the immunogenic epitopes corresponding sites (Figure 1 and Table 2) permitting the field variants to evade the immune response of vaccinated birds and in turn decrease the efficacy of the currently used vaccines. (2) Several mutations were fixed in the RT-qPCR primer specific sites of the H5 gene (Slomka et al., 2007) and to a lesser extent in the conserved M gene (Spackman et al., 2002) resulting in false negatives. In addition, alteration, even a deletion, in the receptor binding domain which could facilitate inter and intraspecies transmission, emergence of less virulent virus in humans and affecting the sensitivity of serological tests was observed. (4) Last but not least, several synonymous and non synonymous mutations were recorded in the proteolytic cleavage site of the H5 gene without adverse effect on the pathogenicity (Abdel-Moneim et al., 2009; Abdelwhab et al., 2010b; Arafa et al., 2010a; Balish et al., 2010; Kilany et al., 2010; Cattoli et al., 2011; Watanabe et al., 2011). Oseltamivir (Tamiflu®) resistant marker (N294S) in the viral neuraminidase was reported in two viruses isolated from a suspected family cluster in 2007 in Egypt (Earhart et al., 2009) and amantadine resistant markers in the M2 gene were also observed in two chicken isolates (Kayali et al., 2011). However, these mutations were not fixed and are rarely seen.

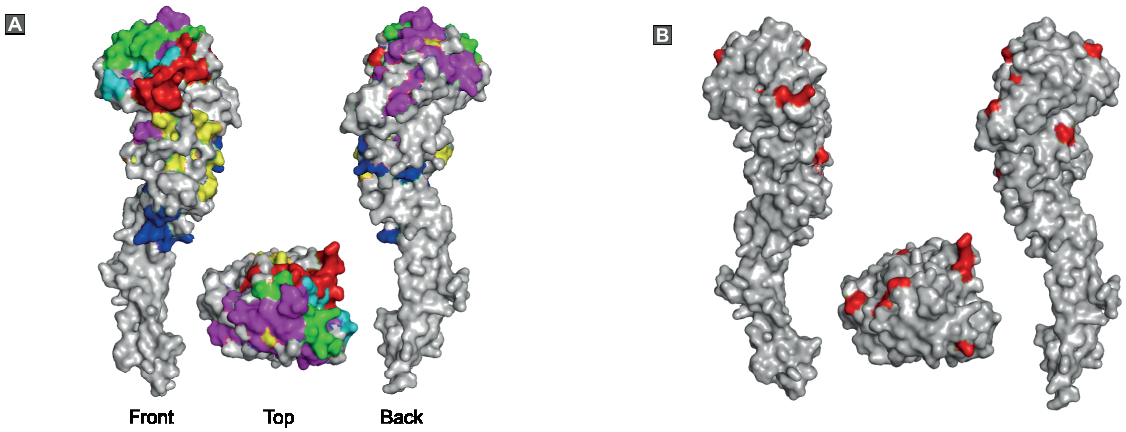

Fig. 2: Location of the amino acid differences in the Egyptian H5N1 variant virus used in this study on the tertiary structure of the HA molecule of the index virus in Egypt.
A. tertiary structure graph of the Egyptian index virus A/chicken/Egypt/06207-NLQP/2006 shows the monomeric H5 molecule with immunogenic epitopes ([Duvvuri et al., 2009]: A – red, B – green, C – blue, D – magenta, E – yellow) and receptor binding domain (cyan) were shown. **B.** Views of the unique peculiar mutations (see table 2) in the HA molecule (red) found in the Egyptian 2.2.1 variant but not in 2.2.1 classic and/or vaccine strains (adapted from Kilany et al., 2011).

Table (2). Amino acid changes in the hemagglutinin of the recent HPAIV H5N1 Egyptian strains in relation to the virus originally introduced, compared with the vaccine strains commonly used in poultry in Egypt (Kilany et al., 2011).

Amino acid position*	Virus strains				Epitope[5]	Antige-nic sites[6]	N-glyco-silation[7]	RBD[8]
	EGYext /H5N1[1]	EGYvar /H5N1[2]	Re1 /H5N1 [3]	Potsdam /H5N2[4]				
45	D	D	N	N	e			
74	P	**S****	P	P	e			
83	I	I	A	D	E			
94	N	N	D	D	e			
97	D	**N**	D	D				
108	I	I	T	T	A			
110	H	**R**	H	H	A			
123	S	**P**	S	S	a			
124	D	D	N	N	A			
126	E	E	D	D	A			
138	Q	Q	H	N		+		
140	R	**G**	R	R	B	+		
141	S	**P**	S	S	B	+		
144	F	**Y**	F	F	B			
154	D	D	N	N	D		+	
162	R	**K**	R	R	d			
165	N	**H**	N	N			+	
184	A	**E**	A	A	d			
189	R	R	K	K		+		+
212	K	K	E	E	D			
217	S	S	P	P	d			
226	M	**V**	M	M	D			
235	S	S	P	P				
252	N	N	Y	Y				
263	T	T	A	A	C			
282	I	I	M	M	C			
320	S	S	T	V				
323	G	G	R	R				

* H5 numbering $D_1Q_2I_{3...}$

**Bold case letters in EGYvar/H5N1 revealed to the unique peculiar mutation found in this group of variant viruses but not in extinct and/or vaccine strains.

1 – A/chicken/Egypt/06207-NLQP/2006 (EU372943)

2- EGYvar/H5N1 = A/chicken/Egypt/086Q-NLQP/2008(ACA29681.1)

3- Re1/H5N1 = A/Goose/Guangdong/1/96(YP_308669.1)

4- Potsdam/H5N2= A/duck/Potsdam/1402-6/1986(ABI84497.1)

3 – A/chicken/Egypt/NLQP-0879/2008 (ACR56243.1); EGYvar/H5N1

5 - 8 – Designation according to Duvvuri et al., (2009); RBD – receptor binding domain
Capital and minor case letters means the mutations occurred in or adjacent to the immunogenic epitopes, respectively according to Duvvuri et al., (2009)

2.4.2 Improvement of diagnostics

Increased specificity and sensitivity of diagnostics is of great importance. A number of mismatches in the current circulating viruses and the primers and probes oligonucleotides specific for H5 (Slomka et al., 2007) and M gene (Spackman et al., 2002) segments were fixed in the recent Egyptian H5N1 viruses (Arafa et al., 2010b). This resulted in false negative results when examined by the corresponding RT-qPCR. The update of RT-qPCR primers is critical in diagnosis of HPAIV H5N1 in Egypt (see publications 1 and 2 in this dissertation).

Antigens used in HI test for routine monitoring of the post vaccination immune response are usually supplied by the company producing the vaccines. However, recent studies showed that serum antibodies produced by these vaccines didn't react with HI antigens prepared from the field strains (Hafez et al., 2010; Grund et al., 2011; Shany et al., 2011). Likewise, serum obtained from birds vaccinated with inactivated vaccines prepared from the Egyptian variant field strains didn't cross-react with H5N2 antigens of commercial vaccines currently applied in poultry in Egypt (Kilany et al., 2010; Grund et al., 2011). Therefore, the use of antigens prepared from circulating field strain in a geographical area for serological evaluation of the current H5 vaccines against possible infection with field strains has been proposed instead of the homologous antigens of the commercial vaccines. Furthermore, monoclonal antibodies based ELISA from Asian H5N1 virus as well as rapid chromatographic strips failed to detect the new Egyptian variants but not the parent virus isolated in 2006 in Egypt (Soliman et al., 2010; Postel et al., 2011).

