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in Kooperation mit dem
Institut für Geflügelkrankheiten des Fachbereichs Veterinärmedizin, der Alexandria Universität, Ägypten

Molekulare Aspekte und chemische Inaktivierung von Influenza H5N1-Viren aus ägyptischen Hühnerbeständen von Ausbrüchen der Jahre 2006 bis 2010

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Molecular aspects and chemical inactivation of Influenza H5N1 viruses isolated from Egyptian chicken flocks during the 2006-2010 outbreaks

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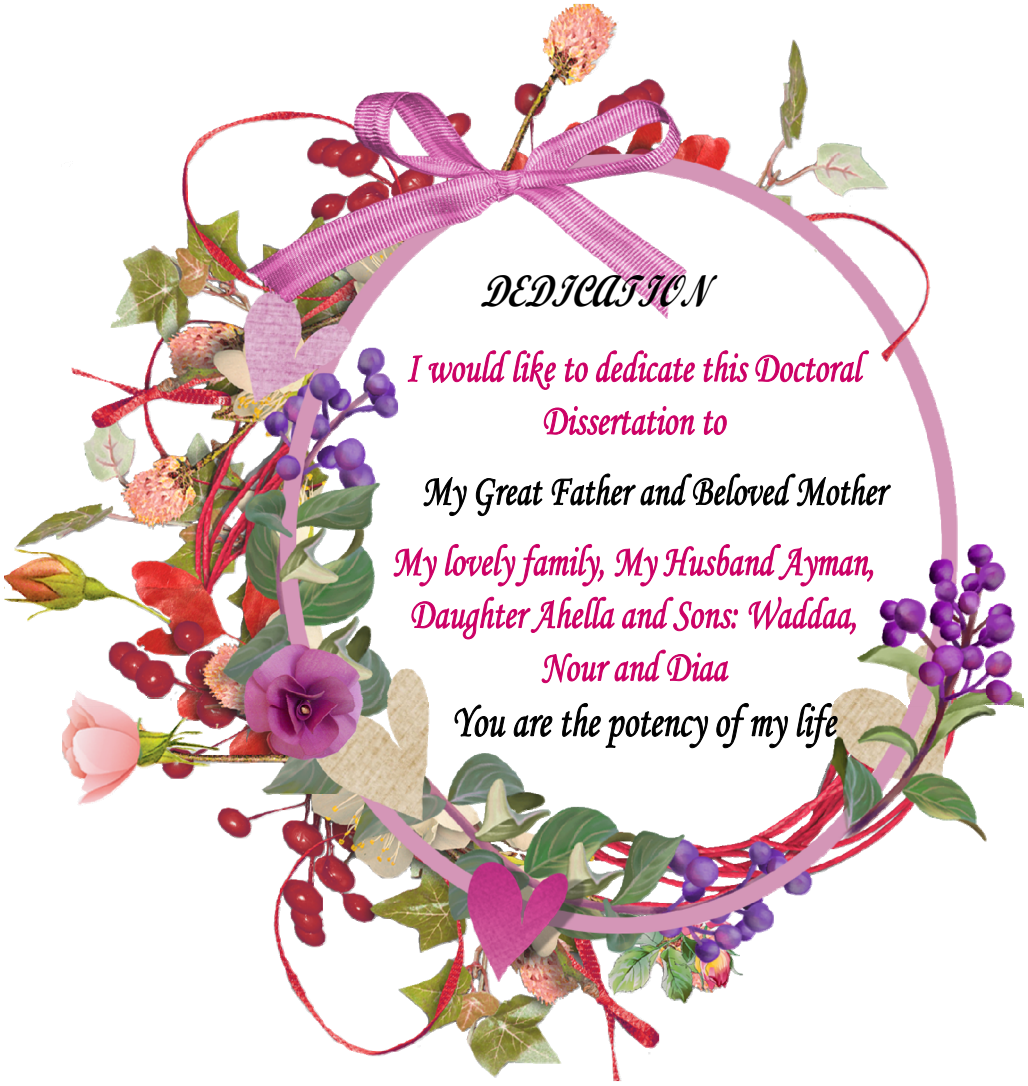
DEDICATION
I would like to dedicate this Doctoral Dissertation to
My Great Father and Beloved Mother
My lovely family, My Husband Ayman, Daughter Ahella and Sons: Waddaa, Nour and Diaa
You are the potency of my life

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LIST OF ABBREVIATIONS

%	Percent
A549	Adenocarcinomic Human Alveolar Basal Epithelial Cells
AF	Allantoic Fluid
AGPT	Agar gel perciptation test
AI	Avian Influenza
AIVs	Avian Influenza Viruses
AusVetPlan	Australian Veterinary Emergency Plan
BCS	Bovine Calf Serum
BHK-21	Baby Hamster Kidney Cells
BLASTN	Nucleotide Basic Local Alignment Search Tool
bp	Base Pair(s)
CAHO	Community Animal Health Outbreak
CDC	Centers for Disease Control and Prevention
cDNA	Complementary Deoxyribonucleic Acid
CEF	Chicken Embryo Fibroblast
CEK	Chicken Embryo Kidney
CEN	European Committee for Standardization
°C	Degree Celsius
DF1	Chicken Fibroblast
DIVA	Differentiate Infected from Vaccinated
DNA	Deoxyribonucleic Acid
dntp	Deoxyribonucleotide Triphosphate
DVG	Deutsche Veterinärmedizinische Gesellschaft or German Veterinary Medical Society
DVV	German Association for the Control of Virus Disease
ECBO	Enteric Cytopathogenic Orphan Virus
ECDC	European Centre for Disease Prevention and Control
ECEs	Embryonated Chicken Eggs
EDS	Egg Drop Syndrome
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EID50	Mean Embryo Infective Dose
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European-Middle Eastern-African
EMBL	European Molecular Biology Laboratory
EPA	Environmental Protection Agency
FCS	Fetal Calf Serum
FHO	Federal Health Office
FAO	Food and Agriculture Organization of the United Nations
HD11	Chicken Bone Marrow Macrophage
g	gram
g/L	gram per liter
GS	Glycosylation Site(s)
h	Hour
HA	Haemagglutination
HA	Haemagglutinin

HI	Haemagglutination Inhibition
HPAIV	Highly Pathogenic Avian Influenza Virus
IU	International Unit
LPAIV	Low Pathogenic Avian Influenza Virus
M	Matrix
MDCK	Madin-Darby Canine Kidney Cells
MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligram
min	Minute
µl	Microliter
ml	Milliliter
MUSCLE	Multiple Sequence Comparison by Log- Expectation
Mv1Lu	Mink Lung Epithelial Cells
NA	Neuraminidase
NDV	Newcastle disease virus
NLQP	National Laboratory for Veterinary Quality Control on Poultry Production
nm	Nanometer
NP	Nucleoprotein
NS	Non-Structural Protein
OECD	Organization for Economic Co-operation and Development
OIE	World Organisation for Animal Health
PA	Polymerase Acidic
PB	Polymerase Basic
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PCS	Proteolytic Cleavage Site
QACs	Quaternary ammonium compounds
QT-35	Quail Fibroblast
RBCs	Red Blood Cells
RNA	Ribonucleic Acid
rpm	Round per Minute
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SPF	Specific Pathogen Free
TAE	Tris Acetate EDTA
TAQ	Thermus Aquaticus
TCID	Tissue Culture Infective Dose
U.S.	United States
USA	United States of America
UV	Ultra Violet
Vero	Kidney of African Green Monkeys
V/V	Volume (of solute) per Volume (of solvent)
WHO	World Health Organization
W/V	Weight/Volume percent

CHAPTER 1: INTRODUCTION

Avian influenza viruses (AIVs), belonging to the family Orthomyxoviridae, are negative-sense, segmented, single stranded, enveloped RNA viruses. The virus genome encodes at least 11 viral proteins, where the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA) are anchored in the lipid-bilayer viral envelop. To date, AIVs have 16 HA and 9 NA subtypes. While all AIVs subtypes cause mild, if any, clinical signs in poultry, a few members of H5 and H7 subtypes cause major and frequently fatal disease in birds (**Webster et al., 1992**). Highly pathogenic avian influenza virus (HPAIV) H5N1 which originated in South East Asia in 1996/1997 has spread across Eurasia since 2003 and entered Africa in 2005 caused magnificent economic losses in the poultry industry, threatened food security and disrupted trade in poultry (**OIE, 2004; Capua and Marangon, 2006; Cattoli et al., 2009**). Additionally, the H5N1 virus poses a significant risk to human health (**WHO, 2008**). Much interest has arisen that the HPAIV H5N1 could evolve into a new form that can transfer from person to person, which poses a potential pandemic threat (**Djunaidi and Djunaidi, 2007**).

In Egypt, the first outbreak of HPAIV H5N1 clade 2.2.1 was reported in February 2006. In spite of a strong preliminary reaction to the disease, including the depopulation of over 40 million birds, HPAIV H5N1 was not totally eliminated. Over $ 1 billion losses were estimated in the commercial (total annual production of 850 million birds) and backyard sectors (250 million birds) due to HPAIV H5N1 in Egypt in 2006 (**Meleigy, 2007; Abdelwhab et al., 2010a**). At the end of 2007, outbreaks were reported in some poultry farms due to vaccination failure, and a variant H5N1 virus was detected. Sequencing of isolated viruses confirmed that this variant was an HPAI virus subtype H5N1 with a significant increase in the number of amino acid substitutions in the HA1 protein **(Abdel-Moneim et al., 2009; Arafa et al., 2010a)**. Since 2008, Egypt declared an endemic status of HPAIV H5N1 and outbreaks are regularly recorded from different regions, particularly in the household sector. In February 2010, out of 113 HPAI H5N1-infected poultry flocks (chicken, ducks and turkeys) from 17 governorates, 93 (82 %) were detected from the backyard poultry sector (**FAO, 2011**).

In order to limit the animal and human health impacts, it is very important to control and/or eradicate the H5N1 virus infection in poultry (**Tiensin et al., 2005; Songserm et al., 2006a**). Therefore, early detection is essential to prevent the spread of infection in poultry and subsequently spillover to humans. Standard methods for isolation and identification of HPAIV are still time-consuming, less sensitive, laborious, need a qualified team and particular laboratory infra-structure. However, molecular diagnostic tools using RT-PCR have considerably enhanced the speed, sensitivity and specificity of AIV detection (**Dhumpa,**

2011). The principles of HPAI prevention and control depend on biosecurity, flock management, preventive vaccination and sanitation (**Zander et al., 1997**).

Biosecurity is one of the most important tools to reduce the microbial infection generally and the level of pathogens particularly in poultry farms. Usually, a hygiene program is supposed to comprise harmless and simple measures outlining the accurate use of detergents and disinfectants in addition to an efficient monitoring system and appropriate use of application equipment (**Spielholz, 1998; Gehan et al., 2009**).

The application of chemical disinfectants has been an essential constituent of disease management programs. Although there are a wide variety of chemical disinfectants available in markets, which considered effective against pathogens, the appropriate disinfectant must be chosen according to the susceptibility of the target virus (**Suarez et al., 2003**). Based on their resistance to chemical agents, viruses are divided into three categories denoted A, B and C (**Noll and Youngner, 1959**). This classification is based on the presence or absence of lipids on the virus and on the virus size itself, which appear to be the most important characteristics that influence the resistance to chemical agents. AIVs are belonging to category A, which includes all the enveloped intermediate to large sized viruses. Therefore, AIV is grouped in the category of viruses that can be inactivated by all the major classes of disinfectants if used properly (**Maris, 1990; Prince and Prince, 2001**). In contrast to considerable published information on the disinfection of poultry pathogens, respective information for HPAIV H5N1 is still scanty. The objectives of the study therefore were:

1- Standard identification and molecular characterization of two isolates of AIV subtype H5N1 isolated from commercial chicken in Egypt during the 2006 and 2010 outbreaks.

2- Comparative sequence and phylogenetic analyses of the HA and NA genes of different HPAIV H5N1 during the 2006 and 2010 outbreaks.

3- Evaluation of the efficacy of some commercial chemical disinfectants in the Egyptian poultry market against the two identified strains of HPAIV H5N1.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Background of the avian influenza virus

2.1.1 Nature of the virus

Influenza viruses are negative single-strand, enveloped RNA viruses that belong to the genus influenza virus in the family Orthomyxoviridae. Within the family, there are three types of influenza: A, B and C (**Scholtissek et al., 1983**). Influenza viruses that cause diseases in animals belong to type A; in contrast, disease in human beings can be caused by types A, B and C. Based on their surface glycoprotein antigens, the type A viruses are classified into subtypes according to the haemagglutinin (HA) and neuraminidase (NA). The HA is classified into sixteen subtypes (H1, H2…H16), whereas NA is classified into nine subtypes (N1, N2…N9) (**Swayne, 2000; Siengsanan-Lamont, 2010**). Most recently, a new H17N10 virus was detected in bats in Guatemala, however isolation of this live virus was not successful so far (**Sun et al., 2013; Zhu et al., 2013**).

The AIV particle is circular in shape with a width of 80-120 nm, although occasionally, it takes a filamentous or polymorphic structure (**Suarez, 2008; Siengsanan-Lamont, 2010**). All type A influenza viruses have eight genome segments that express at least eleven viral proteins, namely PB2, PB1, PB1-F2, PA, HA, NP, NA, M1, M2, NS1, and NS2 (**Chen et al., 2001; Suarez, 2008; Siengsanan-Lamont, 2010**). The surface of influenza A virus consists of a bilayer of a lipid envelope containing big surface glycoprotein spikes (peplomers) that have HA or NA activities, adjoining and strictly linked with an interior layer consisting of matrix (M1) proteins which in turn bind eight helically symmetrical nucleocapsid segments of dissimilar sizes (**Potter, 2004; Siengsanan-Lamont, 2010**). The HA protein exists in ancestor form that has to be cleaved by proteases at the proteolytic cleavage site (PCS) into HA1 and HA2 subunits for infection to continue (**Potter, 2004**). The HA1 has a receptor-binding subunit, immunogenic epitopes, antigenic determinants and most of potentially glycosylated sites. The HA2 has a cell fusion function and viral transmembrane domain. The NA has a role as a receptor-destroying enzyme that facilitates the liberation of mature progeny virions from the infected cell (**Suarez, 2008; Siengsanan-Lamont, 2010**). A third surface protein, the matrix protein (M2) is organized as tetramers to form an ion channel, which bypasses throughout the envelope (**Padtarakoson, 2006; Siengsanan-Lamont, 2010**).

The nucleocapsid composes of genome segments associated with an RNA polymerase complex containing three polymerase proteins (PB2, PB1 and PA) and is enclosed within a capsid of helically arranged nucleoprotein (NP) (**Padtarakoson, 2006; Siengsanan-Lamont,**

2010). The main function of the non-structural protein NS1 is the inhibition of host antiviral interferon α/β production, while the nuclear export protein NS2 is responsible mainly for exportation of viral ribonucleoprotein from the nucleus to the cytoplasm (**Webster et al., 1992**).

2.1.2 Host spectrum and distribution

Avian influenza viruses are able to infect several bird species, either in the wild bird fauna or in domesticated poultry (**ECDC, 2006**; **Fiebig et al., 2011**). All subtypes of AIV have been isolated from more than 90 species of wild birds, which mostly showed no clinical disease (**Ligon, 2005**). Gulls, wild waterfowl and shore birds are the common reservoir of nearly all AIVs. They possibly have carried the viruses, asymptomatically, for thousands of years in an environment that is most favourable for adaptation of the virus to the host. These particular birds are very movable, and mainly wild waterfowl are well known to carry the virus over long distances and to expel large quantities in their faeces (**Ligon, 2005; WHO, 2005**). All other bird species are thought to be susceptible to being infected with AIV, even though some more so than others, with less favourable consequences (**Ligon, 2005**).

Since 1996, the first history of the recent spread of HPAIV H5N1 is thought to originate from the wild bird reservoir into domesticated land-based poultry. The virus was initially reported in Hong Kong, spread in the Far East region and later in some parts of Europe, Africa and the Middle East. At present, the infection is endemic in poultry at least in different areas of Bangladesh, Cambodia, China, Egypt, India, Indonesia and Viet Nam. Moreover, the viruses have also been reported sporadically in poultry and wild birds in other countries, including Europe (**ECDC, 2011**). Infected birds demonstrate a wide variety of symptoms, ranging from mild illness to a highly contagious and rapidly fatal disease. Therefore, avian influenza A virus strains are classified into two pathotypes: high pathogenic AIV (HPAIV) or low pathogenic AIV (LPAIV). The majority of avian influenza A viruses are LPAI viruses that are frequently associated with mild infection in poultry. In contrast, the HPAIV is usually evolving from LPAIV precursors and causes sudden onset, severe illness, rapid death and up to 100% mortality within 48 hours. Some HPAI viruses of subtype H5N1 have been found to cause no illness in some poultry species, such as ducks (**CDC, 2005; Capua and Alexander, 2006; Busquets et al., 2010**). Until now, HPAIV is limited only to viruses of the H5 and H7 subtypes; therefore, both subtypes are notifiable to the World Organization for Animal Health "OIE" (**OIE, 2009**).

In contrast to the LPAIV which is usually limited to the respiratory and eliminatory tracts of birds, the HPAIV causes high viraemia and systemic infection (**Capua and Marangon, 2000; Tiensin et al., 2005; Busquets et al., 2010**).

In humans, LPAIV infections have been also reported, including very mild signs (e.g. conjunctivitis) to influenza-like illness (**CDC, 2005**). Likewise, AIV of the subtypes H5 and H7, including H5N1, H7N7, and H7N3 viruses, have been associated with human infections causing mild (H7N3, H7N7) to severe and fatal disease (H7N7, H5N1). Unlike other avian influenza viruses, HPAIV H5N1 is highly pathogenic for humans. The average human case-fatality rate among recorded diseased cases still varies considerably, it was more than 50% in 2011 (**ECDC, 2011; WHO, 2011**). Nevertheless, the virus remains scantily adapted to humans and transmission from birds to humans is not uncommon (**Tarantola et al., 2010; ECDC, 2011; Fiebig et al., 2011**).

2.2 Worldwide situation of HPAI subtype H5N1

In April 1997, the first outbreak of HPAI subtype H5N1 was recorded in Hong Kong (**Sims et al., 2003; Minh, 2010**). About 1.5 million poultry in all poultry farms and markets in Hong Kong were destroyed as the result of control procedures. From 1999 to 2002, the virus was persistently isolated from poultry farms and markets in Hong Kong (**Sims et al., 2003; Minh, 2010**). In 2001, HPAIVs were isolated from duck meat introduced from China into the Republic of Korea (**Chen et al., 2004**). At the end of 2003, HPAI of subtype H5N1 from poultry outbreaks was distributed throughout Asia. At the beginning of 2004, HPAI outbreaks were concomitantly recorded in nine Asian countries: South Korea, Vietnam, Japan, Thailand, Cambodia, Laos, Indonesia, China, and Malaysia (**Li et al., 2004; Minh, 2010**).

From 2003 to 2010, outbreaks of HPAIV H5N1 were recorded in Asia, Africa, Europe, and the Middle East. The virus spread into household poultry, undomesticated birds, humans and other mammals. In Europe, Germany reported two outbreaks in backyard poultry in December 2007. In the north central part of Turkey on the Black Sea coast, an outbreak of HPAI H5N1 was detected in backyard poultry in mid-January 2008 as well as in commercial laying hens in the Grimean region of Ukraine. Moreover, several cases were detected in carcasses of a small number of wild swans gathered in December 2007 and January 2008 in Dorset (south-central coast of England), United Kingdom (**OIE, 2008**). HPAI outbreaks in birds eventually have been recorded in more than 60 countries. This disease has then affected both wild birds and land-based poultry.

In humans, the incidence of HPAI subtype H5N1 outbreaks in 1997 in Hong Kong has attracted worldwide concern due to the probability that this might supply satisfactory conditions to begin an influenza pandemic. From 2004 to 2005, human infections with HPAIV H5N1 were recognized in Vietnam, Thailand, Indonesia and Cambodia. A great concern is directed to the subtype H5N1 because, at 22 July 2010, it infected 501 humans in 15 different countries (**Minh, 2010; WHO, 2010**).