2.4.3 Vaccines update

Approximately 1.3 billion doses of different H5 vaccines, of which none contained Egyptian field strain, were used until January 2009 (Hafez et al., 2010). The homology of the H5 gene of currently used H5N2 based vaccines and H5N1 reverse genetic modified vaccines share 78% and 94%, respectively, with the currently circulating viruses (Abdelwhab et al., 2009). Insufficient efficacy of these vaccines in protecting chickens and turkeys after experimental infection with the newly emerging variant HPAIV H5N1 in Egypt has been recently demonstrated (Grund et al., 2011; Kilany et al., 2011; Rauw et al., 2011). On the contrary, optimum protection under experimental conditions was achieved by several inactivated tissue culture and/or oil adjuvanted vaccines prepared from the Egyptian field variant strains (Bahgat et al., 2009; Grund et al., 2011; Kilany et al., 2011). Novel vaccines generated by reverse genetics from the current Egyptian immune escape mutants are being considered for licensing. However, regular updates of the vaccinal strains in the face of antigenic drift of the

H5N1 virus is needed annually or every two years to optimize the efficacy of these vaccines against the newly emerging variants (Suarez, 2010).

2.4.4 Maternal immunity

Vaccination of broiler breeders in Egypt immediately after emergence of the disease in early 2006 was rather effective in protection against the early HPAI H5N1 infections as shown by lower number of infected flocks nationwide (Arafa et al., 2008). However, and due to mass vaccination of broiler breeders, existence of maternal antibodies in one day old chicks was inevitable. Field observations in late 2006 and early 2007 revealed 295 out of the 355 (83%) examined broiler flocks possessed maternal immunity (MI), those birds evoked low or no antibody titers after vaccination at 1st and /or 7th day of age (Arafa et al., 2008) and the number of vaccinated infected flocks has been increased. Therefore, vaccination of broilers, in general or at the first days of the newly hatched broiler chicks when the level of maternal antibody is considerable, against HPAIV H5N1 in Egypt was claimed as a possible cause of vaccination failure in chickens in Egypt (Hafez et al., 2010; Kim et al., 2010). Unlike most of other important viral diseases of poultry, the influence of maternal immunity on protection against H5N1 virus and the effect on the potency of vaccination of one day old chicks were inadequately studied. Recently, only 17% protection of 10 days old broilers possessed H5N2 derived MI against Eurasian HPAIV H5N1 infection and interference with early vaccination were obtained (De Vriese et al., 2010) (see publications 3 and 4 in this dissertation).

3. Summary of the results of the dissertation

3.1. Diagnostic update

Publication 1: Abdelwhab EM, Arafa A, Erfan AM, MM Aly, Hafez HM (2010) Modified H5 real-time RT-PCR oligonucleotides for detection of divergent avian influenza H5N1 viruses in Egypt. Avian Diseases 54:1301-1305.

Rapid detection of HPAIV H5N1 in poultry is pivotal for prompt control of the virus and limits its spread to other species, including human. In Egypt, detection of the virus in tracheal and cloacal swab samples obtained from surveyed birds depends mainly on the OIE recommended RT-qPCR assay which targets the H5 gene of AIV. In the first wave of the H5N1 epidemic in Egypt, results obtained by this assay were, so far, in line with clinical observation of suspected flocks. However, it is well known that vaccinated birds excrete lower amount of virus than non vaccinated birds. Moreover, since late 2007, negative RT-qPCR results have been obtained by examination of flocks suffering from signs commonly seen in HPAIV infections. In this study we analyzed 316 H5 gene sequences of Egyptian H5N1 viruses available in the GenBank data base until March, 2010. The alignment indicated divergence of the Egyptian H5N1 virus and several nucleotide substitutions in the primers and probe target the H5 gene. A modified assay by insertion of degenerate oligonucleotides to the primers/probe sets has been validated to match all Egyptian viruses analysed in this study (Table 1 in publication 1).

Validation of the modified assay, in comparison to the generic H5 and matrix genes detection assays, was done by examination of ten H5N1 isolates representing different Egyptian virus groups as well as examination of 433 field cases (chickens, ducks, and geese) representing commercial and backyard sectors. Specificity of the newly modified primers was tested against RNA and DNA from several viruses, bacteria and Mycoplasma spp.

The modified RT-qPCR has had higher sensitivity and efficiency than the original one in detection of Egyptian isolates. Thus, 61 field cases were positive in our assay, while 51 samples tested positive by the generic H5 assay. The modified H5 assay was also more sensitive than the generic matrix gene assay. Meanwhile, 372 field cases were negative by both H5 assays. Both assays, the generic and modified, have had detection limit of 10 EID_{50}. Influenza viruses of H1N1, H3N2, H6N2, H7N3, H13N8 and H9N2 subtypes, and other non-influenza viruses and bacteria were tested negative by both assays.

In this study, modification of the recommended H5 RT-qPCR assay was found to be more sensitive and efficient for detection of the divergent HPAIV H5N1 in Egypt. Nevertheless, evolution of new mutations due to uninterrupted circulation of the virus in poultry in endemic countries requires annual update of the current diagnostics for rapid detection of the lethal H5N1 virus in poultry sectors and reduces the risk of lateral spread of the virus.

3.2. Development of new diagnostic

Publication 2: Abdelwhab EM, Erfan AM, Grund C, Ziller M, Arafa AS, Beer M, Aly MM, Hafez HM, Harder TC (2010) Simultaneous detection and differentiation by multiplex real time RT-PCR of highly pathogenic avian influenza subtype H5N1 classic (clade 2.2.1 proper) and escape mutant (clade 2.2.1 variant) lineages in Egypt. Virol J 7:260.

Efficient control of Egyptian H5N1 virus in poultry necessitates rapid, sensitive and specific diagnostic tools. One of the contemporary challenges to control the disease in Egypt is the accelerated evolution rate of the virus due to immune pressure exerted by the vaccine and/or continuous interspecies and intraspecies transmission. Two major distinct H5N1 lineages co-circulate in poultry in Egypt; classic 2.2.1 (also referred to as 2.2.1 proper) isolated mainly from backyard birds and recent human infections and the variant 2.2.1 sublineage perpetuated mainly in vaccinated commercial poultry farms.

In this study, we developed sensitive multiplex RT-qPCR for detection and typing of the Egyptian H5N1 major sublineages. An 106 bp conserved fragment in the HA1 gene region was used for detection of both subtypes, meanwhile a variable 85 bp fragment in the HA2 gene region was selected for discrimination of the two Egyptian sublineages. To further increase the specificity of certain probes, locked nucleotide chemistry was added (Table 1 Publication 2). Analytical characterization was carried out using 50 Egyptian HPAIV H5N1 strains, 45 other avian influenza viruses, examination of 63 swab samples collected from experimentally infected chickens and 50 AIV-positive field samples obtained from different host species in Egypt.

The developed assay had a detection limit of approximately 2 – 5 RNA copies per reaction. All 50 isolates were detected by the multiplex RT-qPCR; meanwhile 40 isolates only were detected by the generic H5 RT-qPCR assay (Table S1). High discriminatory power was obtained using the developed RT-qPCR, hence 33 out of 50 examined Egyptian H5N1 isolates were assigned to the classic (proper) subclade 2.2.1 and 15 isolates belonged to variant 2.2.1 subclade similar to phylogenetic analysis of HA gene sequences (Table S1). Nevertheless, two isolates were positive for both sublineages which could be due to mixed infections. All swab samples collected from commercial farms (n=11) and backyard poultry holdings (n=39) in Egypt were positive by the multiplex assay while 45 samples only were tested positive in the generic H5 RT-qPCR. Likewise, sublineage specific assays were significantly more sensitive than the generic H5 RT-qPCR to detect swab samples (n=63) collected from experimentally infected birds with A/chicken/Egypt/0879-NLQP/2008 (variant 2.2.1 sublineage) or A/chicken/Egypt/NLQP-0918Q/2009 (classic 2.2 sublineage) 2 or 7 days post infection (Table S3 Publication 2). Moreover, several non-Egyptian H5 viruses could also be detected by the multiplex RT-qPCR with equal or slightly less sensitivity compared to the standard H5 recommended protocol by the OIE. In addition, the assay was specific for detection of H5 viruses only; hence no positive signals were obtained from non-H5 AIV as well as other avian viral or bacterial pathogens (Table S2 Publication 2).

The new multiplex RT-qPCR assay could be useful for rapid high-throughput monitoring for the presence of HPAIV H5N1 in commercial poultry in Egypt. It may also aid in prospective epidemiological studies to further delineate and better control spread of HPAIV H5N1 in Egypt.

3.3. Protection

Publication 3: Abdelwhab EM, Grund C, Aly MM, Beer M, Harder TC, Hafez HM (2011) Multiple dose vaccination with heterologous H5N2 vaccine: Immunresponse and protection against variant clade 2.2.1 highly pathogenic avian influenza H5N1 in broiler breeder chickens. Vaccine 29:6219-6225.

Since late 2007, the incidence rate of HPAIV H5N1 infection of vaccinated poultry flocks has dramatically increased. Antigenic and genetic characterization of the isolated viruses from those vaccinated poultry flocks revealed two different co-circulating sublineages, denoted as classic 2.2.1 (EGYcls/H5N1) and variant 2.2.1 (EGYvar/H5N1) subclades. The classic 2.2.1 subclade had limited amino acid diversity in comparison to the parent H5N1 introduced into Egypt in early 2006. On the contrary, variant 2.2.1 viruses had major genetic and antigenic drift from the H5 vaccine strains and the parent H5N1 virus as well. Protection of broiler breeder chickens vaccinated with multiple doses of the available commercial H5N2 inactivated vaccines in Egypt is unclear.

In this work, 100 broiler breeder chickens received intramuscular Potsdam/H5N2 inactivated vaccine at 6, 12 and 18 weeks of age and 100 broiler breeders were kept without vaccination as control. The randomly collected serum samples from vaccinated birds at 6, 10, 14, 18, 26

and 50 weeks of age were examined using the HI test against different HA antigens. At 50 weeks of age, 25 vaccinated and 25 non-vaccinated breeders were allocated in 3 groups each. One group from vaccinated and one from non-vaccinated breeders were challenged with EGYcls/H5N1 (n=8) or EGYvar/H5N1 (n=8) or Italy/H5N2 (n=9) viruses. All birds were kept under close observation for 10 days. Combined tracheal and cloacal swabs were collected from live birds at 2, 4, 7 and 10 days post infection (dpi) and examined by RT-qPCR for quantification of the excreted virus. Laid eggs (n=40) were collected from infected birds and virus detection was carried out by RT-qPCR from eggshell, albumin and yolk materials; positive samples were confirmed by virus isolation.

Immune response of the breeders was high against the homologous Italy/H5N2 antigen (HI mean titer ≥7.4), meanwhile the HI titer was significantly lower after the first immunization using EGYcls/H5N1 HA antigen (HI mean titer ≥4.2) but increased after booster vaccinations (HI mean titer ≥6.2). On the contrary, no cross-reaction was induced against the antigenically distinct EGYvar/H5N1 virus. All control birds died within 3 dpi and showed cyanotic comb and wattle, facial edema and respiratory distress. Only one bird died from the vaccinated group infected with EGYcls/H5N1 virus, in contrast, 6/8 (75%) and 5/9 (56%) died after infection with EGYvar/H5N1 and Italy/H5N2 viruses, respectively. Vaccinated birds excreted significantly lower virus than non-vaccinated groups. Interestingly, infected vaccinated breeders laid eggs almost throughout the observation period and the virus and/or the viral RNA was detected on the external eggshell and internal content as well. Most of the infected eggs were laid by EGYcls/H5N1 infected breeders.

Findings of this experiment proved that multiple dose vaccination of broiler breeder chickens using a commercial H5N2 inactivated vaccine broadened the antigen profile of induced antibodies, protected chickens against the classic Egyptian strain but not against the newly emerging variant viruses. Thus, there is an urgent need to update the currently used H5N2 vaccines in Egypt. Furthermore, contaminated or infected eggs insight the potential risk of silent spread of HPAIV H5N1 and control measure should be implemented.

3.4. Maternal immunity

Publication 4: Abdelwhab EM, Grund C, Aly MM, Beer M, Harder TC, Hafez HM (2011) Influence of maternal immunity on vaccine efficacy and susceptibility of one day old chicks against Egyptian highly pathogenic avian influenza H5N1. Veterinary Microbiology doi:10.1016/j.vetmic.2011.08.004 (in press).

It is well-stated in the immunology of avian pathogens that passive antibodies transferred from vaccinated breeders to their offspring via yolk sac materials during the gestation period continued to be absorbed from the yolk sac into the serum for 2 – 3 days post-hatching (Brandly et al., 1946; Higgins, 1971). Thereafter, the level of maternal antibodies declined at a steady rate with catabolism of protein and dilution in the increasing volume of body fluid in the growing chicks. Therefore, MI usually protects the new hatch against the lethal effect of

the virulent viruses in the first few days of life; however, MI interferes with vaccination of newly hatched chicks resulting in decreased duration of vaccine-induced immunity for e.g. as shown after Newcastle vaccination using live vaccines (Allan, 1973; Kim et al., 1978; Rahman et al., 2002).

Due to the blanket vaccination policy targeting all poultry sectors including broiler breeder chickens in Egypt, approximately all one-day old chicks possess maternally derived immunity (MI). Field observations as well as preliminary experimental studies claimed that MI might be incriminated as a cause of vaccination failure in broiler flocks. Here, the influence of H5N2 vaccine derived MI in one-day old chicks was investigated as a cause of vaccine failure in broiler chickens in Egypt. One-day old chicks were derived from broiler breeder chickens vaccinated with the commercially available inactivated Potsdam/H5N2 vaccine (Publication 3). Three separate experiments were conducted to investigate the impact of MI on protection of newly hatched chicks against H5 AIV challenge and possible interference with vaccination at the early days of life. In experiment 1, chicks with (MI+) or without (MI-) maternal immunity were infected at 3 days or 10 days of age with either homologous Italy/H5N2 or EGYvar/H5N1 viruses. In experiment 2, MI+ and MI- chicks were vaccinated once with prepared EGYvar/H5N1 inactivated vaccine at 3 or 14 days, then infected with EGYvar/H5N1 virus at 35 days of age. In experiment 3, MI+ and MI- chicks received a single shot of homologous Potsdam/H5N2 commercial vaccine at 3 days of age and were infected with EGYcls/H5N1 virus at 35 days of age. Clinical observation, virus shedding and serological investigations were recorded for 10 days post infection.