2.3 Situation of HPAI H5N1 in Egypt

After Nigeria, Egypt was the second African country to report the infection of poultry with HPAIV H5N1, on 16 February 2006 (**Aly et al., 2008; Hafez et al., 2010**). In the first wave of the disease in 2006, the National Laboratory for Veterinary Quality Control on Poultry Production (NLQP) reported infections of HPAIV H5N1 in 820 commercial poultry farms (1 grandparent, 67 broiler breeders, 332 layers and 366 broilers) and in one quail, 22 ducks and 31 turkey farms. In 20 March 2006, the first case of human infection with H5N1 was reported in Egypt. Between March 2006 and March 2009, the Egyptian Ministry of Health recorded 6355 suspected cases of H5N1 infection (**Kandeel et al., 2010**). In November 2010, 36 fatalities out of 112 laboratory confirmed human cases were reported in Egypt. All confirmed clinical cases of H5N1, except for three, were linked to household poultry possibly infected with H5N1 virus due to either contact with or involvement in the slaughter and de-feathering of backyard birds, approximately one week prior to the beginning of the symptoms (**WHO, 2010; Abdelwhab and Hafez, 2011**).

From 2006 to 2009, the incidence of HPAI H5N1 virus in Lower Egypt was higher than in Upper Egypt in commercial farms, backyards and humans and the outbreaks were concentrated mainly in the Nile delta (northern Egypt) (Table 1) (**Aly et al., 2008; Hafez et al., 2010; Arafa et al., 2012**). In 2006 – 2008, an association of H5N1 outbreaks with winter months was noticed in Egypt. When the temperature increased during the summer and autumn, the occurrence of the disease decreased. On the other hand, in 2009, circulation of the virus all year around has been reported in both commercial poultry and backyard birds (**Abdelwhab and Hafez, 2011; Arafa et al., 2012**).

Table 1: Outbreaks of HPAIV H5N1 recorded in different areas in Egypt from 2006 to 2011*

Region	Total	2005/2006	2006/2007	2007/2008	2008/2009	2009/2010	2010/2011
Delta	1595	708	143	80	161	302	201
Cairo	330	198	24	8	23	51	26
Upper Egypt	513	125	77	31	88	104	88
Other	141	40	38	8	15	14	21
Total	2579	1071	282	127	287	471	336

* Modified from **Arafa et al.** (**2012**)

2.4 Diversifying evolution of HPAIV H5N1

The origin of the Asian HPAIV H5N1 (A/Goose/Gunagdong/96) is thought to be an unidentified ancestral LPAI virus (es) circulating in wild aquatic birds (**Alexander, 2000**). Meanwhile, the current H5N1 virus is mostly a reassortant derived from Goose/GD/96-like virus and non-H5 AIV. Over time, the HPAI of subtype H5N1 virus has varied into numerous phylogenetically distinct lineages, classified according to the WHO/OIE/FAO nomenclature system as clades 0 to 9, which are further sub-diversified into second, third and fourth orders or clusters (**WHO/OIE/FAO, 2008; Fusaro et al., 2010; WHO/OIE/FAO, 2012**).

There are two main prevalent phylogenetic clades: Clade 1 viruses in Cambodia, Thailand, and Vietnam and clade 2 viruses, which moved from China and Indonesia to Europe, the Middle East and Africa. Until now, six distinct subclades of clade 2 have been identified where H5N1 virus of clade 2.2 (designated as European-Middle Eastern-African "EMA" or Qinghai-like in earlier publications) is predominant in central Asia, Europe, the Middle East and Africa (**Chen et al., 2004; Liu et al., 2005; Salzberg et al., 2007; WHO/OIE/FAO, 2009; Fusaro et al., 2010**). In April 2005, the 2.2 clade was firstly discovered during a large outbreak of a phylogenetically distinct H5N1 virus amongst wild bird populations at the Qinghai-Lake in western China and quickly spread west throughout middle Asia and Europe, eventually reaching Africa in 2006 (**Salzberg et al., 2007; Fusaro et al., 2010**). Thereafter, clade 2.2 has diversified into clade 2.2.1 including the Egyptian viruses that recently subgrouped into the clade 2.2.1.1. The later clade includes specifically the antigenic drift variants isolated from vaccinated poultry while the 2.2.1/C "classic" subclade isolated mainly from backyard birds and humans and accidently from vaccinated small-scale commercial poultry (**WHO/OIE/FAO, 2009; Abdelwhab et al., 2012a; WHO/OIE/FAO, 2012)**.

2.5 Laboratory host system for the propagation of AIV

Formerly, two universal systems are used for influenza virus isolation: embryonated chicken eggs (ECEs) and/or tissue/cell culture methods (**Pearson, 2003; El Zowalaty et al., 2011**). Inoculation of ECEs in the allantoic sac of 9 – 11 day-old eggs is the common used route for isolation and propagation of AIV. ECEs are the most sensitive system for AIV propagation due to the growth of viruses with a high titre in eggs, regardless of the host origin of the virus (**Swayne et al., 1998**). Avian, swine, human and equine influenza viruses are usually propagated in ECEs and this method is still widely used for diagnostic purposes and vaccine production (**Swayne and Halvorson, 2003**). Eggs obtained from specific pathogen free (SPF) or AIV-free chicken is the frequent source ever, but eggs from turkeys, muscovy ducks and mallards can support the replication of LPAIV and HPAIV as well (**Capua et al., 2003**). For LPAIV, two or more passages are required to obtain a quantifiable virus, whereas HPAIV kill the embryo within 72 hours after inoculation (**OIE, 2009**). Although ECEs are the most efficient system for growth of influenza viruses, they are expensive and time consuming (**Reina et al., 1997**). Moreover, further identification and subtyping using conventional methods (e.g. haemagglutination inhibition "HI" test) is inevitable which is insensitive, laborious, awkward and can lack specificity (**Pearson, 2003; El Zowalaty et al., 2011**). Importantly, propagation of HPAIV H5N1 to such high titre requires high biosafety containment facilities, which are not available mostly in countries endemic with the virus.

On the other hand, a number of cell cultures and cell lines can successfully support the in-vitro cultivation of the virus. Several cells from different species are used in different laboratories for isolation and propagation of AIV; like primary chicken embryo kidney (CEK) and primary chicken embryo fibroblast (CEF) or cell lines such as Madin-Darby canine kidney (MDCK), chicken bone marrow macrophage (HD11), chicken fibroblast (DF-1), mink lung epithelial (Mv1Lu) cells, quail fibroblast (QT-35), baby hamster kidney (BHK-21), adenocarcinomic human alveolar basal epithelial cells (A549) or kidney of African green monkeys (Vero) (**Suarez, 2008; Moresco et al., 2010**). It is worth mentioning that so far there is no single cell line allows proliferation of all influenza virus subtypes. Moreover, contrary to the HPAIV, addition of trypsin is a prerequisite for isolation of LPAI viruses (**Suarez, 2008**).

2.6 Molecular diagnosis of HPAIV H5N1

Over the previous decade, the use of molecular methods, based on nucleic acid magnification for molecular identification have enhanced the sensitivity and speed for diagnosis and research investigations of AIV (**Pasick, 2008**). Polymerase chain reaction (PCR) is an

influential molecular technique, which used mainly to magnify a single or few copies of DNA to several-million-fold of copies. To use this technique for finding of AIV, a copy of DNA, complimentary (cDNA) to viral RNA, is synthesised using a reverse transcriptase (RT) enzyme and random hexanucleotides or a sequence-specific primer (**Dhumpa, 2011**). The sequence of a target segment within the cDNA is usually amplified using a heat stable polymerase enzyme from the bacterium Thermus aquaticus (TAQ) plus primers. The PCR endpoint analysis is performed by gel electrophoresis. PCR is more sensitive than the traditional virus isolation method. The improved sensitivity is predictable due to the detection of RNA fragments from incompletely packaged virus particles or of viral RNA from contaminated cells (**Carter and Mahy, 1982; Dhumpa, 2011**).

Moreover, molecular subtyping of different AIV serotypes/subtypes/pathotypes could be achieved by specific primers targeting variable or conserved regions along the HA and/or NA genes. Also, simultaneous detection of different HA or NA subtypes has been developed to rapidly identify multiple influenza subtypes in one analytical run (**Hoffmann et al., 2001; Wang et al., 2008; Fereidouni et al., 2009; Gall et al., 2009**). The main challenge for accurate and sensitive diagnosis of AIV using PCR assays is the continuous mutation of the virus. In the event of sequence mismatches, the oligonucleotide primers should be modified to avoid false-negative results from infected flocks (**Ellis and Zambon, 2002**). In Egypt, the 2.2.1.1 clade found in vaccinated commercial birds escaped from the H5-specific PCR assay recommended by the OIE (Slomka et al., 2007). Genetic analysis revealed several nucleotide mismatches in the primers sequon complementary to the viral RNA (**Arafa et al., 2010b**). Modification of those nucleotides improved dramatically the sensitivity of the PCR-assay for detection of the divergent HPAIV H5N1 in Egypt (**Abdelwhab et al., 2010a**). Furthermore, a versatile multiplex PCR assay for simultaneous detection and differentiation of the Egyptian 2.2.1.1 and 2.2.1/C genotypes has been successfully developed (**Abdelwhab et al., 2010b**).

Sequence analysis of influenza genes amplified by PCR may also be done to provide information on novel genetic mutations and/or reassortment of AIV (**Banks et al., 1998**). In many laboratories, sequence analysis of PCR amplicons is a routine practice, particularly of the HA gene, where sequence changes are usually studied in relation to functional aspects by reverse genetics (**Cox and Bender, 1995**). Generated sequences are used to study the phylogenetic relatedness with the circulating or ancestral viruses to better understand the epidemiology of the HPAIV H5N1 (**Sims and Brown, 2008**).

2.7 Serological diagnosis of HPAIV H5N1

Serological assays are inexpensive valuable tools used widely in surveillance activities (**Spackman et al., 2008**). The HI test, agar gel precipitation test (AGPT) and enzyme-linked immunosorbent assay (ELISA) are commonly used tests for detection of antibodies or antigens. HI is a simple test for diagnosis, vaccination monitoring and subtyping of AIV using a panel of subtype-specific antisera or antigens representing the 16 HA subtypes. While the HI and AGPT are found to be more specific for detection of AIV antibodies, ELISA was more sensitive (**Abraham et al., 1986; Swayne et al., 1997; Davison et al., 1998; Lu et al., 2004**). Several commercial ELISA kits are available for the detection of anti-AIV antibodies in serum, plasma, and egg yolk from chicken. ELISA can be more sensitive than the AGPT but may give false-positive results due to poor specificity. The ELISA positive test is routinely confirmed with the AGPT (**Swayne and Halvorson, 2003**). ELISA, based on heterologous (NA) subtypes or on NS1, has been successfully used to differentiate infected from vaccinated birds (DIVA) (**Capua et al., 2003; Zhao et al., 2005**).

In Egypt, monitoring of vaccination efficiency by examination of serum samples collected from vaccinated birds using the HI test is a routine laboratory investigation (**Hafez et al., 2010**). A moderate to strong correlation between the HI titre and the efficiency of H5 vaccines to protect chicken against H5N1 infection was reported (**Tian et al., 2005; Kumar et al., 2007**). In contrast of the original 2.2.1 virus introduced into Egypt in 2006, the new variant 2.2.1.1 clade viruses induce very low titres as shown by testing serum samples obtained from flocks vaccinated with H5N2 vaccines (**Hafez et al., 2010**; **Grund et al., 2011; Kilany et al., 2011; Abdelwhab et al., 2012b; Yoon et al., 2013**). Likewise, a number of H5N1 monoclonal antibodies targeted different epitopes in the HA protein or commercial NP-competitive ELISA were not able to detect anti-H5 or anti-NP antibodies, respectively, in sera obtained from 2.2.1.1 variant vaccinated chicken, indicating a significant antigenic drift of the Egyptian HPAIV H5N1 (**Postel et al., 2011**).

2.8 Prevention and control of HPAIV H5N1

Prevention and control of HPAIV H5N1 are complicated duties that can be achieved by a combination of several measures. No particular set of measures is probably suitable and effective for all countries. The measures are to be selected and modified according to economic situations and the disease condition of each country or area (**Hinrichs et al., 2006**).

The main approaches are: early detection, movement restriction of birds, improvement of biosecurity in poultry farms, stamping out of infected poultry followed by cleaning and

disinfection of poultry farms as well as of live bird markets and associated equipment and facilities (**FAO, 2004; Hinrichs et al., 2006**).

Vaccination of poultry has been recently introduced in several developing countries to mitigate the socioeconomic impact of the endemic HPAIV H5N1 on the poultry industry (**Swayne, 2012**). However, vaccination of poultry as a sole tool is insufficient to eliminate H5N1 in endemic countries and direct control measures must be implemented to support the vaccination campaign (**Capua and Marangon, 2006**). Vaccination against the HPAIV has several benefits: prevention of clinical disease and mortality, decreased shedding of the virus into the environment, increased resistance of the host to infection, reduced bird-to-bird transmission and limited losses in egg production (**Van den Berg et al., 2008; Swayne, 2009**). On the other hand, use of the vaccine masks the field infection, interferes with serological surveillance and increases virus evolution and antigenic drift as the major drawbacks of vaccination against HPAIV (**Capua and Marangon, 2006; Abdelwhab et al., 2011; Grund et al., 2011; Kilany et al., 2011**). Two major types of vaccines have been developed and are used in poultry against HPAIV: (1) inactivated whole virus vaccines, administered by parenteral routes, seeded by the same H5 AIV strain matching the circulating field virus (homologous) or prepared from an unrelated virus of the same subtype (heterologous) (**Swayne and Suarez 2000; Lee et al., 2004**). It has been used extensively to eradicate outbreaks of H5N2 in Mexico, H7N3 in Pakistan and to combat the HPAIV H5N1 in China, India, Indonesia, Viet Nam and Thailand (**FAO, 2011; Swayne, 2012**). (2) Live recombinant vaccines: different viruses have been used as a vector for one or more genes of AIV without interrupting the vector virus replication, like poxvirus, NDV, ILT, Adenovirus, HVT and Vaccinia virus (**Swayne et al., 2000; Lüschow et al., 2001; Qiao et al., 2003; Veits et al., 2008; Swayne et al., 2012)**. Mass application, labour saving, cost-effectiveness and no interference with serological surveillance of vaccinated and infected birds are advantageous (**Swayne et al., 1999; Qiao et al., 2003**). Main disadvantages of the recombinant vaccines, except for the HVT-AIV, are that prior exposure or vaccination of chicken with the vector virus as well as high levels of maternal immunity will lead to vaccination failure (**Rauw et al., 2011**). Non-vaccine alternative approaches for control of HPAIV H5N1 including antiviral therapies, avian-cytokines, RNA interference, genetic breeding and/or development of transgenic poultry as complementary strategies for control of HPAIV H5N1 in poultry were recently reviewed in details by **Abdelwhab and Hafez** (**2012**).

In Egypt, the early control strategy depended on stamping out infected birds, achievement of quarantine measures, movement restriction, cleaning, and disinfection. Nevertheless, the disease spread throughout the country within a short period due to rapid and random movement of live poultry and the lack of geographical barriers between most of the Egyptian

governorates. Consequently, Egypt altered its control policy to include mass vaccination and culling of infected birds. To date, at least 26 H5 vaccines are licensed in Egypt. These vaccines are based on inactivated homologous H5N1 or heterologous H5N2 vaccines as well as on rHVT-H5 recombinant vaccines (**Abdelwhab and Hafez, 2011**).

2.9 Environmental persistence of AIV

A number of studies reported persistence of AIV in faecal materials, water or the environment. In an early experimental study conducted by **Webster et al.** (**1978**), AIV retained infectivity for 32 days in both faecal material and non-chlorinated river water. Persistence of AIV for long periods in water at 4º C, 17º C, and 28º C has been recorded, whereby water temperature, pH, and salinity greatly affected persistence of the virus (**Stallknecht et al., 1990; Brown et al., 2006**). In addition, **Horm et al.** (**2012**) have studied persistence of H5N1 in the environment. They found that H5N1 retained infectivity in rainwater not more than 4 days, but viral RNA was detectable up to 20 days. They also could not detect any infectious virus particles in pond and lake water or mud contaminated with high doses of the virus but viral RNA was detected in water and mud for up to one and two weeks, respectively. Intriguingly, H5N1 remained infectious and viral RNA was detected, although scarcely, in the aquatic fauna and flora. Moreover, **Hénaux et al.** (**2012**) reported continuous circulation of LPAIV during summer in unfavourable environmental conditions in California wetlands. Similar observation has been recently reported in Egypt where a virus of the 2.2.1/C group was isolated from backyard native ducks in mid-summer (**Hassan et al., 2012**).

2.10 Virus stability to physical factors

Several studies have addressed the sensitivity of AIV, particularly HPAIV H5N1, to ultraviolet (UV) light, heat, sunlight, relative humidity and pH. Exposure to UV light was effective for viral destruction on a clean surface, water or in air but not in faecal material, as it protects the virus from direct UV light (**Jeffrey, 1995**; **Lu et al., 2003; Chumpolbanchorn et al., 2006; Lénès et al., 2010**). In contrast, **Shahid et al. (2009)** found that UV light was not effective in inactivating virus completely even after 60 minutes. Moreover, **Birnbaum and O'Brien** (**2008**) mentioned that AIVs are generally sensitive to heat and **Lu et al.** (**2003**) confirmed that heat accelerates the inactivation of the AIV in manure. At temperatures of 40º C, AIV in manure may be killed within short time (**Chumpolbanchorn et al., 2006**). HPAIV H5N1 can persist at 4° C for more than 100 days but the virus was inactivated after 24 hours at 28° C, 30 minutes at 56° C (**Shahid et al. 2009)** and after 3 min at 70° C (**Songserm et al., 2006b).** Likewise, at 4° C infectivity of H5N2 in wet faecal matter was retained after 35 days but was inactivated after incubation at 25° C for 2 days (**Beard et al. 1984**). In addition,

Chumpolbanchorn et al. (**2006**) showed that AIV lost its infectivity within 24 hours at 25° C and at 40° C within 15 minutes. **Lu et al.** (**2003**) reported inactivation of H7N2 virus within less than a week at an ambient temperature of 15-20° C. The virus lost infectivity completely within 30 minutes after direct exposure to sunlight at an ambient temperature of 32 to 35° C. In contrast, infectivity was retained after 4 days in the shade at 25 to 32° C (**Songserm et al., 2006b**). Moreover, **Wood et al.** (**2010**) reported persistence of HPAIV on glass and steel after 13 days at low temperature and relative humidity conditions. Acidic pH (1- 3) and basic pH (11- 13) had virucidal effect after 6 hours contact time for H5N1 (**Shahid et al., 2009**) and pH 2 for H7N2 for 2 minutes (**Lu et al., 2003**); yet, H5N1 virus retained infectivity at pH 5 (18 h), at pH 7 and 9 for more than 24 hours as reported by **Shahid et al.** (**2009**). Principally, **Wanaratana et al.** (**2010**) noticed variation among different H5N1 viruses in sensitivity to pH and temperature.

2.11 Inactivation of AIVs by chemical agents

While considerable published information and disinfection efficacy data regarding bacteria and fungi exist, the efficacy of chemical disinfectants against viruses (**Bieker, 2006**), particularly HPAIV H5N1, is scanty. Chemical disinfectants acting against AIVs can be grouped into soaps and detergents, acids, alkalis, chlorine and chlorine compounds, aldehydes, oxidizing agents, phenol compounds, alcohols and quaternary ammonium compounds (QACs) (Table 2) (**Klein and Deforest, 1965, 1983; Evans et al., 1977; Scott, 1979; Maris, 1990: Maris, 1995; AusVetPlan, 2005; DeBenedictis et al., 2007**). It is worth pointing out that the mechanisms of virucidal activity by chemical agents are not widely understood but theories exist (**Maillard and Russell, 1997; Maillard, 2001; Lambert, 2004**).