As found in experiment 1, MI protected the 10 days old offspring against infection using the antigenically related HPAIV strain Italy/H5N2; however virus replication was not fully suppressed. On the contrary, all chicks infected with the Egyptian HPAIV EGYvar/H5N1 died few days post-infection. In experiment 2, all EGYvar/H5N1 vaccinated chicks survived the infection with EGYvar/H5N1 virus regardless of the maternal immune status and/or age of vaccination. Whereas Potsdam/H5N2 vaccinated MI+ birds in experiment 3 were not protected against EGYcls/H5N1 infection and partial protection was afforded in MI- chicks. Taken together, the results showed that maternal antibodies didn't protect MI+ chicks against infection with the antigenically variant EGYvar/H5N1 virus and so didn't interfere with the heterologous EGYvar/H5N1 vaccine. In contrast, MI interfered with vaccination of one day old chicks with the homologous Potsdam/H5N2 vaccine strain. Single-dose vaccination of broilers at 3 or 14 days of age with EGYvar/H5N1 vaccine was protective against infection with the variant EGYvar/H5N1 virus however; inadequate protection was afforded by single-dose vaccination with Potsdam/H5N2 vaccine after infection with the EGYcls/H5N1.

4. General discussion and conclusion

4.1. Introduction

Fatal outcome in 6 out of 18 cases of the first spill-over infection of an HPAIV H5N1 of avian origin directly from infected poultry to humans in 1997 in Hong Kong marked the starting

point of an unprecedented spread of HPAI across three continents involving more than 63 countries (Sims et al., 2003; Peiris et al., 2007; Webster et al., 2007; Garcia, 2011). Along the temporal and geographical lines of expansion, HPAIV H5N1 revealed remarkable genetic flexibility resulting, through reassortment, in numerous genotypes of which few persisted until today (Peiris et al., 2007; Gao et al., 2009). The HA protein, however, has been subject to significant antigenic drift shaping deeply fissured phylogenetic patterns which to date comprises at least ten clades, some of them with several discernable subclades (Smith et al., 2006; Salzberg et al., 2007). Likewise unprecedented is the expanded host spectrum of this virus which includes, besides numerous genera of poultry and wild bird species, also mammals and humans (Smith et al., 2006). Despite intense global efforts to limit the spread of HPAIV H5N1 and to eradicate the virus where it surfaced, the infection established endemic status in poultry populations in several regions including Indonesia and Egypt.

Incursion of the HPAI H5N1 virus of clade 2.2 into poultry in Egypt in mid February 2006 (Aly et al., 2008), probably by infected wild birds (Saad et al., 2007), resulted in nationwide outbreaks causing more than 1 billion US$ losses for the Egyptian poultry industry. It directly affected 1.5 million people whose livelihood depended mainly on poultry (Meleigy, 2007). Recently, there are, so far, two distinct sublineages in Egypt: 2.2.1 variant viruses isolated from immunized birds and 2.2.1 classic or proper isolated mainly from backyard birds and human (Abdelwhab et al., 2010; WHO, 2011b).

4.2.Detection of HPAIV H5N1 in Egypt

The statutory control measures in Egypt depend mainly on the rapid and accurate detection of the virus by RT-qPCR targeting the HA gene as a starting point for a further control cascade (Aly et al., 2008; Arafa et al., 2010b). However, by late 2007, escape of variant strains from detection by RT-qPCR in samples collected from poultry flocks have had signs and lesions commonly seen in HPAI H5N1 infected birds were observed (Arafa et al., 2010b). Sequence analysis of these variants revealed multiple single nucleotide substitutions in the primers and probe target the H5 gene by real-time RT-PCR (Arafa et al., 2010b). Therefore, optimization of such a fundamental technique in the face of the continuing viral evolution, which is even further accelerated and skewed by vaccination pressure, remains a daily challenge for diagnostic measures which are at the root of all efforts to control the situation (Abdelwhab and Hafez, 2011). In the first publication (chapter 2) after alignment of 316 HA gene sequences available from the Egyptian H5N1 virus from 2006 to March 2010 we identified the point mutations in the primer and probes of the used RT-qPCR which are recommended by the OIE (Slomka et al., 2007). The modified RT-qPCR assay was more sensitive than the original one in detection of Egyptian isolates with 104% amplification efficiency. Sixty-one field samples were found positive in our assay, but only 51 samples tested positive by the original H5 protocol and more sensitive than the matrix gene RT-qPCR detection assay. A detection limit of 10 EID50 with the updated oligonucleotides primers and probe set was found which was similar to the generic assays.

In the second publication (chapter 3) we developed a versatile, sensitive and lineage-specific multiplex RT-qPCR for detection and typing of H5N1 classic as well as variant strains in Egypt. The developed assay was shown to have a detection limit of 2 – 5 cRNA copies per reaction. Based on a phylogenetic analysis of 50 isolates tested (Arafa et al., 2010a; Balish et al., 2010), the assay is fully specific with regard to assigning the Egyptian H5N1 isolates to either phylogenetic cluster. No unspecific reactivity with either non-H5 AIV or other avian pathogens was evident. However, the assay cannot be used for generic detection of subtype H5 viruses as a large percentage of non-Egyptian H5 subtype strains could not be detected. Field application was conducted using oropharyngeal or cloacal swabs collected from commercial farms (n = 11) and backyard poultry holdings (n = 39) in Egypt. A total of 50 field samples pretested by M-specific RT-qPCR (Spackman et al., 2002) to be positive for AIV was examined using the H5 multiplex RT-qPCR assay and the generic H5 protocol (Slomka et al., 2007). The multiplex RT-qPCR found all 50 samples positive for subtype H5. Among them, 32 were assigned to clade 2.2.1 classic and 15 to lineage 2.2.1 variant while three field samples were positive for both, which might indicate mixed infection by both lineages. Only 45 samples tested positive in the generic H5 RT-qPCR using the original protocol by Slomka et al. (2007), and on average the Ct values produced with the newly developed multiplex assay were significantly lower ($p << 0,001$) than those obtained in both the M and the H5 generic RT-qPCRs. A detection limit of 400 cRNA copies per ml sample matrix was found. Higher diagnostic sensitivity of the multiplex assay in comparison to other generic H5 or M-gene based RT-qPCR assays were found by examination of 63 swab samples from experimentally infected chickens by the Egyptian H5N1 clade 2.2.1 variant and the classic viruses.

4.3. Protection of birds against Egyptian HPAI H5N1 viruses

Vaccination against HPAIV H5N1 has been stated in developing countries as an alternative tool for the pre-emptive culling of infected birds to decrease the socioeconomic impact of the disease (Capua and Marangon, 2007; Van den Berg, 2009). Earlier studies have shown that heterologous and distantly related vaccines protected birds efficiently against infections with AI viruses isolated from several decades (Swayne et al., 2000). This was merely true because of the short life span of birds, infrequent vaccination and rapid control of emerging AI outbreaks in developed countries limited the tendency of the virus for serious antigenic drift (Pfeiffer et al., 2010). However, and recently, endemicity of HPAI H5N1 virus in developing countries and adoption of the vaccine to confront the disease in poultry enhanced the antigenic drift of the circulating virus from the vaccine strains to mimic the situation of seasonal influenza in humans (Boni, 2008). Therefore, annually or biennially reappraisal of the protectiveness and efficiency of the vaccines in the face of the newly emerging variants has been recently recommended (Lee et al., 2004; Pfeiffer et al., 2010).