Lu et al. (**2003**) mentioned that H7N2 virus was inactivated after contact with 70% ethanol in less than 30 minutes. **Wanaratana et al.** (**2010**) showed very low, if any, resistance of different HPAIV H5N1 against QAC, chlorine and phenol (Table 3). **Lénès et al.** (**2010**) reported effectiveness of ozone, chlorine and chlorine dioxide in inactivating HPAIV H5N1, whereas monochloramine required relatively higher doses and extended contact times to induce significant reductions. **Muhmmad et al.** (**2001**) confirmed that AIV is very sensitive to detergents, probably due to the destruction of the fat-containing virus envelop. **Shahid et al.** (**2009**) observed that soap (lifebuoy®), detergent (surfexcel®) and alkali (caustic soda) destroyed infectivity of H5N1 after 5 min at 0.1, 0.2 and 0.3% dilution and all commercially available disinfectants inactivated virus at recommended concentrations (Table 3).

Table 2: List of available chemical disinfectants against HPAIV (modified from **DeBenedictis et al., 2007**)

Chemical product	Recommended Concentration	Mode of action	Recommended contact time	Reference
Alkalis Sodium hydroxide Sodium carbonate (washing soda) Calcium hydroxide	2% 10% 3%	Breaking down the cell walls of viruses **(**lipid-containing virus has up to several layers of fat that sheaths the nucleic acid that the virus uses to infect the host cell. Alkaline disinfectants stop the growth of these viruses**)**	10 min 30 min	**Birnbaum and O'Brien (2008)**
Acids Hydrochloric acid (inorganic acid) Citric acid (organic acid)	2–5% 0.2%	Inhibition of enzymatic reactions. Proteins and nucleic acid denaturize	10 min 30 min	**Yilmaz et al. (2004)**
Chlorine compounds Calcium hypochlorite Sodium hypochlorite (household bleach)	2–3% 2–3%	Protein denaturizing and oxidizing	10-30 min 10-30 min	**AusVetPlan (2005)**
Oxidizing agents Hydrogen peroxide Virkon®	3–6% 1-2%	Denaturizing activity on lipids and DNA	30 min	**Muhammed et al. (2001)**
Aldehydes Formalin Glutaraldehyde Formaldehyde	8% 1–2% 40%	Alchilation of amino and sulphydrilic groups of protein and nitrogen of the purinic basis	10-30 min 10-30 min 15-24 h	**AusVetPlan (2005)**

2.12 Factors affecting the efficacy of chemical disinfectants

Different environmental aspects such as humidity, pH, temperature, and organic load considered to have a high impact on the efficacy of commercially available chemical disinfectants against AIV (**Maris, 1995; Sattar and Springthorpe, 1999; Prince and Prince, 2001; Quinn and Markey, 2001; Maillard, 2004; Bieker, 2006**). The rate of chemical reaction can be enhanced in warm climate conditions, but the stability of chemical disinfectants can be affected by very high temperatures. In contrast, cold environmental temperatures will reduce the efficacy of chemical agents, and very cold climate can freeze liquid-based chemical disinfectants. Factors like pH have the major impact on acidic- and alkaline-based chemical disinfectants (**Bieker, 2006**). These chemical agents need specific pH ranges (acidic for the acid based disinfectants and basic for the alkaline compounds) and are sometimes affected by the presence of heavy organic soiling (**Prince and Prince, 2001;**

Quinn and Markey, 2001). Additional factors can come into play depended on the proposed application of a chemical agent. Several chemical disinfectants also have corrosive properties of undesirable effects on some materials being treated. **Quinn and Markey** (**2001**) and **Bieker** (**2006**) found that the deterioration of metal surfaces that were treated with bleach is a primary example of corrosive properties of chemical disinfectants. Exposure time of the disinfectant on sensitive surfaces or materials will also influence the degree of corrosion. Furthermore, chemical disinfectants possess their own inherent stability properties and the disinfectant product shelf life can be an important issue (**Quinn and Markey, 2001; Bieker, 2006**).

2.13 Suspension and carrier tests used for inactivation studies of influenza viruses

Different methods used for testing the virucidal efficacy of chemical disinfectants exist both nationwide and worldwide in the form of guiding principles and published standards. The most important two methods used for evaluating the virucidal efficacy of different types of chemical disinfectants are suspension and carrier tests (**Bieker, 2006**). Suspension tests evaluate the virus as liquid inoculum, while carrier tests are designed for evaluating virus inoculum onto various materials. The standard protocols for testing the virucidal activity of various biocides in suspension as well as carrier tests have been described in detail (**Maillard, 2004; CEN, 2005; OECD, 2009**). In Germany, the virucidal testing of chemical disinfectants used in the veterinary field is performed according to the guidelines of the German Veterinary Medical Society "DVG" (**Anonymous, 2007**). The basic protocols involve the use of Newcastle disease virus (NDV) as well as the Vaccinia virus as representatives of enveloped viruses and the enteric cytopathogenic orphan (ECBO) virus as well as the Reovirus as representatives of non-enveloped viruses as test organisms. Testing of virucidal activity of chemical disinfectants by DVG methods using organic soiling and surface porosity may under field conditions significantly interfere with the inactivating potency of chemical substances. According to the guidelines, tests have to be performed at room temperature (20–22º C). However, several experiments stated that temperature is one of the most important factors to be considered when using chemical disinfectants (**Herbst et al., 1990; Haneke, 1991; Yilmaz et al., 2004**); especially the efficacy of aldehydes and organic acids is reduced at temperatures under 20º C (**Yilmaz and Kaleta, 2003**). In the European Committee for Standardization (CEN), 10º C is designated as the test temperature. Additional testing temperatures at room temperature, 0º C, 4º C and 10º C are optional. Another difference between the guidelines is the protein load. According to DVG-guidelines, the suspension test is run using 40% foetal calf serum (FCS) as a protein load, whereas the CEN protein load consists of yeast extract and bovine serum albumin (BSA) (low level 0.4% and high level 2%) (**Yilmaz and Kaleta, 2003**).

Yilmaz et al. (**2004**) studied the efficacy of two commercial disinfectants against AIV using suspension tests and poplar wood carrier tests loaded with serum to simulate the field conditions considering organic soiling and surface porosity. The tests were carried out at 20° C for reaction times of 15-120 min and additionally at 10 and 4° C for reaction times of 5 and 10 min. Both disinfectants were initially effective but showed losses of efficacy when organic load increased and temperature decreased. Both disinfectants were suitable at 20° C but for safe inactivation at 4° C the contact time had to be extended up to 120 min. To evaluate the virucidal activity of six commercial disinfectants against LPAI viruses, **Lombardi et al.** (**2008**) used materials made up of metal, plastic and wood as carriers, which are typically present in a poultry house. They noticed that all tested disinfectants were effective at maximum concentrations, although not all of the tests on porous surfaces were conclusive and a lower neutralization index for wood was believed to be due to better recovery from the media rather than poor inactivation on the surface.

Table 3: Chemical disinfectants used for inactivation of highly pathogenic H5N1 avian influenza viruses

Subtype (clade)	Virus	Disinfectant (Concentration)	Source	Temperature	Contact time	Results	Reference
H5N1	A/crow/Kyoto/T2/04	Povidone-iodine copound products (0.2, 0.5, 0.25, and 0.23%)	Allantoic fluid	25° C	10 sec	Virus was inactivated	**Ito et al. (2006)**
H5N1 (Clade 2)	A/chicken/Hong Kong/D-0947/2006, A/WhooperSwan/Mongolia /244/2005	Chlorine	Water	5° C	1 min	Both viruses were inactivated by chlorine	**Rice et al. (2007)**
H5N1	Local Pakistani strain (not mentioned)	Formalin (Formaldehyde; Merck), Phenol crystals (Merck), Iodine crystals (Merck), CID 20 (CID LINES®, Belgium), Virkon®-S (Antec™ International, UK), Zeptin 10% (Nawan laboratories, Pakistan), KEPCIDE 300 (KEPRO B.V., Holland), and KEPCIDE 400 (KEPRO B.V., Holland)	Amnio-allantoic fluid	28° C	15, 30, 45 and 60 min	(1) The virus was inactivated by soap (lifebuoy®), detergent (surf excel®) and alkali (caustic soda) after 5 min at 0.1, 0.2 and 0.3% dilution. (2) The virus was inactivated with all tested commercial disinfectants at recommended concentrations	**Shahid et al. (2009)**
H5N1 (Clade 1)	A/Cambodia/408008/2005	Chlorine-based disinfectants	Water	22±2° C	5 min	Virus was inactivated	**Lénès et al. (2010)**
H5N1	A/chicken/Chonburi/Thailand/CU-7/04, A/chicken/Nakorn Patom/Thailand/CU-K2/2004, A/chicken/Ratchaburi/Thailand/CU-68/04	20% glutaraldehyde (Glu), 50% hydrogen peroxide (HP), 10% quaternary ammonium compounds (QAC), 10% Glu + QAC, 3% iodine, 1% chlorine, formalin and phenol	Allantoic fluid	25 and 37° C	10 min	(1) All viruses were resistant to HP and iodine (2) All viruses were relatively sensitive to Glu + QAC, chlorine and phenol at both temperatures (3) Viruses showed variable sensitivity to QAC alone	**Wanaratana et al. (2010)**

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Avian influenza virus

Two H5N1 isolates, A/chicken/Egypt/0626/2006 (designated here as EGY06) and A/chicken/Egypt/1094/2010 (designated as EGY10) were obtained from the repository of the Department of Poultry Diseases and Hygiene, Faculty of Veterinary Medicine, Alexandria University, Egypt. They were selected according to the NLQP database of official outbreaks reported to the General Organization of Veterinary Services in Egypt, which indicated that the most frequent outbreaks were recorded in 2006 and 2010 with 1071 and 471 outbreaks, respectively. The two strains were isolated from cloacal and tracheal swabs of broiler flocks with high mortality during the 2006-2010 HPAIV H5N1 outbreaks in two different areas along the Northern Coast of Egypt. The first strain, EGY06 was isolated from a non-vaccinated flock in February 2006 in the Alexandria governorate. While the second strain, EGY10, was isolated from a vaccinated flock in November 2010 in the Marsa Matrouh governorate.

3.1.2 Embryonated chicken eggs

Specific Pathogen Free (SPF) chicken embryonated eggs were purchased from Kom Oshiem Farm, Fayoum province, Egypt.

3.1.3 Chemical disinfectants

No.	Disinfectant	Contents	Concentration	Company
1	Formalin	Formalin	38%	Alpha Chemical (39 Melsa Buildings, Cairo, 11361, Egypt
2	Glutaraldehyde	Glutaraldehyde		ADWIC, Abu Zaabal Area, 491, Cairo, Egypt
3	Virkon®-S	Potassium Peroxymonosulfate	21.41%	Antec International- A DuPont Company (Sudbury, Suffolk C010 2XD, UK)
		Sodium Chloride	1.5%	
		other ingredients	77.09%	
4	TH4®	Didecy Dimethyl Ammonium Chloride	18.75g	Sogeval (Laboratoire Sogeval, 200, avenue Mayenne, 53022 Laval Cedex 9- France)
		Octyldecyl Dimethyl Ammonium Chloride	37.5g	
		Alkyl Dimethyl Benzyl Ammonium Chloride	50g	
		Glutaraldehyde	62.5g	

3.1.4 Wood and Gauze carriers

Source	Description	Carrier
The poultry farm of the Faculty of Veterinary Medicine, Alexandria University, Egypt	2 cm^2 large and 1 mm thick	Pieces of poplar wood
Tiba pharma company, Alexandria, Egypt	2 cm^2 large and 4 layers thick	Gauze

3.1.5 Solutions and reagents

3.1.5.1 Phosphate buffer saline (PBS)

Weight	Reagent
8.0 g	NaCl
0.2 g	KCl
1.15 g	Na_2HPO_4
0.2 g	KH_2PO_4
Add to 1 liter	Distilled water

Autoclave at 12° C for 15 minutes

3.1.5.2 Bovine calf serum (BCS)

Sigma-Aldrich Chemie GmbH, Germany Product NO.12133C Bovine calf serum

3.1.5.3 Chicken red blood cells (RBCs)

Blood was collected from the wing vein of chicken in sterile tubes containing 3.8% sodium citrate solution.

3.1.5.4 Antibiotic solution for 1 liter PBS

$2x10^6$ IU	Penicillin G
200 mg	Streptomycin
$0.5x10^6$ IU	Mycostatin
250 mg	Gentamycin

3.1.5.5 RT-PCR reaction mix

RT-PCR amplification was carried out using Qiagen® Onestep kit as mentiond in point 3.2.2.2.2.

3.1.5.6 Agarose gel (1.5%)

Agarose gel medium (1.5%) was prepared as agarose powder (ABgene) 1.5 gm plus 100 ml of 1× Tris Acetate EDTA (TAE) buffer (Serva, Germany). The agarose was melted in the microwave until it was completely melted.

3.1.5.7 Ethidium bromide solution

Ethidium bromide solution (10 mg/ml) was prepared from Ethidium bromide powder (Sigma) 10 mg and 1.0 ml of sterile distilled water.

3.1.6 Instruments and equipments

3.1.6.1 Bio-Rad iCycler Thermal Cycler (2×48 PCR Machine)

PCR amplification of the two isolates of H5N1 was done by using the iCycler thermal cycling instrument (iCycler, Biorad, USA), which provides optimum performance for PCR and other thermal cycling techniques.

3.1.6.2 Applied Biosystems 3130 genetic analyzer

A sequencer machine (Applied Biosystems 3130 genetic analyzer, 4 capillaries system; 80 cm, Hitachi, Japan) was used after adjustment of its software for genetic analysis of H5N1.

3.1.6.3 Microtiter plates

V-shaped 96 Well Microtiter Microplates were obtained from the Sigma Aldrich Chemicals Company (Eschenstrasse 5, 82024 Taufkirchen, Germany). These plates were used in HA and HI tests.

3.1.6.4 Multichannel micropipette

The multichannel micropipette from 5-50 µl (Biohit Proline®, Helsinki, Finland) was used in the current study for HA and HI tests.

3.1.6.5 Vortex-Genie

The vortex-Genie Model K 550-GE (USA) was used for mixing the samples.

3.1.7 Kits

Kit	Cat-No.	Manufacturer
QIAamp viral RNA Mini Kit	52904	Qiagen GmbH, Hilden, Germany
Qiagen one step RT-PCR Kit	210212	Qiagen GmbH, Hilden Germany
QIAquick Gel Extraction kit	28704	Qiagen GmbH, Hilden Germany
BigDye® Terminator V3.1 Cycle Sequencing kit	4336935	Applied Biosystems, Foster City, USA
Centri·Sep spin columns	CS-901	Princeton Separations Inc., Adelphia, New Jersy, USA

3.1.8 Software

Software	Version	Reference
DNA sequencing analysis software	5.1	Applied Biosystems
SecScape	2.5	Applied Biosystems
BioEdit	7.1.9	Hall (1999)
Multiple Evolutionary Genetics Analysis (MEGA 5)	5.0	Tamura et al. (2011)
Multiple Sequence Alignment (MUSCLE)	3.5	Edgar (2004)
Inksacpe	0.48	Inkscape.org

3.1.9 Primers used for RT-PCR amplification of HA and NA segments of HPAIV H5N1

Table 4: Primers used for amplification of HA and NA genes of HPAIV H5N1

Segment/ Fragment (size)	Primer designateon	Primer sequence *	Reference
H5/A (1085 bp)	4FV2	^{21}AGCAGGGGTTCAATCTGTCAAAA^{43}	**Starick et al. (2008)**
	H5-1064R	^{1105}ARCCTGCTATAGCYCCAAAT^{1086}	**NLQP****
H5/B (EGY06) (976 bp)	KH1	^{835}CCTCCAGARTATGCMTAYAAAATTGTC^{861}	**Slomka et al. (2007)**
	HR	^{1794}ATATCGTCTCGTATTAGTAGGAAACAAGGGTGTTTT^{1773}	**Hoffmann et al. (2001)**
H5/B (EGY10) (977 bp)	H5-F4	^{817}AGTAATGGAAATTTCATTGCTCCAGAA^{843}	**NLQP**
	4RV2	^{1794}AGTAGAAACAAGGGTGTTTTTAACTA^{1768}	**Starick et al. (2008)**
N1/A (815 bp)	6FV2	^{17}AGCAAAAGCAGGAGTTTAAAATGA^{40}	**Starick et al. (2008)**
	N1-R2	^{807}GGCATCAGGATAACAGGAGCACTCC^{831}	**NLQP**
N1/B (804 bp)	N1-608	^{609}AATAACAGACACCATCAAGAGTTG^{632}	**NLQP**
	6RV2	^{1396}TAGAAACAAGGAGTTTTTTGAACAAAC^{1370}	**Starick et al. (2008)**

*Upper-case numbers refer to the sequence corresponding to the binding sites of the primers for the selected reference viruses A/chicken/Egypt/06541-NLQP/2006 for the HA gene and A/chicken/Egypt/12378N3-CLEVB/2006 for the NA gene. Nucleotide numbering begins with the signal peptide in the HA.

** Unpublished internal primers are designated by NLQP in Egypt. All primers were purchased from Metabion International AG, Martinsried, Germany.

3.2 Methods

3.2.1 Virus propagation and isolation

H5N1 virus was propagated in SPF ECEs via the allantoic sac route of inoculation. Eggs were kept in the egg incubator at 37º C with humidity 40-60% for 9-11 days. The eggs were inoculated with suspected fluid and then incubated at 37º C for 72 hours. The allantoic fluid (AF) was then harvested and kept at -80º C until use.

3.2.2. Identification of AIV subtype H5N1

3.2.2.1 Classical methods

Classical methods (HA and HI tests) for the identification of the two isolates were carried out in the Department of Poultry and Hygiene, Faculty of Veterinary Medicine, Alexandria University, Egypt. The allantoic fluids were tested primarily by the HA test and confirmed by HI test, using monospecific antiserum against AIV H5N1 for the presence or absence of haemagglutinating viruses (**OIE, 2009**).

3.2.2.1.1 Rapid slide haemagglutination test

Blood was collected from the wing vein of chicken in sterile tubes containing 3.8% sodium citrate solution. An equal volume of PBS was added and the blood was centrifuged at 3000 round per minute (rpm) for 10 minutes. After 3 wash cycles, 0.5% RBCs in PBS was used for HA and HI tests. Briefly, 0.1 ml of harvested fluid was taken from an SPF embryonated chicken egg and placed in a glass slide plus an equal amount of 10% washed chicken RBCs was used for the rapid slide agglutination test.

3.2.2.1.2 Haemagglutination test

The test was conducted as previously described (**OIE, 2009**). Each well of a microtitre 96-well V-bottomed plate was initially filled with 25 µL of PBS. Then, 25µl of a virus sample was added in each well in the first line of the plate and a two fold dilutions was made across each line for the whole plate. Lastly, 25 µL of 0.5% chicken RBCs were added to each well and the plate was incubated for about 45 min at room temperature. Reading of the results was done by sloping the plate 45 degrees and the HA activity was detected by the formation of tear shaped streaming of RBCs. One HA unit in the haemagglutinin titration is the smallest amount of virus that will cause complete agglutination of the RBCs. The last well that demonstrates complete agglutination is the well that contains one HA unit.

3.2.2.1.3 Haemagglutination inhibition test (HI)

The HI was done after (**OIE, 2009**) using a V-bottom disposable plate. A total of 25 µL of allantoic fluid from each sample was dispensed into the first well of the microwell plate and then two-fold dilutions were started with 25 µL PBS. Negative and positive control allantoic fluid samples were included on one plate. Then 25 µl of monospecific AI H5N1 antiserum (prepared by the Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo, Egypt) (4 HA units) were added to each well. Then, 25 µl of 0.5% chicken red blood cell suspension were put in each well. The sides of the plate were gently tipped for mixing. A cover was placed on the plate. The plate was allowed to stand for 45 minutes at room temperature. The results were observed and recorded.

3.2.2.2 Molecular characterization and phylogenetic analysis of HPAIV H5N1

Molecular identification and genetic analysis were conducted in the gene analysis unit of the National Laboratory for Veterinary Quality Control on Poultry Production (NLQP), Egypt.

3.2.2.2.1 Viral RNA extraction

Total RNA extraction was done by using the QIAamp Viral RNA Mini Kit (Cat no. 52904, GmbH, Hilden, Germany) according to the manufacturer guidelines using 140 µl sample and the RNA was eluted in 60 µl AVE buffer provided by the kit, then stored at 0-4° C when used within 2-3 days, otherwise kept at -80° C.

3.2.2.2.2 RT-PCR

Reverse transcription and PCR reaction for amplification of the HA and NA genes of H5N1 viruses were done in one step using the Qiagen® Onestep RT-PCR Kit. Each gene was divided into two overlapped fragments to be easy amplified, using primers described in point 3.1.9 in a total volume of 25 µl as following:.

Reagent	Volume (µL)/reaction
Buffer 5x containing 12.5 mM MgCl2	5
QIAGEN One Step RT-PCR Enzyme Mix	1
Forward primer	1
Reverse primer	1
dNTP Mix, 10 mM	2
RNase-free water	10
Template RNA	5

The thermal profile of the RT-PCR was as following:

Step	Temperature	Time	Cycle
RT	50° C	30 min	1 x
	95° C	15 min	
PCR	95° C	30 sec	40 x
	56° C	45 sec	
	72° C	2 min	
	72° C	10 min	1 x
Storage	4° C		

To amplify full HA and NA gene segments, two overlapping regions (denoted A and B) were amplified separately for each gene segment (H5 and N1 gene). For the HA gene, primer pairs 4FV2 and H5-1064R were used for amplification of fragment H5/A for both viruses, EGY06 and EGY10, with expected fragment size of 1085 bp. Primer pairs KH1 and HR and primers H5-F4 and 4RV2 were used to amplify 976 and 977 bp of fragment H5/B of EGY06 and EGY10, respectively. For the NA gene of both viruses, primers 6FV2 and N1-R2 were used to amplify 815 bp of segment N1/A and primers N1-608 and 6RV2 for amplification of 804 bp of the overlapping N1/B gene fragment.

3.2.2.2.3 Agarose gel electrophoresis

Agarose gel medium (1.5%) was prepared, melted in the microwave until it was completely clear and mixed with ethidium bromide solution (0.1- 0.5 µg/ml). The PCR products were inoculated (8 µl of product mixed with 3 µl of loading dye) in agarose gel. For determination of the DNA fragment size in parallel 5 µl 100-bp DNA ladder was loaded. Then the gel was covered with 1X TAE buffer and run at 95 volt for 40 min. The gel was examined by a UV transilluminator for DNA visualization.

3.2.2.2.4 Purification of the PCR products

The purification of the PCR products was done using the QIAquick Gel Extraction kit according to the manufacturer recommendations, where the DNA was finally eluted in 50 or 30 µl of the provided buffer EB.

3.2.2.2.5 Sequencing reaction

The sequencing reaction of the purified one step RT-PCR products was performed using the Terminator V3.1 kit following the instructions provided by the producing company. Each reaction was adjusted to a total volume of 20 µl as following:

Reagent	Volume (µL)
Big dye terminator v.3.1	2
Primer	1
Template DNA	1 to 10
PCR grade water	Add to 20

Then the reaction was loaded to the genetic analyzer following this thermal profile:

Cycle	Time	Temperature
1 x	1 min	96˚ C
25 x	10 sec	96˚ C
	5 sec	50˚ C
	2 min	60˚ C

3.2.2.2.6 Purification of sequence reaction

Using the Centri-sep spin columns kit according to the manufacturer protocol, the sequence reactions were purified from the unincorporated dye terminators as well as possible salts and protein contamination which may interfere with the electrokinetic injection of the instrument.

3.2.2.2.7 HA and NA genes sequencing

Once the cycle sequencing reaction was completed and purified, the sample was analyzed using a DNA Sequencer. The purified sequence reaction was mixed well with 10 µl of Hi-Di formamide. The mixture was arranged in 96 well plates and was loaded to the sequencer machine.

3.2.2.2.8 Sequence analysis

The obtained sequences were firstly viewed and edited by the DNA sequencing analysis software version 5.1. Then, partial overlapping generated sequences of both HA and NA genes were assembled by SecScape V 2.5 to obtain full gene sequences. Using the nucleotide Basic Local Aligment Search Tool (BLASTN) our query sequences were entered, the highly similar sequences (megablast)" option was selected and other advanced options for blast search were set as default. Similar sequences were identified and retrieved from the GenBank database. Also both viruses were compared with the available gene HA and NA sequences of A/chicken/Egypt/06207-NLQP/2006 (HA accession number: EU372943.1), one of the earliest viruses introduced into Egypt in 2006 and designated here as the parent virus, as well as with isolated viruses from the Alexandria and Marsa Matrouh provinces. BioEdit software was

used to generate alignment between the retrieved and generated sequences and were further manually edited. Potential N-linked glycosylation sites of the HA and NA proteins were predicted using the NetNGlyc 1.0 Server, where N-X-S/T and X can be any amino acid residue except proline (**Gupta et al., 2002**). Moreover, potential O-linked glycosylation amino acid residues were predicted by the NetOGlcy 1.0 server (**Julenius et al., 2005**). Amino acids mutations found in EGY10 in comparison to EGY06 were imposed on the tertiary structure of the H5 and N1 proteins using 3D-JIGSAW (**Contreras-Moreira and Bates, 2002**), then viewed and edited by RasTop version 2.7.1. Amino acid numbers mentioned in this study are according to the H5 and N1 numbering systems of the EGY06 virus. Phylogenetic relatedness of our viruses to representative viruses from Egypt and Asia were done by the Neighbor-Joining and bootstrap analysis was performed with 1000 replications as implemented in MEGA5. Evolutionary trees were further edited using the Inkscape software 0.48 for improving the quality.

3.2.3 Inactivation of HPAIV H5N1 using commercially available disinfectants

Virucidal efficacy of chemical disinfectants using suspension test with and without protein and a wood carrier test were carried out according to the DVG guideline (**Anonymous, 2007**).

3.2.3.1 Titration of HPAIV H5N1 for infectivity

The mean embryo infectious dose (EID_{50}) of HPAI H5N1 viruses was measured by ten-fold serial dilutions (10^{-1} – 10^{-9}) of the allantoic fluid suspension. Each dilution was inoculated in 9 – 11 day-old SPF ECEs via the allantoic sac. Eggs were incubated for 3 days at 37º C. After 3 days incubation, the AF was harvested from each egg and tested for HA activity to determine the presence or absence of AIV. The results were tabulated and the EID_{50} was then calculated by the formula of **Reed and Muench** (**1938**).

3.2.3.2 Suspension test with or without protein

In the suspension test without protein a solution with 1 ml virus suspension, 0.8 mL PBS and 0.2 ml of the ten-fold concentration of each disinfectant was used at room temperature (20-22° C). In the suspension test with protein the PBS was replaced by 0.8 ml BCS. Then, 0.1 ml aliquots of this mixture were taken after 15, 30, 60 and 120 min and diluted in 9.9 ml of PBS. The HA activity of H5N1 was tested on slides for the presence or absence of the virus. Estimation of the HA titre was done using a microtitre plate incubated at room temperature.

3.2.3.3 The wood and gauze carrier test

A suspension of 1 ml H5N1 virus, 0.8 ml of bovine calf serum (BCS) and 0.2 ml ten-fold concentrated disinfectants (Formalin, Glutaraldehyde, TH4® and Virkon®S) was prepared. Briefly, 0.1 ml of the protein loaded virus suspension was dropped onto each wood and gauze carriers. The carriers were dried in sterile petri dishes for 60 min at room temperature. The infected carriers were then immersed in selectable disinfectants in their final concentrations of 0.5, 1% and 2% and left at room temperature for 15, 30, 60 and 120 min afterwards; each carrier was homogenized, and then placed in 9.9 ml PBS. Vortex was used for centrifuging the samples. The HA activity of H5N1 was tested on slides for the presence or absence of the virus. Estimation of the HA titre was done using a microtitre plate incubated at room temperature.

CHAPTER 4: RESULTS

The primary objective of this study focused on rapid molecular diagnostic methods for the identification and subtyping of HPAIV in Egypt. The second aim was to determine in vitro the efficacy of four different commercially available chemical disinfectants on the two isolates.

4.1 Detection and identification of AIV subtype H5N1

4.1.1 Classical methods

Both isolates showed positive results when titered in the microplate HA test. The HA titre of **EGY06** was estimated to be 1:64 (Log_2 ^{6}) while the HA titre of **EGY10** was 1:128 (Log_2 ^{7}).

4.1.2 Molecular identification and phylogenetic analysis of HPAIV subtype H5N1

4.1.2.1 Detection of HPAIV subtype H5N1 by RT-PCR

The full HA and NA gene sequences were amplified using the generic primers described in Table 5. We succeeded to amplify the whole HA and NA gene segment of the two H5N1 viruses. As shown in Figure 1, each gene fragment was amplified in two different RT-PCR reactions and bands were at the expected molecular weight.

Table 5: Description of results obtained by RT-PCR to detect HA and NA full genes of the selected isolates of AIV subtype H5N1

No.	Sample name	Primers used	base pairs (bp) length
1	HA, fragment A of EGY06	4FV2 + H5-1064R	1085
2	HA, fragment A of EGY10	4FV2 + H5-1064R	1085
3	Negative control	-	-
4	HA, fragment B of EGY06	KH1 + HR	976
5	HA, fragment B of EGY10	H5 F4 + 4RV2	977
6	Negative control	-	-
7	NA, fragment A of EGY06	6FV2 + N1 R2	815
8	NA, fragment A of EGY10	6FV2 + N1 R2	815
9	Negative control	-	-
10	NA, fragment B of EGY06	N1 608 + 6RV2	804
11	NA, fragment B of EGY10	N1 608 + 6RV2	804
12	Negative control	-	-

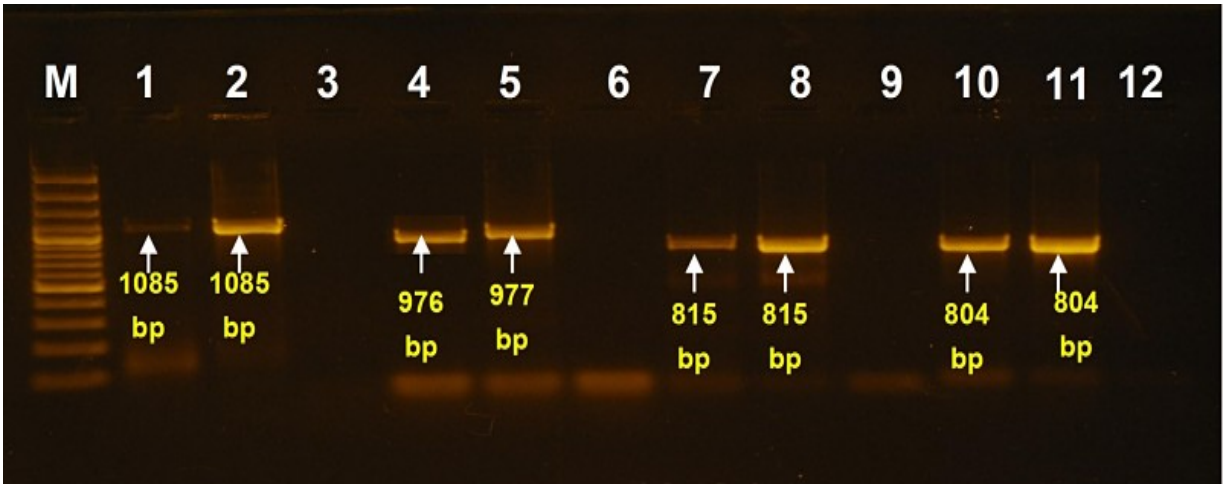


Figure 1: Amplification of HA and NA genes of EGY06 and EGY10 H5N1 using RT-PCR
Shown: lane M, DNA molecular marker (100 bp ladder); lanes 1 and 4, fragments H5/A and H5/B of HA gene for EGY06 (1085 and 976 bp, respectively); lanes 2 and 5, fragments H5/A and H5/B of HA gene for EGY10 (1085 and 977 bp, respectively); lanes 3, 6, 9 and 12, negative controls; lanes 7 and 10, fragments N1/A and N1/B of NA gene for EGY06 (bps 815 and 804, respectively); lanes 8 and 11, fragments N1/A and N1/B of NA gene for EGY10 (bps 815 and 804, respectively)

4.1.2.2 Sequence analysis of the hemagglutinin of HPAI H5N1 EGY06 and EGY10

Sequences of the HA genes of EGY06 and EGY10 were submitted to the GenBank and their accession numbers are KF178948 and KF178950, respectively. The length of the EGY06 HA gene is 1707 nucleotides which encodes 568 amino acids (aa); 16 aa in the signal peptide and 552 aa in the mature HA protein. The length of HA of EGY10 is 1704 which encodes 567 aa (16 aa in the signal peptide and 551 aa in the mature HA protein). The HA length of EGY06 is similar to the parent-2006 virus, while the EGY10 has three-nucleotide-deletion ^{433}TCA^{435} which encodes the aa serine at position 145 (S145) (H5 numbering). This deletion (denoted as S145Δ) is shown in Figure 2 as black bold arrow. Compared with the parent 2006 virus the HA of EGY06 has only 3 nucleotide differences with 99.82% homology, while the EGY10 has 31 nucleotide differences compared with both parent and EGY06 viruses, with 98.18% homology.

A total of 11 amino acid substitutions were found in EGY10 compared to both the parent and EGY06 viruses with 98.06% identity. One mutation occurred in the signal peptide through substitution of the leucine (in the parent virus) by isoleucine in EGY10 virus as shown in Figure 2. Ten mutations were observed in the HA1 subunit, namely D59N, S136D, S145Δ,

I167T, D170N, N171D, R178K, N209K, G288S and R341K. All strains have 6 potential GS. Four potential GS are located at the HA1 subunit: ^{26}NNS^{28}, ^{39}NVT^{41}, ^{182}NTN^{184}, ^{302}NSS^{304} and two GS in the HA2 subunit: ^{500}NGT^{502} and ^{559}NGT^{561}. The PCS of all viruses have multiple basic amino acids where the EGY06 is identical to the parent virus with the E**R**RRKKR*GLF motif but EGY10 has the E**K**RRKKR*GLF motif. All mutations in the HA1 subunit occurred in "coil" secondary structures, except I167T which occurred in "strand" structure (Figure 2).

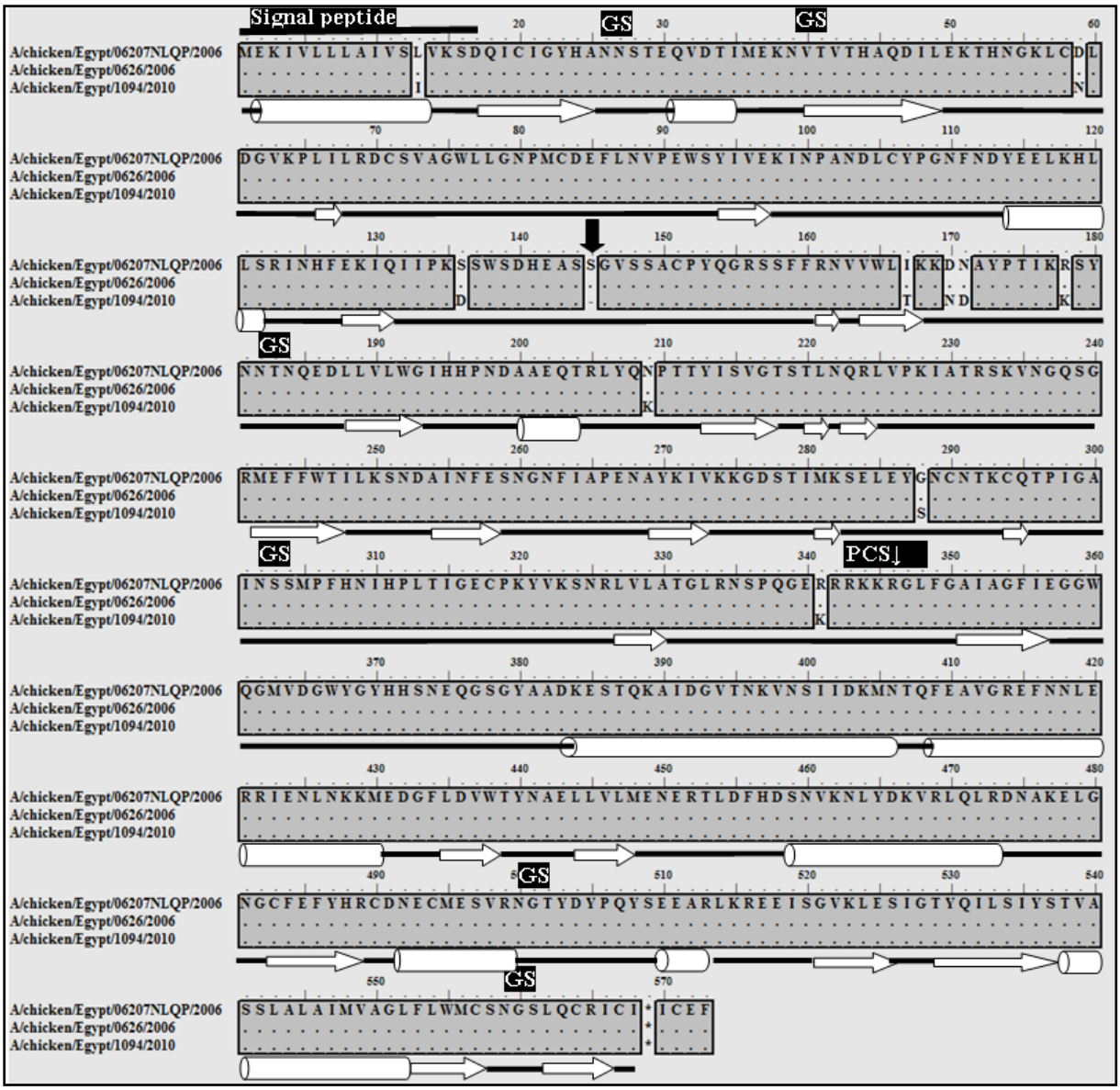


Figure 2: Amino acid sequences alignment of the HA protein of EGY06 and EGY10 in comparison to the parent A/chicken/Egypt/06207-NLQP/2006 virus

Amino acids M^{1} to S^{16} are the signal peptide of the HA protein. No amino acid differences between EGY06 and the parent virus but 11 novel amino acid substitutions were found in the

2010 virus: one mutation occurred in the signal peptide, 10 mutations in the HA1 subunit and no mutations were found in the HA2 subunit. The black bold arrow refers to the deletion at the receptor binding residue S145Δ. GS refers to a total of 6 potenital N-linked glycosylated sites (N-X-S/T) while PCS refers to the proteolytic cleavage site motif: E**R**RRKKR*GLF. The secondary structure of the HA was predicted and illustrated: white cylinders refer to predicted "helix" structures, white arrows to "strand" structures and black-bold lines to "coil" structures. No O-linked GS was found. Dots indicate residues that are identical to the corresponding residues in the parent virus.

As shown in Figure 3, all mutations occurred in the globular head domain except D59N, G288S (in stalk domain) and R341K in the PCS.