Continuous circulation of HPAI H5N1 virus in poultry flocks in Egypt with a history of multiple and variable vaccination regime was reported (Arafa et al., 2010a; Hafez et al., 2010;

Kilany et al., 2010). After 18 months of the blanket vaccination policy in Egypt, there was a debate whether the current vaccines can protect birds against the circulating field strains, particularly variant viruses isolated from vaccinated birds and characterized by extensive alteration in the antigenic epitopes of their hemagglutinin (Pyres et al., 2009; Arafa et al., 2010a; Hafez et al., 2010; Kilany et al., 2010). In the third (chapters 4) and fourth (chapters 5) publications we studied the protection of vaccinated birds against two antigenically distinct HPAI H5N1 viruses co-circulating in Egypt in comparison to a European HPAI A/chicken/Italy/8/98 (H5N2) virus which is phylogenetically and antigenically closer to the used H5N2 vaccine strain. In the third publication (chapters 4) we showed that multiple dose vaccination of broiler breeder chickens at 6, 12 and 18 weeks age with a commonly used H5N2 vaccine broadened the immune response but did not induce protection against challenge with an Egyptian highly pathogenic avian influenza H5N1 virus of clade 2.2.1 variant (mortality rate 6/8). Significant clinical protection, in comparison to the non-vaccinated control, was surprisingly observed only against the classic Egyptian strain2.2.1 but still 1 out of 8 chickens died and less protection was found against the European strain Italy/H5N2 (mortality rate 5/9). Virus shedding was reduced in all groups compared to non-vaccinated controls. Nevertheless, extensive virus shedding through oropharyngeal and cloacal swabs was observed in all vaccinated birds from 2 to 10 days post infection (in the surviving birds).

In chapter 5, broilers vaccinated with the H5N2 vaccine at 3 days old were not protected against classic 2.2.1 HPAI H5N1 virus infection at 35 days age (mortality 4/7). On the contrary, broilers vaccinated at 3 or 14 days age with an inactivated vaccine prepared from the variant Egyptian H5N1 virus survived the challenge with the same variant virus at 35 days age and the virus excretion was significantly lower than in the infected non-vaccinated birds. These findings support results obtained by Veits et al. (2008), De Vriese et al. (2010) and Tian et al., (2010), that the antigenic match between the vaccine strain and the circulating field virus is one of the most decisive factors in determining the vaccine efficacy to prevent replication of H5N1 virus in vaccinated birds. Therefore, the currently used vaccines in Egypt, at least if based on Eurasian H5 isolates, should be updated and newly developed ones seeded with field virus strain are greatly needed.

4.4.Dissemination of HPAI virus in eggs of infected breeders

It is well established that an infection with HPAI H5N1 virus decreased the egg-production and reduced the quality of eggs (Bean et al., 1985). Furthermore, since the clinical course of HPAI in chickens is extremely short (1-3 days until death), it has been considered unlikely that the virus could be disseminated via infected/contaminated eggs. However, detection of HPAIV H5N1 from internal contents of eggs obtained from vaccinated and naturally infected layer flocks was recently reported in Egypt (Kilany et al., 2010) and other birds worldwide (Li et al., 2006; Promkuntod et al., 2006). In publication 3 (chapter 4) virus excretion was detected in eggs which some of the chicken breeders continued to lay also after experimental

infection. Especially the group challenged with the 2.2.1 proper proved to continue egg laying activity. These results are in accordance with the recent isolation of HPAIV H5N1 2.2.1 proper from albumin and egg shell of eggs obtained from a layer flock in Egypt vaccinated three times with an inactivated vaccine based on an H5N1 strain at 1, 7, and 16 weeks of age (Kilany et al., 2010). Further previous reports exist which showed that other HPAIV viruses (H5N2) were also found in chicken eggs (Bean et al., 1985; Cappucci et al., 1985). Contamination of the eggshell surfaces with the virus is not uncommon in infected flocks, and has been identified as a means of indirect transmission between flocks (Pillai et al., 2010) but may also be a problem in the hatcheries, should hatching eggs be affected from contamination. Our results enforce the need for adequate safeguards to mitigate the potential hazards of hatchery contamination, infection of newly hatched chicks and/or spread of the virus in the environment by movement of contaminated eggs and egg trays.

4.5.Role of maternal immunity

All one-day old chicks in Egypt have a considerable antibody titer due to transfer of the maternal immunity (MI) from sera of vaccinated breeders through egg yolk to their progeny which could interfere with the early vaccination regimes of broilers (Kim et al., 2010). Our findings in publication 4 (chapter 5) revealed that clinical protection, but not virus replication, could be afforded by maternal immunity at least 10 days post-hatch against homologous H5N2, but not heterologous variant H5N1, virus infections. There was little, if any, interference of H5N2 derived maternal immunity with vaccination of chicks with variant HPAIV H5N1 at 3 or 14 days age. All birds survived the infection with mild, if any, clinical signs while excretion of the virus was recorded from the 2nd day post-challenge until 10 days post challenge (the end of the experiment) regardless of the vaccination day. Nevertheless, vaccination at 3 days age with homologous H5N2 vaccine in presence or absence of maternal immunity didn't protect chickens against classic HPAIV H5N1 infection.

Taken together, maternal immunity of H5N2 vaccinated breeders will not protect their progeny against circulating variant H5N1 in Egypt. Single vaccination of broilers with H5N1 vaccine could clinically protect broiler birds against homologous H5N1 variant infection. Maternal immunity derived from H5N2 vaccinated breeders can interfere with vaccination of broilers with the same vaccine at 3 days age in the face of classic H5N1 virus infection. These observations indicate interference of the maternal antibodies with efficacy of an early vaccination of hatched chicks. This is also in accordance with field observations from Egypt in late 2006 and early 2007 where 295 out of 355 (83%) examined broiler flocks possessing MI did not seroconvert upon vaccination with vaccines similar to those received by the breeders (Arafa et al., 2008). A one-dose vaccination of broilers apparently is not sufficient to abort virus excretion.

This study did not set out to exactly define the half life of maternally derived H5-specific antibodies and thus only two time points were available for estimation by HI assay. Using

homologous H5N2 antigens, half life was approximately 4.2 – 4.4 days based on an initial mean titre of ≥ 5. This value is in agreement with results (4.2 days) obtained by Sarfati-Mizrahi et al. (2010) and is lower than that (7 days) reported by De Vriese et al. (2010). Notwithstanding clinical protection, in none of the vaccination schemes sterile immunity could be induced in the chicks. Prolonged excretion of virus RNA was observed, however, at reduced amounts as compared to non-vaccinated MI- chicks. Therefore, at least one booster vaccination should be useful, as previously recommended (De Vriese et al., 2010), to further reduce virus excretion of broiler chicks (Gharaibeh, 2008; Grund et al., 2011).