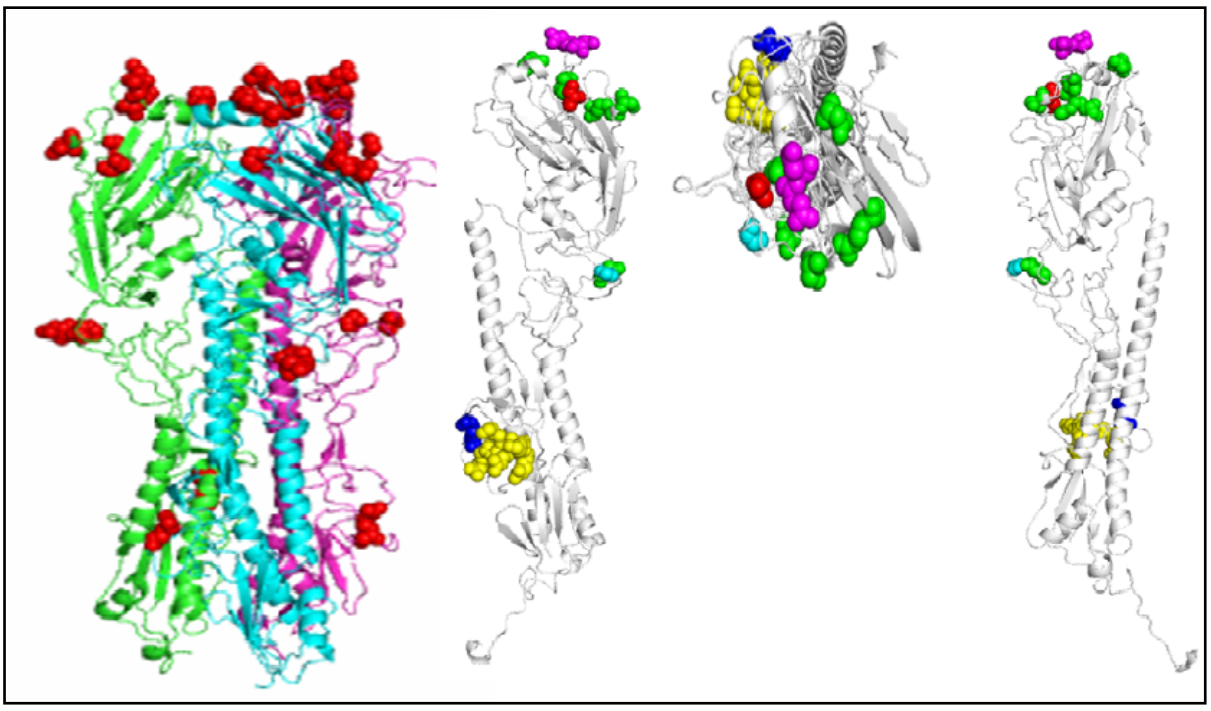


Figure 3: Position of amino acid substitutions found in EGY10 on the predicted tertiary structure of the HA of the parent A/chicken/Egypt/06207-NLQP/2006 virus.
Shown is the trimer structure of the HA of the parent virus (left); mutations found in the HA of EGY10 virus were imposed on the monomer of the HA protein of the parent virus (right): front view, head view and back view. Mutations in or adjacent to the immunogenic epitopes are illustrated in green, deletion within the receptor binding domain in red, substitution in potential GS in magenta, substitution in the PCS in yellow, mutation with unknown function in cyan and the PCS is depicted in blue. Protein modeling was generated by JIGSAW (**Contreras-Moreira and Bates, 2002**) and edited by RasTop version 2.7.1.

4.1.2.3 Sequence analysis of the neuraminidase of HPAI H5N1 EGY06 and EGY10

Sequences of the NA genes of EGY06 and EGY10 were submitted to the GenBank and their accession numbers are KF178949 and KF178951, respectively. The length of the NA gene of both EGY06 and EGY10 is 1363 nucleotides which encode 449 aa, equal to the length of the parent virus. Compared with the parent 2006 virus, the NA of EGY06 has six nucleotide differences with 99.56% homology. While the EGY10 has 30 nucleotide differences compared with the parent virus and 34 nucleotide differences to EGY06 with 97.8% and 97.5% homology, respectively. Only the S436Y substitution was unique for EGY06 compared to the parent and EGY10 viruses. In contrast, 11 amino acid substations were found in EGY10 compared to the parent and EGY06 viruses as shown in Figure 4. Two mutations were observed in the transmembrane domain: V20A, M29I, two in the stalk: A46D, P48S while seven mutations occurred in the globular head domain: R91K, L204M, S319F, M353I, S366N, D378E, and S430G (Figure 4). The NA protein of EGY06 has a 99.78% identity rate to the parent virus and 97.32% to the EGY10 virus. Also, the NA protein of EGY10 shares 97.55% identity with the parent virus. Three potential GS are located at the stalk (^{68}NSS^{70}) and head domain (^{126}NGT^{128} and ^{215}NGS^{217}) as shown in Figure 4. No O-linked GS was found.

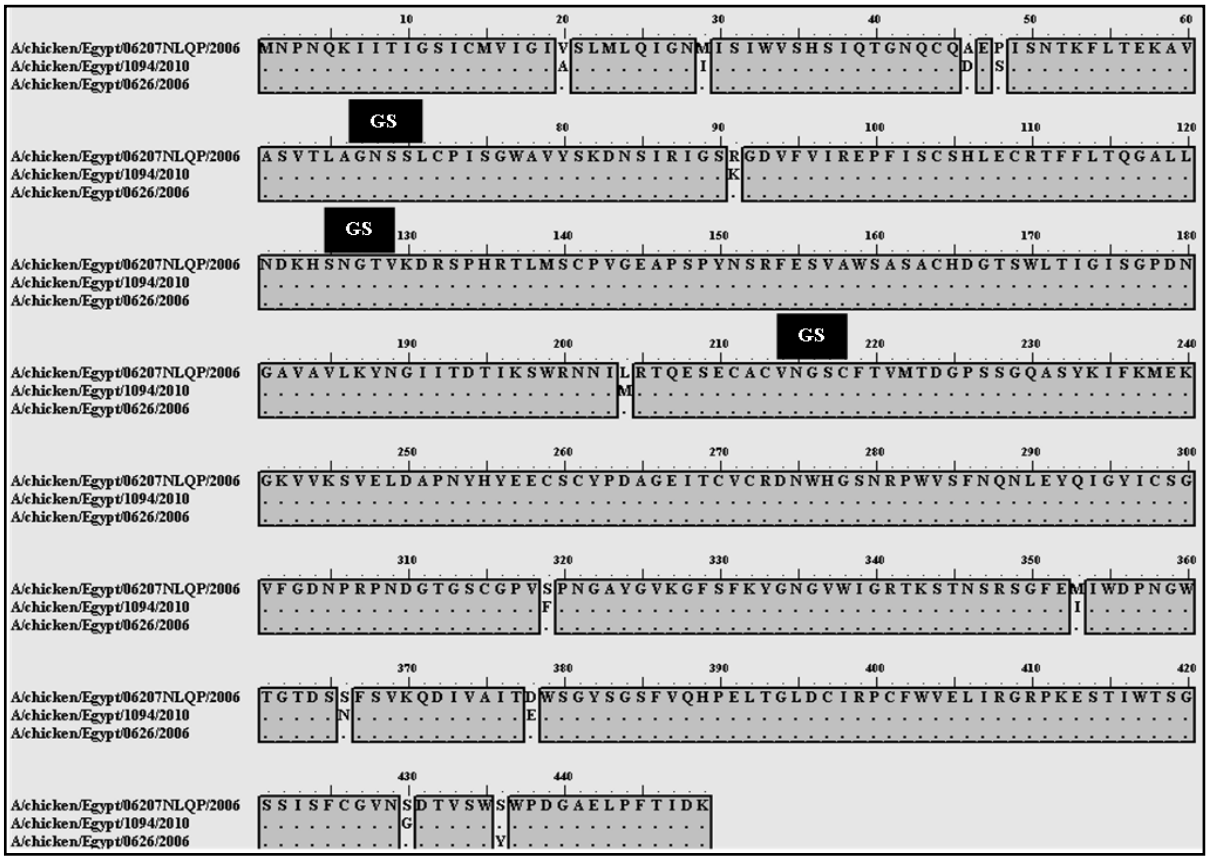


Figure 4: Amino acid sequences alignment of the NA protein of EGY06 and EGY10 in comparison to the parent A/chicken/Egypt/06207-NLQP/2006 virus

Amino acids M^1 to K^6 are the cytoplasmic domain, I^7 to S^{35} are the transmembrane domain, H^{36} to S^{70} are the stalk region while the rest is the head domain according to **Munier et al. (2010)**. Only one aa difference between EGY06 and the parent virus but 11 novel amino acid substitutions were found in EGY10. GS referes to the three potenital N-linked glycosylated sites (N-X-S/T). Dots indicate residues that are identical to the corresponding residues in the parent virus.

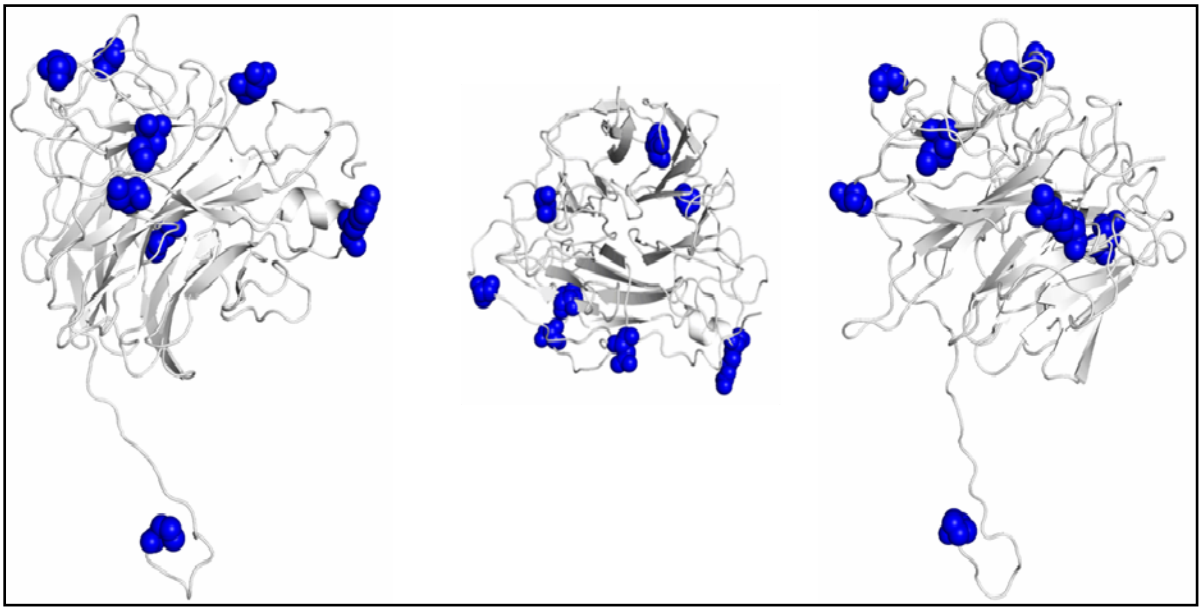


Figure 5: Tertiary structure of the NA protein of the parent A/chicken/Egypt/06207-NLQP/2006 virus

Shown are right side view, head view and left side view of the NA monomer. The stalk and head domains are only predicted. Mutations in the stalk region and head are illustrated. Protein modeling was generated by JIGSAW (**Contreras-Moreira and Bates, 2002**) and edited by RasTop version 2.7.1.

4.1.2.4 Phylogenetic relatedness of the HA and NA genes of EGY06 and EGY10

Phylogentic analysis of HA genes revealed that EGY06 is closely related to the predecessor parent virus of clade 2.2.1, introduced into Egypt in early 2006 (Figure 6). In contrast, EGY10 clustered within the classic 2.2.1/C group that contains recent human-origin H5N1 viruses and viruses isolated from chicken in small- scale vaccinated commercial farms. The same topology was also observed in the phylogenetic tree of the NA genes (Figure 6).

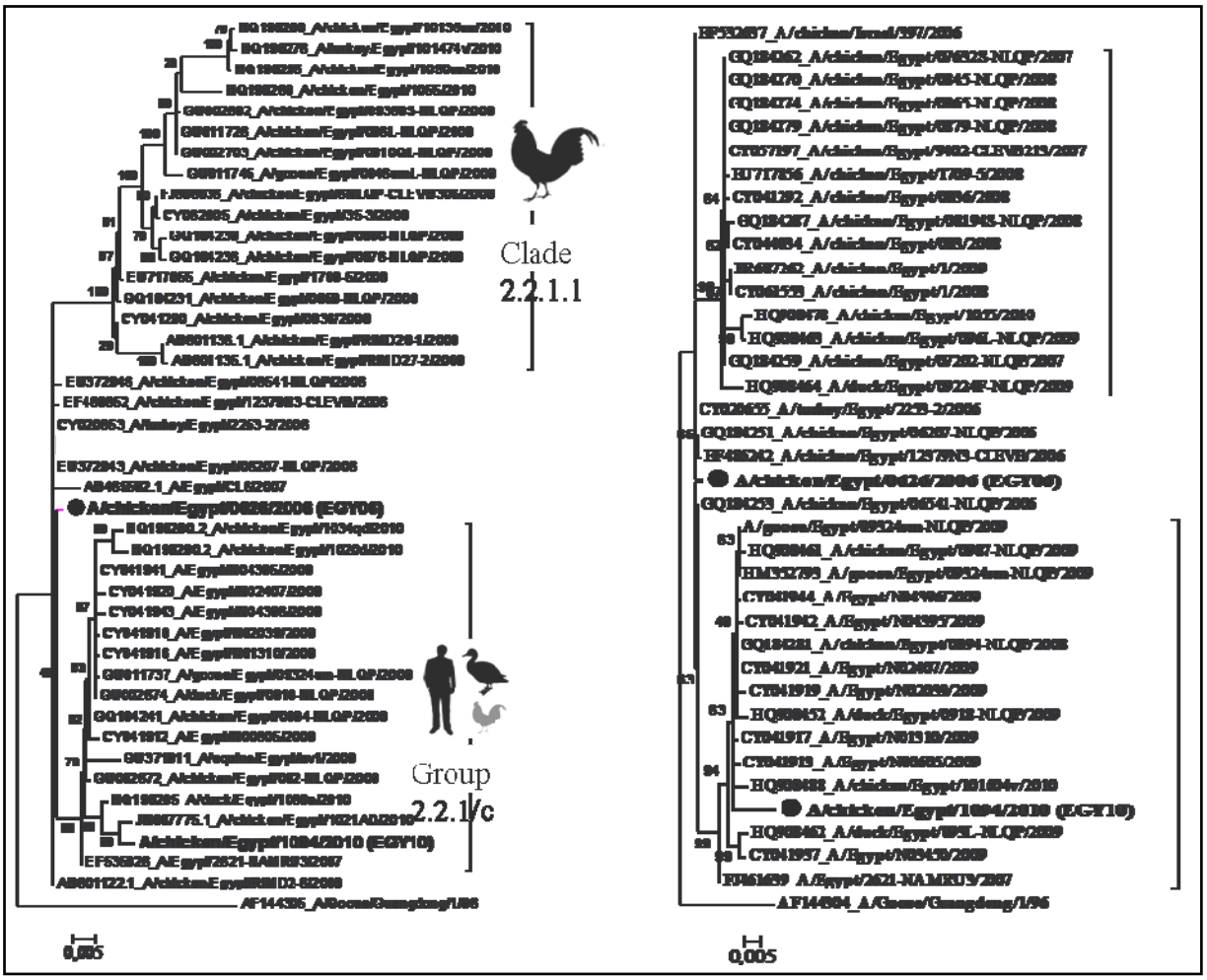


Figure 6: Phylogenetic relatedness of HA gene (left) and NA gene (right) of EGY06 and EGY10 to other Egyptian H5N1

The phylogenetic trees of the HA and NA were generated using the Neighbor-Joining Method with 1000 bootstrap replicates by the Maximum Composite Likelihood Model implemented in MEGA5. Viruses obtained in this dissertation are written in black-bold lines. The EGY06 virus was close to the parent virus introduced into Egypt in 2006, meanwhile EGY10 clustered with H5N1 viruses isolated from human-origin and small-scale vaccinated commercial farms in the 2.2.1/C group. Both viruses distinguish from the variant 2.2.1.1 clade commonly isolated from medium to large-scale vaccinated commercial poultry farms. Trees were rooted to the corresponding sequences of the origin of the Eurasian H5N1 viruses, A/Goose/Guangdong/1/1996.

4.2 Inactivation of two strains of HPAIV subtype H5N1 using four disinfectants

4.2.1 Propagation and titration of HPAIV subtype H5N1

Propagation of EGY06 and EGY10 strains was done by inoculation in 9 – 11 day-old SPF embryonated chicken eggs and incubation at 37º C for 72 hrs. The harvested AF was tested by slide and plate agglutination. The HA titre of the two strains were $Log_2^{\ 6}$ and $Log_2^{\ 7}$, respectively. To determine the mean EID_{50}, serial dilutions of 10^{-1} to 10^{-9} were then inoculated into the allantoic sacs of 4 – 5 SPF embryonated eggs. The EID50 was estimated according to the **Reed and Muench** (**1938**) to be $Log_{10}^{\ 7.15}$ and $Log_{10}^{\ 8.13}$ EID_{50}/ 0.1 ml for EGY06 and EGY10, respectively.

4.2.2 Inactivation of HPAIV of subtype H5N1 EGY06 by four chemical disinfectants

4.2.2.1 Suspension test with or without protein load

Different commercially available chemical disinfectants commonly used in the Egyptian poultry market, using Formalin, Glutaraldehyde, TH4® and Virkon®S were tested for their capability of inactivating HPAIV subtype H5N1 at concentrations of 0.5%, 1% and 2% at different contact times (15, 30, 60 and 120 min). Use of **Formalin** 1% and 2% in the presence as well as absence of protein load led to a complete inactivation of the H5N1 strain EGY06 at all contact times. In contrast, use of Formalin 0.5% with protein load did not inactivate the virus at 15 min contact time and the HA titre decreased to $Log_2^{\ 1}$ (Tables 6+7, Figure 7). In contrast, Formalin 0.5% without protein load led to complete inactivation of the tested strain at all contact times. Use of 0.5% **Glutaraldehyde** without protein load and 1% as well as 2% with or without protein load inactivated the virus completely at all contact times. In contrast, Glutaraldehyde at a concentration of 0.5% with protein load at 15 min contact time failed to inactivate the virus completely and the remaining HA titre was $Log_2^{\ 2}$. Glutaraldehyde 0.5% with protein load inactivated this strain at contact times of 30, 60 and 120 min. Use of **TH4®** with and without protein load was highly efficient to inactivate the avian influenza virus even at low concentration (0.5%) at all contact times. When the virus was tested against **Virkon®**S 0.5% in the presence or absence of protein load, the virus still survived at all contact times except after 120 min exposure time without protein load. Treatment with Virkon®S 1% and 2% with and without protein load led to complete inactivation of the virus strain at all contact times.

In summary: TH4® and then Formalin were the best disinfectants even at lowest concentrations (0.5%), leading to inactivation of EGY06. Glutaraldehyde and Virkon®S led to complete inactivation of the virus only at higher concentrations (Tables 6 and 7, Figure 7).

Table 6: Use of the slide agglutination test after treatment of **EGY06** H5N1 with four commercially available chemical disinfectants (suspension test)*

Disinfectant concentrations	**Protein load**	**Slide agglutination test after using suspension test with different effect times in minutes**			
		15	**30**	**60**	**120**
Formalin **0.5%**	without	-	-	-	-
	with	+	-	-	-
Formalin **1%**	without	-	-	-	-
	with	-	-	-	-
Formalin **2%**	without	-	-	-	-
	with	-	-	-	-
Glutaraldehyde **0.5%**	without	-	-	-	-
	with	+	-	-	-
Glutaraldehyde **1%**	without	-	-	-	-
	with	-	-	-	-
Glutaraldehyde **2%**	without	-	-	-	-
	with	■	■	■	■
TH4 **0.5 %**	without	-	-	-	-
	with	-	-	-	-
TH4 **1%**	without	-	-	-	-
	with	-	-	-	-
TH4 **2%**	without	-	-	-	-
	with	-	-	-	-
Virkon ®-s **0.5 %**	without	+	+	+	-
	with	+	+	+	+
Virkon ®-s **1%**	without	-	-	-	-
	with	-	-	-	-
Virkon ®-s **2%**	without	-	-	-	-
	with	-	-	-	-

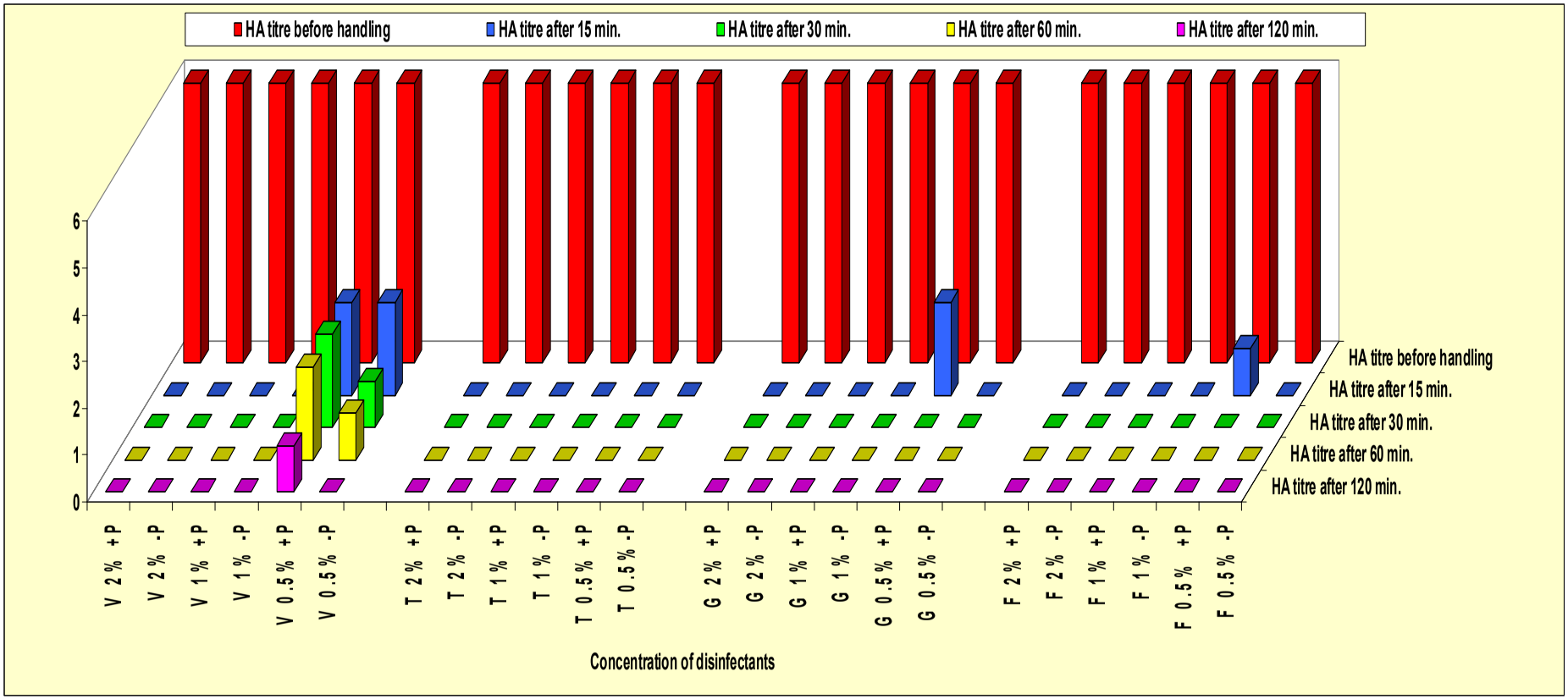
* EID50 before handling was Log_{10} $^{7.15}$/0.1 mL virus ■ = Gel formation

Table 7: Estimation of HA titres of **EGY06** H5N1 after treatment with four commercially available chemical disinfectants using the plate agglutination test*

Disinfectant concentrations	**Protein load**	**HA titre in microtitre plate after using suspension test with different contact times in minutes**			
		15	**30**	**60**	**120**
Formalin **0.5%**	without	-	-	-	-
	with	1	-	-	-
Formalin **1%**	without	-	-	-	-
	with	-	-	-	-
Formalin **2%**	without	-	-	-	-
	with	-	-	-	-
Glutaraldehyde **0.5%**	without	-	-	-	-
	with	2	-	-	-
Glutaraldehyde **1%**	without	-	-	-	-
	with	-	-	-	-
Glutaraldehyde **2%**	without	-	-	-	-
	with	■	■	■	■
TH4 **0.5 %**	without	-	-	-	-
	with	-	-	-	-
TH4 **1%**	without	-	-	-	-
	with	-	-	-	-
TH4 **2%**	without	-	-	-	-
	with	-	-	-	-
Virkon ®-s **0.5 %**	without	2	1	1	-
	with	2	2	2	1
Virkon ®-s **1%**	without	-	-	-	-
	with	-	-	-	-
Virkon ®-s **2%**	without	-	-	-	-
	with	-	-	-	-

* HA titre (Log_2) before handling $=2^6$ ■ = Gel formation

Figure 7: Estimation of HA titres of **EGY06** H5N1 after treatment with four commercially available chemical disinfectants using plate agglutination test (suspension test)



F = Formalin **G** = Glutaraldehyde **V** = Virkon®-S **T** = TH4®

+P = Sample with protein load **-P** = Sample without protein load

HA titre (Log_2) before handling $=2^6$

4.2.2.2 Effect of four chemical disinfectants on EGY06 by use of carrier tests

4.2.2.2.1 Gauze carrier test

As shown in Table 8, treatment of the strain EGY06 of EID50 Log_{10} $^{7.15}$ with 0.5%, 1% and 2% Formalin, Glutaraldehyde (1%, 2%) and TH4® (1%, 2%) led to complete inactivation of the virus at all contact times. In contrast, using 0.5% of Glutaraldehyde and TH4® showed a minimal response of the virus which still survived at 15 min, giving Log_2 ^{1} HA titres in both trials. After 30, 60 and 120 min the virus was completely inactivated by TH4® 0.5% but also after 30 min contact time; Glutaraldehyde 0.5% failed to inactivate this strain and the remaining HA titre was Log_2 ^{1} (Table 9). The efficacy of Virkon®S on EGY06 by using the gauze carrier test gave unsatisfactory results (Figure 8). Virkon®S inactivated the virus only after 60 min at a concentration of 0.5% and in 30 min at a concentration of 1%. Only 2% concentration led to complete inactivation of this strain at all contact times.

4.2.2.2.2 Wood carrier test

Formalin 1% and 2% inactivated the virus at all contact times in both trials (Tables 10+11) while use of Formalin 0.5% failed to inactivate this strain, resulting in a HA titre Log_2 ^{1} in the first trial at 15 min contact time (Table 11). For **Glutaraldehyde** 1 and 2%, the virus did not survive at all contact times in both trials (Figure 9). Glutaraldehyde 0.5% decreased the HA titre of this strain into Log_2 ^{2} at 15 min contact time in both trials and to Log_2 ^{1} at 30 min in the first trial only. Use of **TH4®** 1 and 2% at all contact times led to the complete inactivation of this strain, whereas with TH4® 0.5% the virus survived until at 15 min and led to a still remaining HA titre of Log_2 1. **Virkon®S 2%** performed differently, it did inactivate the virus at all contact times of 15, 30, 60 and 120 min, while at 0.5% the tested strain survived at 15 min contact time, still showing Log_2 ^{3} HA titre in the first trial and Log_2 ^{2} in the second trial. The HA titre also still was Log_2 ^{2} in both trials at 30 min contact time (Figure 9). In case of Virkon®S 1%, the tested virus failed to resist at 30, 60 and 120 min contact time in both trials, while at 15 min, the HA titre also was Log_2 ^{1} in both trials.

Table 8: Effect of four different chemical disinfectants on the strain **EGY06** of subtype H5N1 by use **gauze** as a carrier (slide haemagglutination test)

Disinfectant concentrations	Virus control (EID50) in Log $_{10}$	Trial number	Detection of the remained virus confirmed by slide haemagglunation test after different contact times in minutes			
			15	30	60	120
Formalin **0.5%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-
Formalin **1%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-
Formalin **2%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-
Glutaraldehyde **0.5%**	7.15	1	+	+	-	-
	7.15	2	+	+	-	-
Glutaraldehyde **1%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-
Glutaraldehyde **2%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-
TH4 **0.5 %**	7.15	1	+	-	-	-
	7.15	2	+	-	-	-
TH4 **1%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-
TH4 **2%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-
Virkon ®-s **0.5 %**	7.15	1	+	+	-	-
	7.15	2	+	+	-	-
Virkon ®-s **1%**	7.15	1	+	-	-	-
	7.15	2	+	-	-	-
Virkon ®-s **2%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-

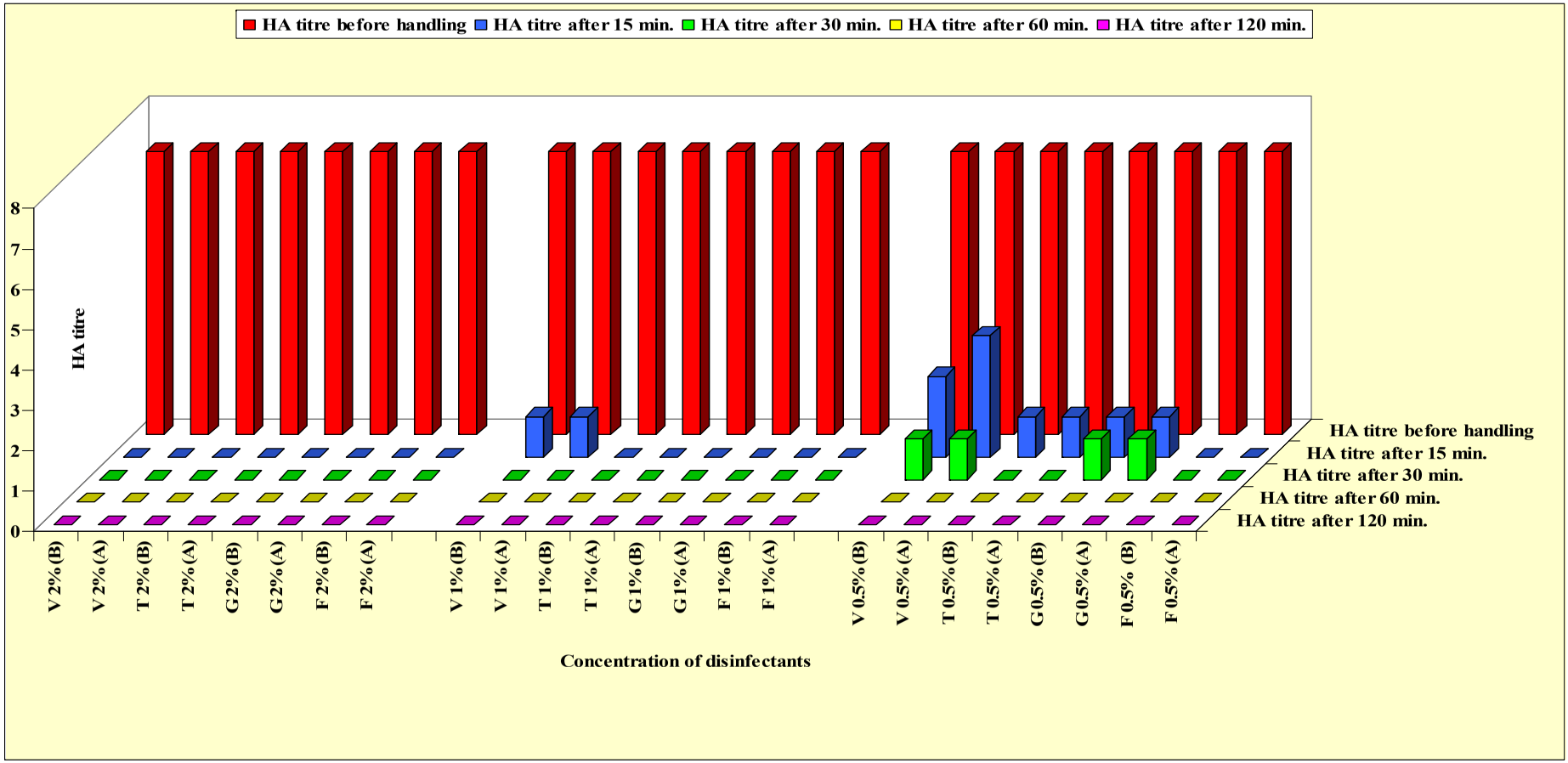
EID50 before handling was $Log_{10}{}^{7.15}$/0.1 mL virus

Table 9: Estimation of HA titre of strain **EGY06** subtype H5N1 in the **gauze** carrier test using the plate agglutination test*

Disinfectant concentrations	Trial number	HA titre in microtitre plate after using Gauze carrier test with different contact times in minutes			
		15	30	60	120
Formalin **0.5%**	1	-	-	-	-
	2	-	-	-	-
Formalin **1%**	1	-	-	-	-
	2	-	-	-	-
Formalin **2%**	1	-	-	-	-
	2	-	-	-	-
Glutaraldehyde **0.5%**	1	1	1	-	-
	2	1	1	-	-
Glutaraldehyde **1%**	1	-	-	-	-
	2	-	-	-	-
Glutaraldehyde **2%**	1	-	-	-	-
	2	-	-	-	-
TH4 **0.5 %**	1	1	-	-	-
	2	1	-	-	-
TH4 **1%**	1	-	-	-	-
	2	-	-	-	-
TH4 **2%**	1	-	-	-	-
	2	-	-	-	-
Virkon ®-s **0.5 %**	1	3	1	-	-
	2	2	1	-	-
Virkon ®-s **1%**	1	1	-	-	-
	2	1	-	-	-
Virkon ®-s **2%**	1	-	-	-	-
	2	-	-	-	-

* HA titre (Log_2) before handling was $=2^6$

Figure 8: Estimation of HA titres of strain **EGY06** subtype H5N1 in gauze carrier test using plate agglutination test



F = Formalin **G** = Glutaraldehyde **V** = Virkon®-S **T** = TH4®

HA = Haemagglutination titre **A** = First trial **B** = Second trial

HA titre (Log_2) before handling was $=2^6$

Table 10: Effect of four chemical disinfectants on the strain **EGY06** of subtype H5N1 by use **wood** as a carrier (slide haemagglutination test)

Disinfectant concentrations	**Virus control (EID50) in Log $_{10}$**	**Trial number**	**Detection of the remained virus confirmed by slide haemagglunation test after different contact times in minutes**			
			15	**30**	**60**	**120**
Formalin **0.5%**	7.15	1	+	-	-	-
	7.15	2	-	-	-	-
Formalin **1%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-
Formalin **2%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-
Glutaraldehyde **0.5%**	7.15	1	+	+	-	-
	7.15	2	+	-	-	-
Glutaraldehyde **1%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-
Glutaraldehyde **2%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-
TH4 **0.5 %**	7.15	1	+	-	-	-
	7.15	2	+	-	-	-
TH4 **1%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-
TH4 **2%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-
Virkon ®-s **0.5 %**	7.15	1	+	+	-	-
	7.15	2	+	+	-	-
Virkon ®-s **1%**	7.15	1	+	-	-	-
	7.15	2	+	-	-	-
Virkon ®-s **2%**	7.15	1	-	-	-	-
	7.15	2	-	-	-	-

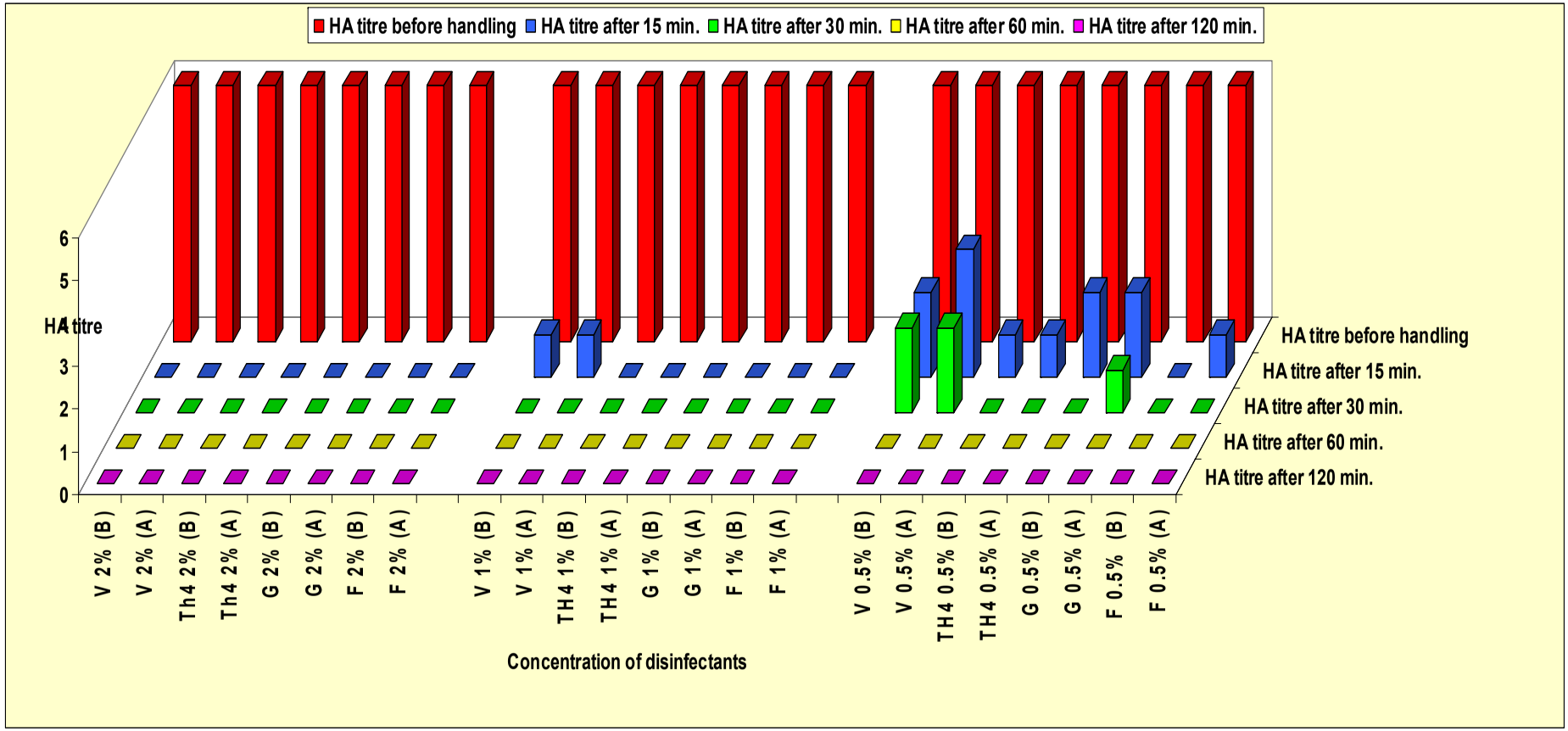
EID50 before handling was $Log_{10}{}^{7.15}$/0.1 mL virus

Table 11: Estimation of HA titre of strain **EGY06** subtype H5N1 in the wood carrier test using the plate agglutination test*

Disinfectant concentrations	Trial number	HA titre in microtitre plate after using the wood carrier test with different contact times in minutes			
		15	30	60	120
Formalin **0.5%**	1	1	-	-	-
	2	-	-	-	-
Formalin **1%**	1	-	-	-	-
	2	-	-	-	-
Formalin **2%**	1	-	-	-	-
	2	-	-	-	-
Glutaraldehyde **0.5%**	1	2	1	-	-
	2	2	-	-	-
Glutaraldehyde **1%**	1	-	-	-	-
	2	-	-	-	-
Glutaraldehyde **2%**	1	-	-	-	-
	2	-	-	-	-
TH4 **0.5 %**	1	1	-	-	-
	2	1	-	-	-
TH4 **1%**	1	-	-	-	-
	2	-	-	-	-
TH4 **2%**	1	-	-	-	-
	2	-	-	-	-
Virkon ®-s **0.5 %**	1	3	2	-	-
	2	2	2	-	-
Virkon ®-s **1%**	1	1	-	-	-
	2	1	-	-	-
Virkon ®-s **2%**	1	-	-	-	-
	2	-	-	-	-

* HA titre (Log_2) before handling was $=2^6$

Figure 9: Estimation of HA titres of strain **EGY06** subtype H5N1 in the wood carrier test using the plate agglutination test



F = Formalin **G** = Glutaraldehyde **V** = Virkon®-S **T** = TH4®

HA = Haemagglutination titre **A** = First trial **B** = Second trial

HA titre (Log_2) before handling was $=2^6$

4.2.3 Inactivation of H5N1 EGY10 using four disinfectants

4.2.3.1 Suspension test with or without protein load

The same disinfectants were tested for their capability of inactivating the other strain of HPAIV-H5N1 EGY10.