4.6.Serological evaluation of the immune response after vaccination

HI titers are considered to be of predictive value concerning protectivity if suitable matching pairs of HI antigen and challenge virus are used (Brugh and Stone, 1986; EFSA, 2008; Swayne, 2009). Serological monitoring of H5 vaccinated flocks by the HI test using the homologous vaccine antigen is a routine laboratory procedure to evaluate vaccination efficacy of poultry in Egypt and elsewhere (Hafez et al., 2010). Titres > 4 $\log_2$ have been claimed to be an indicator for clinical protection and titres > 6 $\log_2$ for prevention of viral shedding as stated by Tian et al. (2005) and Kumar et al. (2007). The results obtained in the current study confirmed that antibody titres differed according to the antigen used. Attempts to broaden the specificity of the humoral immune response by repeated boosting vaccination were successful in that titre means against four distinct H5 antigens were raised above the threshold of 5 $\log_2$. However, no HI seroreactivity against the 2.2.1 variant Egyptian virus was induced which correlated with absence of protection. Conversely, high HI titres measured against the 2.2.1 proper Egyptian strain also correlated with significant, although not complete, protection. This mirrors the field situation in Egypt where the commercial H5N2-based vaccines seem to provide clinical protection against 2.2.1 proper viruses only (Hafez et al., 2010). On the other hand, only minor protection was seen against the Italian H5N2 HPAI isolate despite high HI titres against this strain.

Accordingly, the use of homologous antigen only to measure the HI titer against the commercial vaccine H5N2 antigen may result in an erroneous prediction of protection against currently circulating H5N1 viruses in Egypt. In countries endemically infected with divergent HPAI H5N1 viruses, antigens from the circulating field viruses must be used for HI assay-based predictions. Therefore prospective temporal and/or geographical bases of the field viruses could be useful in selection of HI antigens used for evaluation of vaccine immune response.

4.7.Conclusion

The real challenge of the HPAI H5N1 virus in Egypt is the continuous replication of the virus under immune pressure in vaccinated poultry. Consequently, point mutations were accumulated in the primer and probes specific sites decreasing the sensitivity of the current

used RT-qPCR for detection of the virus, resulting in silent spread of the virus in and among different hosts. On the other hand, accumulation of such mutations in the immunogenic and antigenic sites of the HA gene was common which could permit establishment of the virus in poultry despite of the blanket vaccination campaign. Therefore, in order to eradicate the disease effectively, continuous reappraisal and development of new diagnostic tools and vaccines should be done in Egypt annually or biennially.

CHAPTER 2

Diagnostic update

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Research Note—

Modified H5 Real-Time Reverse Transcriptase–PCR Oligonucleotides for Detection of Divergent Avian Influenza H5N1 Viruses in Egypt

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CHAPTER 3

Development of new diagnostic

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Simultaneous detection and differentiation by multiplex real time RT-PCR of highly pathogenic avian influenza subtype H5N1 classic (clade 2.2.1 proper) and escape mutant (clade 2.2.1 variant) lineages in Egypt

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CHAPTER 4

Protection

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Multiple dose vaccination with heterologous H5N2 vaccine: Immune response and protection against variant clade 2.2.1 highly pathogenic avian influenza H5N1 in broiler breeder chickens

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CHAPTER 5

Maternal immunity

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Influence of maternal immunity on vaccine efficacy and susceptibility of one day old chicks against Egyptian highly pathogenic avian influenza H5N1

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Summary

Highly pathogenic H5N1 avian influenza virus epidemic in Egypt: Detection and protection studies

Poultry is the major source of animal protein in under-resourced countries. Introduction of highly pathogenic avian influenza virus (HPAIV) of subtype H5N1 into such countries had a severe negative impact on the poultry industry and posed a serious threat to public health. Vaccination of poultry was used successfully to reduce the socioeconomic losses during the first wave of the disease in Egypt. However, vaccines as a sole tool to eradicate the HPAIV H5N1 are insufficient to delineate circulation of the virus in vaccinated birds, on the contrary replication of the virulent virus in presence of antibodies induced by the vaccine lead to increase in the mutation rate in genes, such as the hemagglutinin (HA). This is also had negative effects on the sensitivity of molecular diagnostic tools aiming at the HA gene. Second, passive transfer of maternal immunity through eggs to the newly hatched chicks could interfere with their vaccination in the early stage of life.

In this study, to match the divergent Egyptian HPAI H5N1 viruses, we adapted primers and probes of the internationally recommended RT-qPCR assay targeting the H5 gene. As a result the sensitivity to detect the Egyptian variant and classical strains was dramatically increased. Furthermore, phylogenetic analyses of the H5 gene of the Egyptian viruses indicated that two major groups of H5N1 viruses are currently co-circulating in poultry in Egypt. We developed a versatile multiplex real time RT-PCR assay to detect all Egyptian HPAI H5N1 viruses and differentiate between the classic 2.2.1 group (isolated mainly from backyard birds and humans) and the genetic variant 2.2.1 group (isolated mainly from commercial vaccinated chickens). Furthermore, we showed that multiple dose vaccination of broiler breeder chickens with an inactivated H5N2 vaccine induced broad humoral immune responses against different antigens; yet, sufficient clinical protection against the newly emerging variant Egyptian HPAIV H5N1 was not induced. Moreover, we demonstrate that the virus is disseminated in and on eggs of infected vaccinated birds after challenge. H5N2 maternal derived immunity didn't protect chicks challenged with the Egyptian variant HPAI H5N1 virus and interfered with vaccination of 3 days old chicks with homologous H5N2 vaccine. However, vaccine seeded with the recent Egyptian variant H5N1 strain was more highly effective.

In countries with endemic status of HPAIV H5N1 in poultry, regular optimization and development of sensitive molecular diagnostics is a real challenge to ensure early detection and control of infected birds. Vaccination of poultry could be a part of a comprehensive control plan but biosecurity measures should be enforced rigorously. The vaccines should be re-evaluated according to their antigenic match with circulating field viruses. Eggs obtained from infected flocks should be considered as a risk factor for silent virus transmission. Maternal immunity could reduce the vaccine efficacy and should be considered when formulating vaccination regimes against HPAIV H5N1.

Zusammenfassung

Epidemie von hochpathogenenem H5N1 Aviäre Influenza Virus in Ägypten: Studien über Nachweis und Impfschutz

Geflügel ist die wichtigste Quelle für tierische Proteine in Entwicklungsländern. Das Auftreten von HPAIV H5N1 in diesen Ländern hat schwerwiegende negative Auswirkungen auf deren Geflügelwirtschaft und stellt eine ernsthafte Bedrohung für die öffentliche Gesundheit dar. Zur Eindämmung ökonomischer Verluste wurde in Ägypten die gesamte Geflügelpopulation während der ersten Infektionswelle geimpft. Allerdings sind Impfstoffe als einziges Mittel zur HPAIV H5N1 Bekämpfung unzureichend, um die Zirkulation des Virus in geimpften Beständen vollständig zu unterbinden. Darüber hinaus führt das Vorhandensein von Antikörpern zu einem Selektionsdruck auf das Zielprotein, insbesondere das Hämagglutinin (HA), was eine erhöhte Mutationsrate des HA Gens verursacht. Dies führt zur Verminderung der Sensitivität der zur Zeit verwendeten molekulargenetischen Nachweismethoden. Des Weiteren verhindert die Impfung von Elterntieren durch die Übertragung der maternalen Antikörper auf die Nachkommen eine erfolgreiche Impfung der Nachkommen in den ersten Lebenstagen.