Formalin 0.5% without protein load led to complete inactivation of the virus after all contact times, 15, 30, 60 and 120 min (Tables 12+13). The same concentration with protein load failed to inactivate the virus at 15 min. contact time, resulting in a low HA titre (Log_2 ^{1}). After 30, 60 and 120 min, the virus was completely inactivated by 0.5% Formalin, even in the presence of protein load. 1% and 2% of Formalin with and without protein load led to complete inactivation of the virus at all contact times.

Glutaraldehyde 0.5% without protein showed a complete inactivation of the virus even after the shortest contact time (15 min), while the presence of a protein load protected the virus at 15 minutes, giving a higher HA titre of Log_2 ^{3}. This titre was higher than for Formalin 0.5% with a protein load at the same contact time. In contrast, the virus did not survive after being treated with the 0.5% concentration at 30, 60, 120 minutes contact times. Glutaraldehyde with a concentration of 1% with and without protein load led to a strong inactivation of the virus at each contact time. Use of Glutaraldehyde 2% without protein load was efficient enough to inactivate the virus, while the presence of a protein load led to gel formation and reading of its HA titre was difficult (Tables 12+13).

For **TH4®** at 0.5%, 1% and 2% in the presence or absence of a protein load, strain EGY10 was completely inactivated at all contact times.

For **Virkon®S 0.5%** without protein load, this strain could survive for 15, 30 and 60 min at Log_2 ^{2}, Log_2 ^{1} and Log_2 ^{1} HA titres, respectively. At 120 min with and without protein load the tested strain was completely inactivated, while it survived at Virkon®S 0.5% in the presence of protein load; this strain could resist at 15, 30 and 60 min with resultant HA titres of Log_2 ^{3}, Log_2 ^{2} and Log_2 ^{1}. On the other hand, use of Virkon®S 2% with and without protein load and Virkon®S 1% without protein load at 15, 30, 60 and 120 minutes contact times was efficient enough to inactivate this strain completely. At 1% concentration, Virkon®S in the presence of a protein load failed to inactivate this strain at 15 min contact time, the HA titre was Log_2 ^{1} (Tables 12+13 and Figure 10).

Table 12: Use of the slide agglutination test after treatment of **EGY10** H5N1 with four commercially available chemical disinfectants (suspension test)*

Disinfectant concentrations	**Protein load**	**Slide agglutination test after using suspension test with different effect times in minutes**			
		15	**30**	**60**	**120**
Formalin **0.5%**	without	-	-	-	-
	with	+	-	-	-
Formalin **1%**	without	-	-	-	-
	with	-	-	-	-
Formalin **2%**	without	-	-	-	-
	with	-	-	-	-
Glutaraldehyde **0.5%**	without	-	-	-	-
	with	+	-	-	-
Glutaraldehyde **1%**	without	-	-	-	-
	with	-	-	-	-
Glutaraldehyde **2%**	without	-	-	-	-
	with	■	■	■	■
TH4 **0.5 %**	without	-	-	-	-
	with	-	-	-	-
TH4 **1%**	without	-	-	-	-
	with	-	-	-	-
TH4 **2%**	without	-	-	-	-
	with	-	-	-	-
Virkon ®-s **0.5 %**	without	+	+	+	-
	with	+	+	+	-
Virkon ®-s **1%**	without	-	-	-	-
	with	+	-	-	-
Virkon ®-s **2%**	without	-	-	-	-
	with	-	-	-	-

* EID50 before handling was Log_{10} $^{8.13}$/0.1 ml virus

Gel formation = ■

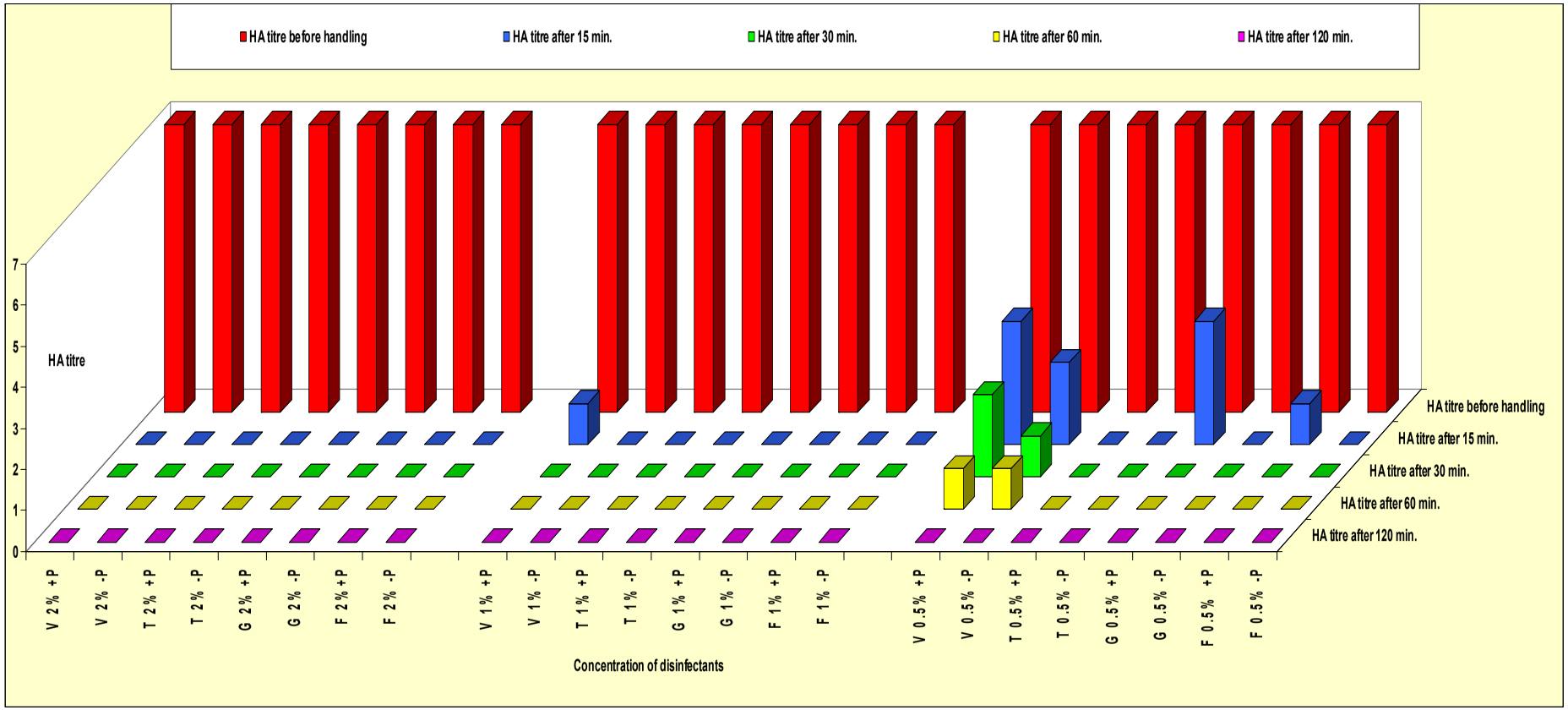
Table 13: Estimation of HA titres of **EGY10** H5N1 after treatment with four chemical disinfectants using the plate agglutination test*

Disinfectant concentrations	Protein load	HA titre in microtitre plate after using suspension test with different contact times in minutes			
		15	30	60	120
Formalin **0.5%**	without	-	-	-	-
	with	1	-	-	-
Formalin **1%**	without	-	-	-	-
	with	-	-	-	-
Formalin **2%**	without	-	-	-	-
	with	-	-	-	-
Glutaraldehyde **0.5%**	without	-	-	-	-
	with	3	-	-	-
Glutaraldehyde **1%**	without	-	-	-	-
	with	-	-	-	-
Glutaraldehyde **2%**	without	-	-	-	-
	with	■	■	■	■
TH4 **0.5 %**	without	-	-	-	-
	with	-	-	-	-
TH4 **1%**	without	-	-	-	-
	with	-	-	-	-
TH4 **2%**	without	-	-	-	-
	with	-	-	-	-
Virkon ®-s **0.5 %**	without	2	1	1	-
	with	3	2	1	-
Virkon ®-s **1%**	without	-	-	-	-
	with	1	-	-	-
Virkon ®-s **2%**	without	-	-	-	-
	with	-	-	-	-

* HA titre (Log_2) before handling was $=2^7$

Gel formation = ■

Figure 10: Estimation of HA titres of **EGY10** H5N1 after treatment with four chemical disinfectants using the plate agglutination test (suspension test)



F = Formalin **G** = Glutaraldehyde **V** = Virkon®-s **T** = TH4®

HA = Haemagglutination titre **A** = First trial **B** = Second trial

HA titre (Log_2) before handling was $=2^7$

4.2.3.2 Effect of four chemical disinfectants on EGY10 H5N1 by use of carrier tests

4.2.3.2.1 Gauze carrier test

Treatment of EGY10 H5N1 of a high EID50 of Log_{10} $^{8.13}$/ 0.1 ml of virus with **Formalin 0.5**% showed complete inactivation of the virus in both trials, even at the shortest contact time (15 min). Formalin 1% and 2% gave the same results.

After treatment of the strain with **Glutaraldehyde 0.5%** in the first trial, the virus was inactivated at contact times of 30, 60 and 120 min, while at 15 min contact time, the virus was not affected by the disinfectant (HA Titer Log_2 ^{1}). In the second trial, the virus survived till 30 min (HA Titer Log_2 ^{1}) and was inactivated completely at 60 and 120 min (Table 14).

TH4® at 1 % and 2% concentrations and **Virkon ®-s** at 2% concentration were able to inactivate this strain at all contact times without problems. In contrast, **TH4® 0.5%** failed to inactivate the virus at 15 min contact time (Figure 11) resulting in HA titre of Log_2 ^{1}.

In case of **Virkon ®-s 0.5%**, the respective strain resisted till 30 min in both trials at a HA titre of Log_2 ^{3} at 15 min contact time and Log_2 ^{2} at 30 min contact time in the first trial. In the second trial at 15 and 30 min contact times the resultant HA titre was Log_2 ^{2}. Treatment with Virkon ®-s 1% led to complete inactivation at 30, 60 and 120 min contact times in both trials, while after 15 min a HA titre of Log_2 ^{1} remained in both trials (Table 15).

Table 14: Effect of different different chemical disinfectants on the strain **EGY10** of subtype H5N1 by use **gauze** as a carrier (slide haemagglutination test)

Disinfectant concentrations	Virus control (EID50) in Log $_{10}$ /ml	No. of trials	Detection of the remained virus confirmed by slide haemagglunation test after different contact times in minutes			
			15	30	60	120
Formalin **0.5%**	8.13	1	-	-	-	-
	8.13	2	-	-	-	-
Formalin **1%**	8.13	1	-	-	-	-
	8.13	2	-	-	-	-
Formalin **2%**	8.13	1	-	-	-	-
	8.13	2	-	-	-	-
Glutaraldehyde **0.5%**	8.13	1	+		-	-
	8.13	2	+	+	-	-
Glutaraldehyde **1%**	8.13	1	-	-	-	-
	8.13	2	-	-	-	-
Glutaraldehyde **2%**	8.13	1	-	-	-	-
	8.13	2	-	-	-	-
TH4 **0.5 %**	8.13	1	+	-	-	-
	8.13	2	+	-	-	-
TH4 **1%**	8.13	1	-	-	-	-
	8.13	2	-	-	-	-
TH4 **2%**	8.13	1	-	-	-	-
	8.13	2	-	-	-	-
Virkon ®-s **0.5 %**	8.13	1	-	-	-	-
	8.13	2	+	+	-	-
Virkon ®-s **1%**	8.13	1	+	+	-	-
	8.13	2	+	-	-	-
Virkon ®-s **2%**	8.13	1	+	-	-	-
	8.13	2	-	-	-	-

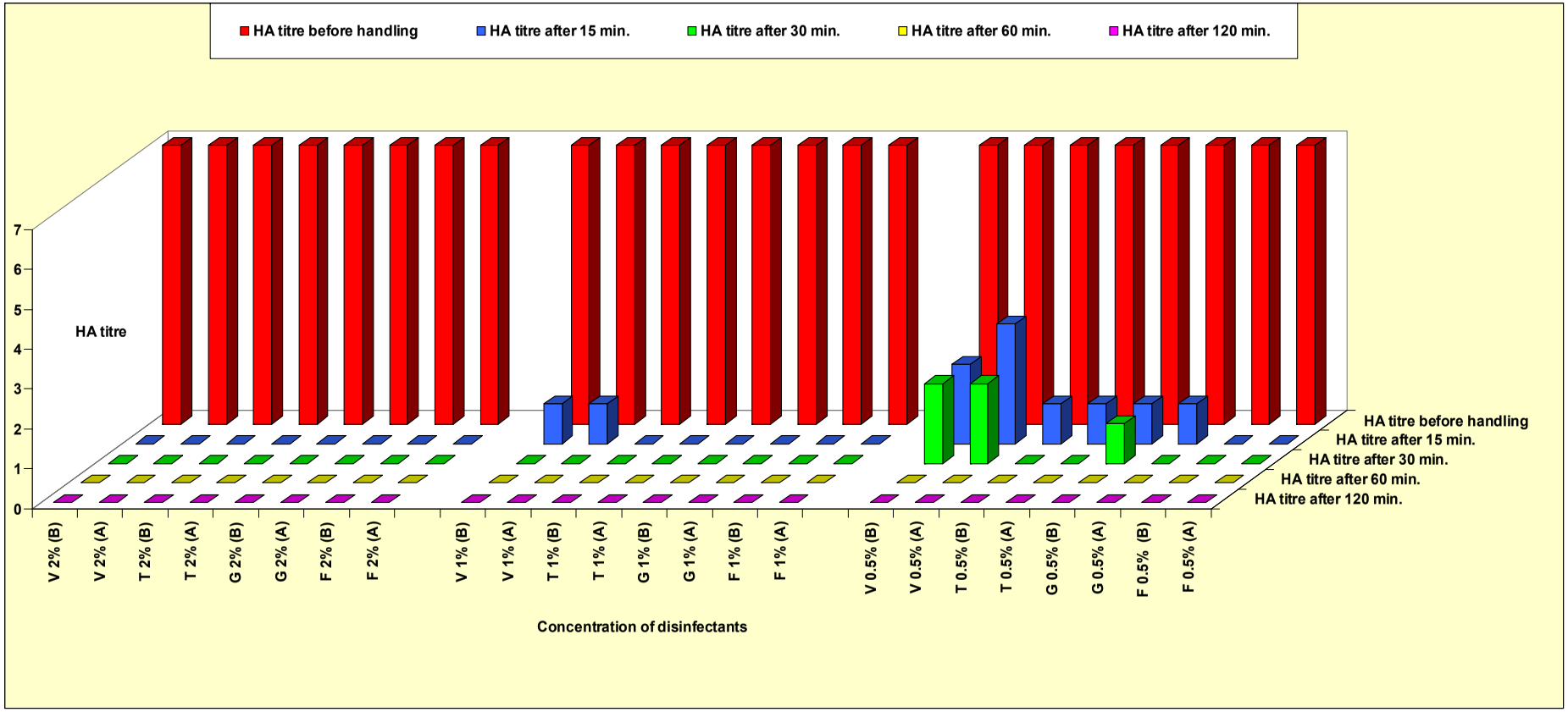
EID50 = Log_{10} $^{8.13}$/0.1 ml virus

Table 15: Estimation of HA titre of the rest **EGY10** H5N1 virus after treatment with chemical disinfectants by the gauze carrier test (plate agglutination test) *

Disinfectant concentrations	Trial number	HA titre in microtitre plate after using the Gauze carrier test with different contact times in minutes			
		15	30	60	120
Formalin **0.5%**	1	-	-	-	-
	2	-	-	-	-
Formalin **1%**	1	-	-	-	-
	2	-	-	-	-
Formalin **2%**	1	-	-	-	-
	2	-	-	-	-
Glutaraldehyde **0.5%**	1	1	-	-	-
	2	1	1	-	-
Glutaraldehyde **1%**	1	-	-	-	-
	2	-	-	-	-
Glutaraldehyde **2%**	1	-	-	-	-
	2	-	-	-	-
TH4 **0.5 %**	1	1	-	-	-
	2	1	-	-	-
TH4 **1%**	1	-	-	-	-
	2	-	-	-	-
TH4 **2%**	1	-	-	-	-
	2	-	-	-	-
Virkon ®-s **0.5 %**	1	3	2	-	-
	2	2	2	-	-
Virkon ®-s **1%**	1	1	-	-	-
	2	1	-	-	-
Virkon ®-s **2%**	1	-	-	-	-
	2	-	-	-	-

* HA titre (Log_2) before handling was $=2^7$

Figure 11: Estimation of HA titres of **EGY10** H5N1 after treatment with four chemical disinfectants using the plate agglutination test (gauze carrier test)



F = Formalin **G** = Glutaraldehyde **V** = Virkon®-s **T** = TH4®

HA = Haemagglutination titre **A** = First trial **B** = Second trial

HA titre (Log_2) before handling was $=2^7$

4.2.3.2.2 Wood in carrier test

Using **Formalin** 0.5% in both trials, the strain EGY10 survived only at 15 min contact time with a Log_2 1 HA titre (Figure 12) and was completely inactivated at 30, 60 and 120 min contact times. At Formalin 1% and 2%, the tested virus was inactivated completely at all contact times.

Treatment of the strain with **Glutaraldehyde** at 0.5% concentration led to virus inactivation after 30, 60 and 120 min contact times, while the virus survived 15 min in the presence of Glutaraldehyde 0.5% at a HA titre of Log_2^{1} in the first trial. Additionally, in the second trial, the virus survived after 15, 30 min, the HA titre of the virus decreased from 2^{7} to Log_2^{1} (Table 17). After 60 and 120 min contact times, the virus was completely inactivated. Glutaraldehyde at 1% and 2% concentrations led to complete inactivation of the H5N1 virus at all contact times and in both trials.

Concerning **TH4®** 1% and 2% and Virkon ®-s 2%, the tested virus did not resist these disinfectants concentrations all contact times. When the strain was treated with TH4® 0.5%, it maintained a low HA titre (Log_2^{1}) at 15 min contact time in both trials.

For **Virkon ®-S** 0.5%, the HA titre decreased from 2^{7} to Log_2^{3} at 15 min contact time, decreased further to a Log_2^{2} HA titre after 30 and 60 min, while after 120 min contact time the virus was completely inactivated. Also for Virkon ®-s 1%, the HA titre of Log_2^{2} was only maintained at 15 min (Table 17+ Figure 12).

In conclusion, the results revealed that there were no significant differences between the two EGY06 and EGY10 isolates when treated with different types of chemical disinfectants. 1% of Formalin, Glutaraldehyde and TH4® most likely are efficient to achieve complete sanitation of poultry houses and farms, even in the presence of organic matter.