In dieser Studie wurden Primer und Sonde der international empfohlenen RT-qPCR für das H5-Gen den Sequenzen der stark divergierenden ägyptischen HPAI H5N1-Viren angepaßt. Dies führte zu einer dramatisch verbesserten Sensitivität in der Detektion ägyptischer H5N1 Stämme. Phylogenetische Analysen des H5-Gens der ägyptischen Viren zeigten, dass zwei großen Gruppen divergenter H5N1-Viren derzeit bei Geflügel in Ägypten zirkulieren. Deswegen wurde ein RT-qPCR Multiplex-Assay entwickelt, der alle ägyptischen HPAI H5N1 Viren detektiert und gleichzeitig zwischen der klassischen 2.2.1 Gruppe (isoliert hauptsächlich aus Kleinsthaltungen von Geflügel sowie von Menschen) und der genetischen Variante 2.2.1 (isoliert aus kommerziellen geimpften Hühnern) differenziert.

Ferner wurde gezeigt, dass wiederholte Impfungen von Masthähnchen-Elterntieren mit einem inaktivierten H5N2-Impfstoff eine breite humorale Immunantworte gegen unterschiedliche H5 Antigene induzierte. Allerdings konnte hierdurch kein klinischer Schutz gegen die neu entstandene Variante ägyptischer HPAI H5N1 Viren induziert werden. Zudem wurde festgestellt, dass das Virus in und auf Eiern, die von geimpften, infizierten Vögeln gelegt wurden, verbreitet wird. Maternale Immunität infolge einer H5N2 Impfung der Elterntiere vermittelte den Küken keinen Schutz gegen die ägyptische Variante des HPAI H5N1 Virus. Dagegen beeinträchtigte diese maternale Immunität den Erfolg der Impfung von 3 Tage alten Küken mit homologem H5N2-Impfstoff. Eine Impfung mit der aktuellen H5N1 Variante war dagegen effektiver.

In Ländern, in denen HPAI H5N1 bei Geflügel endemisch ist, ist eine regelmäßige Optimierung und Fortentwicklung sensitiver molekularbiologischer Testmethoden schwierig, aber für die Früherkennung und Kontrolle infizierter Vögel unabdingbar erforderlich. Die

Impfung der Geflügelbestände kann Teil eines umfassenden Kontrollplanes sein, jedoch müssen flankierende Biosicherheitsmaßnahmen rigoros durchgesetzt werden. Die Impfstoffe sollten entsprechend der Antigenprofile der aktuell zirkulierenden Feldviren neu bewertet werden. Eier aus infizierten Herden sind ein Risikofaktor für eine stumme Übertragung des Virus. Maternale Immunität kann die Wirksamkeit von Impfstoffen in sehr jungen Küken negativ beeinflussen.

List of published articles

A) Publications in peer-reviewed journals

1. **Lotfi A, Abdelwhab EM and Hafez HM** (2011) Persistence of *Histomonas Meleagridis* in and on materials used in poultry houses. Avian Diseases [In press].
2. **Abdelwhab EM, Grund C, Beer M, Harder TC, Aly MM and Hafez HM** (2011) Influence of maternal immunity on vaccine efficacy and susceptibility of one day old chicks against Egyptian highly pathogenic avian influenza H5N1. Veterinary Microbiology [In press].
3. **El-Zoghby EF, Abdelwhab EM, Arafa A, Selim AA, Kholousy SG, Kilany WH, Safwat M, Hassan MK, El-Kanawati Z, Aly MM, Aly MM and Hafez HM** (2011) Active surveillance of avian influenza virus in backyards in Egypt. The Journal of Applied Poultry Research [In press].
4. **Abdelwhab EM, Grund C, Beer M, Harder TC, Aly MM and Hafez HM** (2011) Multiple dose vaccination of broiler breeder chickens with an H5N2 vaccine does not induce protection against challenge with an Egyptian highly pathogenic avian influenza H5N1 virus of clade 2.2.1. Vaccine 29:6219-6225.
5. **Abdelwhab EM, Abdelmagid MA, El-Sheibeny LM, El-Nagar HA, Arafa A, Selim A, Nasef SA, Aly MM and Hafez MH** (2011) Detection and molecular characterization of Mycoplasma gallisepticum field infection in ts-11 vaccinated broiler breeder flock in Egypt: case report. The Journal of Applied Poultry Research 20:390-396.
6. **Abdelwhab EM, Lüschow D, Harder TC, and Hafez HM** (2011) The use of FTA ® filter paper in diagnosis of avian influenza virus. Journal of Virological Methods 174:120-122.
7. **Abdelwhab EM and Hafez HM** (2011) An overview of the epidemic of highly pathogenic H5N1 avian influenza virus in Egypt: Epidemiology and control challenges. Epidemiology and Infection 139:647-657.
8. **Grund C, Abdelwhab EM, Arafa A, Ziller M, Hassan MK, Aly MM, Hafez HM, Harder TC and Beer M** (2011) Highly pathogenic avian influenza virus H5N1 from Egypt escapes vaccine-induced immunity but confers clinical protection against a heterologous clade 2.2.1 Egyptian isolate. Vaccine 29:5567-5573.
9. **Kilany WH, Abdelwhab EM, Arafa A, Selim A, Safwat M, Nawar AA, Erfan AM, Hassan MK, Aly MM and Hafez HM** (2010) Protective efficacy of H5 inactivated vaccines in meat turkey poults after challenge with Egyptian variant highly pathogenic avian influenza H5N1 virus. Veterinary Microbiology 150:28-34.
10. **Abdelwhab EM, Arafa A, Erfan AM, Aly MM and Hafez HM** (2010) Modified H5 real-time RT-PCR oligonucleotides for detection of divergent avian influenza H5N1 viruses in Egypt. Avian Diseases 58:1301-1305.
11. **Aly ZEA, Ibrahim MM, Abdelwhab EM and Hafez HM** (2011) Development of duplex and triplex conventional RT-PCR for the detection of H5N1 avian influenza virus. Archiv für Geflügelkunde 75: (accepted).
12. **Abdelwhab EM, Erfan AM, Grund C, Arafa A, Beer M, Hafez HM, Aly MM, and Harder TC** (2010) Simultaneous detection and differentiation by multiplex real time

RT-PCR of highly pathogenic avian influenza subtype H5N1 classic (clade 2.2.1 proper) and escape mutant (clade 2.2.1 variant) lineages in Egypt. Virology Journal 7:260.

13. **Kilany WH, Hassan MK, Arafa A, Selim A, Safwat MA, Erfan AM, Abdel-haliem A, Aly MM, Hafez HM and Abdelwhab EM** (2010) Isolation of highly pathogenic avian influenza H5N1 from internal contents of eggs obtained from vaccinated layer chickens. Avian Diseases 54:1115-1119.
14. **Abdelwhab EM, Hafez HM, Aly MM, Grund C, and Harder TC** (2010) Increasing prevalence of unique mutation patterns in H5N1 avian influenza virus HA and NA glycoproteins from human infections in Egypt. Sequencing, vol. 2010, Article ID 450823, doi:10.1155/2010/450823.
15. **Hafez MH, Arafa A, Galal S, Selim A, Hassan MK, Abdelwhab EM and Aly MM** (2010) Avian influenza H5N1 infections in vaccinated commercial poultry and backyard birds in Egypt. Poultry Science 89:1609 – 1613.
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18. **Abdelwhab EM, Lebdah ME, El-Sisi MA, Khoddary RM, Abdel-Wanees SA and Aly MM** (2007) Bacteriological and histopathological studies on *Ornithobacterium rhinotracheale* (ORT) in broilers. Journal of Egyptian Veterinary Medical Association 67:273–290.

The following above-mentioned papers have evolved from my doctoral thesis: 2, 4, 10 and 12 in peer-reviewed journals.

B) Publications in academic conferences

1. **Abdelwhab EM, Grund C, Beer M, Harder TC, Aly MM and Hafez HM** (2011) Multiple dose vaccination of broiler breeder chickens with an H5N2 vaccine against variant clade 2.2.1 highly pathogenic avian influenza H5N1 virus infection. The 17th World Veterinary Poultry Association Congress, Mexico, Cancun, 14 – 18 August.
2. **Abdelwhab EM, Grund C, Beer M, Harder TC, Aly MM and Hafez HM** (2011) The influence of maternal antibodies on protection and vaccination against highly pathogenic avian influenza. The 17th World Veterinary Poultry Association Congress, Mexico, Cancun, 14 – 18 August.
3. **Abdelwhab EM, Lüschow D and Hafez HM** (2011) Development and validation of a real-time PCR assay for detection of *Ornithobacterium rhinotracheale* in poultry. The 17th World Veterinary Poultry Association Congress, Mexico, Cancun, 14 – 18 August.
4. **Abdelwhab EM, Lüschow D, Harder TC, and Hafez HM (**2010) The use of FTA® filter paper in diagnosis of avian influenza virus. Proceedings of the 8th International Symposium on Turkey Diseases. 27th - 29th May, Germany 2010, Berlin. (Ed. H. M. Hafez). Mensch & Buch Verlag. ISBN. 978-3-86664-872-2. pp. 232-238.
5. **Lotfi A, Abdelwhab EM and Hafez MH** (2010) Persistence of *Histomonas Meleagridis* in and on materials used in poultry houses. Proceedings of the 8th International Symposium on Turkey Diseases 27th - 29th May, Germany, Berlin. (Ed. H. M. Hafez). Mensch & Buch Verlag. ISBN. 978-3-86664-872-2. pp. 77-81.
6. **Abdelwhab EM and Hafez HM** (2009) Avian influenza in Egypt: Current and future control approaches. Doktorandensymposium des Fachbereichs Veterinärmedizin 6th November, Freie Universität Berlin. Germany pp. 52.
7. **Aly MM, Hassan MK, Arafa A, Selim A, Kilany WH, Abdelwhab EM and Hafez MH** (2009) Isolation of highly pathogenic avian influenza H5N1 from eggs of naturally vaccinated infected layer flocks in Egypt. The 16th World Veterinary Poultry Association Congress Marrakesh, 08th – 12th November.
8. **Aly MM, Hassan MK, Arafa A, Selim A, Kilany WH, Abdelwhab EM and Hafez MH** (2009) Role of feral pigeons in Egypt in spread of highly pathogenic avian influenza H5N1. The 16th World Veterinary Poultry Association Congress Marrakesh, 08th – 12th November.
9. **Abdelwhab EM, Arafa A, Selim A, Samaha H, Kilany WH, Shereen G, Hassan MK, Aly MM and Hafez HM** (2009) Highly pathogenic avian influenza H5N1 in Egypt: Current situation and challenges. Proceedings of the 5th International Meeting of the Working Group 10 (Turkey) of WPSA (Ed. Hafez, H.M). 28th – 30th May 2009, Berlin . Berlin. Mensch & Buch Verlag. ISBN. 978-3-86664-701-5. pp. 308 – 316.
10. **Aly MM, Arafa A, Selim A, Abdelwhab EM, El-Kanawati Z, Hassan MK and Hafez HM** (2008) Highly pathogenic avian influenza H5N1 in vaccinated turkey flock in Egypt: case report. Proceedings of the 7th International Symposium on Turkey Diseases. 19th - 21st June 2008, Berlin. (Ed. H.M.Hafez). Verlag der DVG Service GmbH- ISBN. 978-3-939902-96-6. pp. 222-229.

11. **Hafez HM, Abdelwhab EM, Arafa A and Aly MM** (2008) Highly pathogenic *avian influenza* H5N1 outbreaks in vaccinated poultry in Egypt. 72. Fachgespräch über Geflügelkrankheiten. Hannover, 10th - 11th, May. Deutsche Veterinärmedizinische Gesellschaft:Tagung der Fachgruppe Geflügelkrankheiten: Referatesammlung: Fachgespräch der Fachgruppe Geflügelkrankheiten. S. ISBN:978-3-939902-50-8. pp. 48 – 51.
12. **Arafa A, Kanawaty Z, Abdelwhab EM, Hassan MK, and Aly MM** (2008) Avian influenza H7 surveillance on backyard birds around El-Abassa Lake in Egypt, 2007. In: 2nd International Conference of Virology, Emerging and Exotic Viral Infection Challenging Threats of Human, Animal and Plant Health 5th – 6th April, Organized by the Egyptian Society of Virology, Cairo, Egypt.
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14. **Aly MM, Hassan MK, Selim A, and Abdelwhab EM** (2007) Serological investigation on avian influenza H5 vaccines in Egypt. In: Proceedings of the 7th International Symposium of Faculty of Veterinary Medicine, Cairo University.
15. **Aly MM, Hassan MK, Arafa A and Abdelwhab EM** (2006) Emergence of first outbreak of avian influenza (H5N1) in meat turkeys flocks in Egypt in early 2006. In: Proceedings of the 6th International Symposium on Turkey Diseases Berlin (Ed. Hafez, H. M.). DVG-Service-GmbH, Giessen - ISBN: 3-939902-04-7. pp. 310-318.

C) Oral presentations

1. **Abdelwhab EM** (2011) Challenges of vaccination for control of HPAIV H5N1. Joint symposium on avian influenza and Newcastle Disease in Northern Africa and the Middle East, January 18th -22nd, Dokki, Giza, Egypt.
2. **Abdelwhab EM** (2011) Virulence determinants of Italian H7N1 avian influenza virus. Scientific meeting, 17th January, Friedrich-Loeffler-Institute, Isles of Riems, Greifswald, Germany.
3. **Hafez HM and Abdelwhab EM** (2009). Egypt culls pigs - a religious affair? Scientific meeting, May 13th, Hannover, Germany.
4. **Abdelwhab EM** (2009) Molecular diagnosis of avian influenza viruses. Oral presentation in workshop of "Capacity building for the *avian influenza* through technology transfer and training" CONFLUTECH EU-FP6 project funded by the EU technical workshop for veterinarians in the Middle East on current status, sampling, isolation, surveillance & diagnostic techniques for *avian influenza,* January 18th -22nd, Amman, Jordan.
5. **Abdelwhab EM** (2009) Surveillance startegy of avian infleunza in Egypt 2009. Oral presentation in workshop of "Capacity building for the *avian influenza* through technology transfer and training" CONFLUTECH EU-FP6 project funded by the EU technical workshop for veterinarians in the Middle East on current status, sampling, isolation, surveillance & diagnostic techniques for *avian influenza,* January 18th -22nd, Amman, Jordan.

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Selbständigkeitserklärung:

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

Berlin, den 16.12.2011 Unterschrift: El-Sayed M. Abd El-Whab