Table 16: Effect of different chemical disinfectants on the strain **EGY10** of subtype H5N1 by use **woord** as a carrier (slide haemagglutination test)

Disinfectant concentrations	**Virus control (EID50) in Log 10 /ml**	**No. of trials**	**Detection of the remained virus confirmed by slide haemagglunation test after different contact times in minutes**			
			15	**30**	**60**	**120**
Formalin **0.5%**	8.13	1	+	-	-	-
	8.13	2	+	-	-	-
Formalin **1%**	8.13	1	-	-	-	-
	8.13	2	-	-	-	-
Formalin **2%**	8.13	1	-	-	-	-
	8.13	2	-	-	-	-
Glutaraldehyde **0.5%**	8.13	1	+		-	-
	8.13	2	+	+	-	-
Glutaraldehyde **1%**	8.13	1	-	-	-	-
	8.13	2	-	-	-	-
Glutaraldehyde **2%**	8.13	1	-	-	-	-
	8.13	2	-	-	-	-
TH4 **0.5 %**	8.13	1	+	-	-	-
	8.13	2	+	-	-	-
TH4 **1%**	8.13	1	-	-	-	-
	8.13	2	-	-	-	-
TH4 **2%**	8.13	1	-	-	-	-
	8.13	2	-	-	-	-
Virkon ®-s **0.5 %**	8.13	1	+	+	+	-
	8.13	2	+	+	+	-
Virkon ®-s **1%**	8.13	1	+	-	-	-
	8.13	2	+	-	-	-
Virkon ®-s **2%**	8.13	1	-	-	-	-
	8.13	2	-	-	-	-

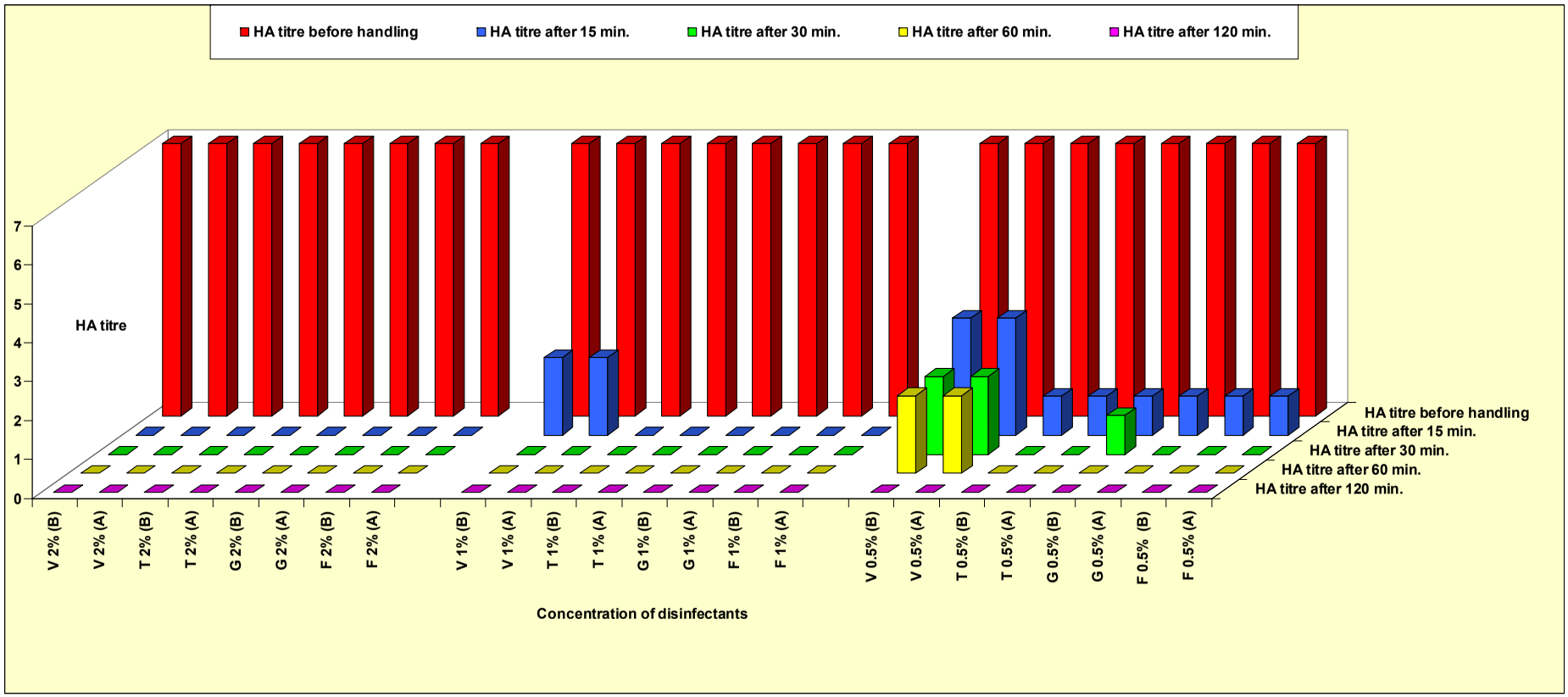
EID50 = Log_{10} $^{8.13}$/0.1 ml virus

Table 17: Estimation of HA titre of the rest EGY10 H5N1 virus after treatment with chemical disinfectants by using the wood carrier test (plate agglutination test) *

Disinfectant concentrations	Trial number	HA titre in microtitre plate after using wood carrier test with different contact times in minutes			
		15	30	60	120
Formalin **0.5%**	1	1	-	-	-
	2	1	-	-	-
Formalin **1%**	1	-	-	-	-
	2	-	-	-	-
Formalin **2%**	1	-	-	-	-
	2	-	-	-	-
Glutaraldehyde **0.5%**	1	1	-	-	-
	2	1	1	-	-
Glutaraldehyde **1%**	1	-	-	-	-
	2	-	-	-	-
Glutaraldehyde **2%**	1	-	-	-	-
	2	-	-	-	-
TH4 **0.5 %**	1	1	-	-	-
	2	1	-	-	-
TH4 **1%**	1	-	-	-	-
	2	-	-	-	-
TH4 **2%**	1	-	-	-	-
	2	-	-	-	-
Virkon ®-s **0.5 %**	1	3	2	2	-
	2	3	2	2	-
Virkon ®-s **1%**	1	2	-	-	-
	2	2	-	-	-
Virkon ®-s **2%**	1	-	-	-	-
	2	-	-	-	-

* HA titre (Log_2) before handling was $=2^7$

Figure 12: Estimation of HA titres of the rest **EGY10** H5N1 virus after treatment with chemical disinfectants by the wood in carrier test (plate agglutination test)



F = Formalin **G** = Glutaraldehyde **V** = Virkon®-s **T** = TH4®

HA = Haemagglutination titre **A** = First trial **B** = Second trial

HA titre (Log_2) before handling was $=2^7$

CHAPTER 5: DISCUSSION

5.1 Detection and identification of AIV subtype H5N1

In the present study, two strains of AIV subtype H5N1 were isolated from infected poultry broiler farms suffering from respiratory distress with high mortality rates in two different Egyptian Governorates, namely the Alexandria and Marsa Matrouh Governorates during the highly frequent and most severe outbreaks of HPAIV H5N1 from 2006 to 2010. The isolation was carried out in SPF. Additional tests as the HA test on fluids from eggs inoculated with the tested samples was used to confirm the presence of haemagglutinating agents. Positive samples for HA were then examined by the HI test to distinguish AIV from other haemagglutinating viruses, for instance of Newcastle Disease (ND) and of Egg Drop Syndrome (EDS) (**Beard, 1980**; **Nooruddin et al., 2007**). Subsequently, HI tests confirmed the AIV subtype H5 by excluding other haemagglutinating viruses. Finally, the RT-PCR method used particular sets of primers to identify H5 and N1 genes of the AIV.

5.2 Molecular characterization

Although virus isolation in ECEs and/or cell culture is the common way for the detection of AIV, these methods are still time consuming. As a result, other molecular diagnostic techniques such as the one-step RT-PCR have shown to get more fast and responsive diagnostic results (**Van Aarle et al., 2006**). In the current study, RT-PCR was carried out to the whole length of both NA and HA genes, wherever they were sequenced directly after gel purification.

In February 2006, Egypt had reported the first outbreaks of HPAI H5N1 in poultry due to infection with an H5N1 virus of clade 2.2.1. Continuous infections of vaccinated poultry and non-vaccinated backyard birds resulted in establishment of two distinct genetic lineages. The variant 2.2.1.1 clade viruses that have been isolated exclusively from vaccinated commercial poultry, mainly chicken and the 2.2.1/C group that have been isolated from humans and small scale commercial farms. Sequence and phylogenetic analysis indicated that EGY06 is very close to the predecessor parent virus. Although, the first reported cases in Egypt in early 2006 were in Cairo and closer provinces, however EGY06 has been isolated from broiler farm in Alexandria with high identity to 2006 viruses. That may indicate the wide spread of H5N1 virus in poultry in Egypt due to rapid, random and uncontrolled movement of live birds and/or by products (**Abdelwhab and Hafez, 2010**). On the contrary, EGY10 belonged to the 2.2.1/C group and was isolated from vaccinated commercial broiler chicken in Marsa Matrouh province. Although, experimental studies showed that the available vaccines in Egypt may

protect chicken against viruses of 2.2.1/C (**Grund et al., 2011; Abdelwhab et al., 2011**), however isolation of these group in the field has been reported from vaccinated chicken (**Kilany et al., 2010; Arafa et al., 2012; El-Zoghby et al., 2012**). It is well known that under field conditions several factors can influence the vaccine efficacy such (1) defects in vaccine storage, transportation or manipulation (2) failure of protection due to improper vaccination (3) or due to prior/concurrent infections with immunosuppressive and/or immunedepressive agents (**Abdelwhab and Hafez, 2012**).

In this study, the HA protein of EGY10 has 11 amino acid substitutions compared with the parent virus. One mutation occurred in the signal peptide through substitution of the leucine (in the parent virus) by isoleucine in EGY10 virus. Ten mutations were found in the HA1 subunit: D59N, S136D, S145Δ, I167T, D170N, N171D, R178K, N209K, G288S and R341K. Although both viruses have polybasic cleavage site motif PQGERRRKKR*GLF in EGY06 and PQGEKRRKKR*GLF in EGY10, however single amino acid substitution (R341K) has been fixed and exclusively found in the 2.2.1/C group (**Abdelwhab et al., 2012a**). Mutations in this position have been recently confirmed to modulate virulence of the Egyptian H5N1 virus in 2.2.1.1 clade (**Yoon et al., 2013**) and it remains to be elucidated in the 2.2.1/C group. Importantly, group 2.2.1/C including EGY10 has a unique deletion S145 deletion which is characteristic for the Egyptian viruses of this genetic group. This deletion was firstly described by Abdelwhab et al. (2010) in viruses of human and backyard origin as well as in all human seasonal H1N1 and H3N2 isolated in Egypt (**Abdelwhab et al., 2010b**). This result was similar to that obtained by **Abdel-Moneim et al. (2010)**, who clarified that the deletion in amino acid serine S145 is also present in all virus groups belonging to 2.2 sublineages A1. Moreover, this position is close to a domain modulating receptor interaction of a typical HA gene of H5N1 clade 2.2.1 isolated from equine. The most important concern is: strains with this deletion appear to evolve through a receptor usage that resembling human H1N1. Thus, a concern has been raised about possible evolution of this Egyptian genotype to use H1N1-like receptors to efficiently replicate in the upper respiratory tract of humans that may induce subclinical "silent" infections and/or possible human-to-human transmission (**Veljkovic et al., 2009**; **Van Kerkhove et al., 2011)**. Significant conformational changes due to Δ145 occurred around the binding pocket of the viral HA, consequently contact angel between sialic acid receptor and the viral HA has been modified which might produced more stable adjustment for binding human receptors. Using reverse genetics, **Watanabe et al.** (**2011**) found that recombinant Egyptian H5N1 viruses carry a double mutations Δ145/I167T enhanced binding affinity of the virus for human receptors and retained simultaneously their avian receptor specificity and increased viral tropism to the human lower respiratory tract epithelium. In addition, lower titer of those mutants was required to establish severe infection in mice model

in comparison to the original Egyptian H5N1 genotype, which was not highly pathogenic to mice. In addition to alteration in glycosylation pattern via D170N and N171D substitutions are characteristics for the recent human viruses of 2.2.1/C sublineage. The impact of these de-novo changes should be investigated by reverse genetics. Similar findings were obtained by **Rockman et al. (2012)**, who compared the antigenic structure of the haemagglutinin sequences of two highly pathogenic strains of H5N1 (A/Vietnam/1194 and A/Indonesia/5/05), belonging to clade 1 and clade 2.1 with the A/Bar-headed Goose/Qinghai/65/05 clade 2.2 virus. Numerous substitutions at positions 140, 145, 171 and 172 were present, all of which are near to the receptor binding site on the distal globular head membrane of the HA. These antigenic sites propose that these amino acid locations relate to the antigenic development of the H5 clade 1 and clade 2.2 viruses. Consequently, these substitutions may be helpful for the universal design of vaccines against the various H5 HA sublineages newly found in circulation.

Meanwhile the NA protein of EGY10 has 11 amino acid substitutions. Two mutations were observed in the transmembrane domain: V20A, M29I, two in the stalk: A46D, P48S while seven mutations occurred in the globular head domain: R91K, L204M, S319F, M353I, S366N, D378E, and S430G. Four distinguished substitutions at A46D, L204M, S319F and S430G were described by **Abdelwhab et al. (2012a)** as less frequently noticed in chicken isolates in comparison to recent backyard and human origin viruses. They mentioned that residue 319S is a part of an immunogenic epitope (C) of the NA protein meanwhile other residues had no unknown function. The impact of these mutations on the NA enzymatic activity of the NA should be elucidated. Nevertheless, it is well known that, compensatory changes in the NA are required to facilitate viral entry and release.

Taken together, the Egyptian viruses are evolving toward more stable infection in human, which is of great concern for the scientific community (**Neumann et al., 2012**). Therefore, as a parallel line to guard and prevent the spread of disease and avoid more economic losses to the poultry industry and spill-over to human, the current study concentrates on prophylaxis and sanitation by comparing some of antimicrobial agents and their effects on HPAI H5N1 Egyptian strains.

5.3 Chemical inactivation of HPAIV subtype H5N1

The global threat for outbreaks of AIV has extremely increased. Consequently, it is most important to have effective procedures, such as disinfection, to prevent the further spread of infection between flocks. Former studies on AIV disinfection were carried out with various substances in suspension tests with and without organic load or on carriers (**King, 1991**).

Nevertheless, the majority of chemical disinfectants tested in these studies are not common today, and the techniques applied were not actually appropriate for testing the capability of a disinfectant under veterinary field conditions. Particularly in animal husbandry, the necessities put on a chemical disinfectant are very high, as many factors like high organic soiling even after accurate cleaning, dissimilar materials with often-permeable surfaces, low temperatures and short contact times can harmfully influence its effectiveness.

In the present study, the efficacy of four chemical disinfectants to inactivate the two HPAI was firstly carried out using suspension tests with or without protein load as well as carrier tests with protein.

The obtained results in the present study showed that the use of Glutaraldehyde, Formalin, TH4® 0.5%, without protein load in suspension tests led to complete inactivation of the virus at all 15, 30, 60 and 120 min contact times. Use of Virkon®S 0.5% with and without protein load led to survival of the virus even after 60 min. In contrast, using Formalin and TH4® (1% and 2%) with and without protein load led to complete inactivation of the virus even at the shortest contact time, 15 min. Similar results were obtained after using Glutaraldehyde 1%, while treatment of H5N1 with Glutaraldehyde 2% led to gel formation. After treatment of infected carriers (poplar wood and gauze) with Formalin, Glutaraldehyde and TH4® 0.5%, the virus was inactivated after 30 min. Concentration of 1% of the three disinfectants was sufficient to inactivate the two isolates at 15 min contact time, except in case of Virkon®S. Use of 1% of Formalin, Glutaraldehyde and TH4® will be efficient to achieve a complete sanitation of poultry houses and farms even in the presence of organic matter.

This study indicated that the four chemical disinfectants could efficiently inactivate the two tested H5N1 viruses when used at higher concentration than the manufactures recommended. Such fast response to chemical inactivation of AIV was also obtained by **Klein and Deforest** (**1983**), **Capua and Marangon** (**2006**) and **Shahid et al.** (**2009**) who indicated that enveloped viruses are most susceptible to chemical disinfectants (Formalin, Phenol Crystals, Iodine Crystals, Virkon®-S, Zeptin 10%, KEPCIDE 300, KEPCIDE 400, Lifebuoy, Surf Excel and Caustic Soda), compared to non-enveloped viruses. This is mainly due to the relatively simple disruption of the lipid envelope by the chemical disinfectants used in this study, and not by the disruption of other viral targets, including the protein or nucleic acid (**Thurmann and Gerba, 1988; Maris, 1995; Davison et al., 1999; Sattar and Springthorpe, 1999; Shahid et al., 2009**). Similar results were also demonstrated by **Songserm et al.** (**2005**) where the Thailand strain of HPAI H5N1 at a titre of $10^{6.3}$ ELD50/ml was completely inactivated following exposure to Glutaraldehyde, Phenol, Peracitic Acid, Ammonium Chloride or Acid Hyperchloride for 10 min. **Shahid et al.** (**2009**) found that different types of chemical

disinfectants, including Formalin, Virkon®-S, Zeptin 10% and KEPCIDE 300 were effective in completely destroying H5N1 virus at recommended dilutions of 0.6%, 1 %, 1% and 2% after 15 min, respectively. In addition, **Wanaratana et al.** (**2010**) tested three strains of HPAIV subtype H5N1 in Thailand during the 2004 outbreak against disinfectants including Glutaraldehyde, QACs, Formaline, Chlorine and Phenol. They found that all these disinfectants could efficiently inactivate all the three isolates of HPAIV subtype H5N1 after 10 min contact time. **Elschner et al.** (**2012**) found that use of 1% and 2% of the chemical disinfectant Virkon®-S led to complete inactivation of HPAIVs subtype H5N1 at 15 min contact time.

The present results were in agreement with those obtained by **Yilmaz et al.** (**2004**) who tested two commercial available disinfectants (Venno FF super, Venno Vet 1 super) which were recorded in the list of the DVG as suitable disinfectants against AIV. Both disinfectants demonstrated a noticeably superior effect against AIV, but showed losses of efficacy in the presence of organic load. Similar results were also observed by **Bieker** (**2006**) in his investigation on the efficacy of various disinfectants **(**DF-200d, 10% bleach, 1% Virkon® S, and 70% ethanol) on HPAIV of subtype H5N1. The presence of organic material is well known to disrupt the mechanism of chemical disinfectants and their overall efficacy by different mechanisms (**Maillard and Russell, 1997; Quinn and Markey, 1999; Sattar and Springthorpe, 1999; Prince and Prince, 2001; Bieker, 2006**). Direct neutralization of the chemical activity of the biocide by compounds of the organic material could disrupt efficacy **(Bieker, 2006)**. Organic matter may also disrupt the efficiency of viral inactivation by diluting the disinfectant and lowering the overall effect (**Bieker, 2006**). A further theory is that the organic material supplies a physical protection of viral particles, which prevents the interaction between chemical disinfectants and target virus. **DeBenedictis et al.** (**2007**) noticed that the virucidal action of the majority of chemical disinfectants is partly or totally inhibited by the interaction with organic material and that is why sanitation procedures must include accurate cleaning prior to disinfection.

CHAPTER 6: SUMMARY

Molecular aspects and chemical inactivation of Influenza H5N1 viruses isolated from Egyptian chicken flocks during the 2006-2010 outbreaks

The primary objective of the current study was to identify two of the highly pathogenic avian influenza virus (HPAIV) isolates of subtype H5N1 genotypically using one step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), followed by sequence and phylogenetic analyses. A further objective was to determine in vitro the virucidal efficacy of four types of chemical disinfectants, namely Formalin, Glutaraldehyde, TH4® and Virkon®S at different concentrations and contact times on the two HPAI isolates. A/chicken/Egypt/0626/2006 (EGY06) and A/chicken/Egypt/1094/2010 (EGY10) were isolated from cloacal and tracheal swabs from broiler during HPAI H5N1 outbreaks in Egypt in 2006 and 2010. The first strain, EGY06, was isolated from a non-vaccinated flock in February 2006 in the Alexandria governorate. The second strain, EGY10, was isolated from a vaccinated flock in November 2010 in the Marsa Matrouh governorate.

Classical identification of the two isolates was carried out in the Department of Poultry and Hygiene, Faculty of Veterinary Medicine, Alexandria University, Egypt. Molecular identification and genetic analyses were conducted in the Gene Analysis Unit of the National Laboratory for Veterinary Quality Control on Poultry Production (NLQP), Egypt.

Using RT-PCR with specific sets of primers for H5 and N1 genes of AIV it was confirmed that the two isolates belonged to AI subtype H5N1. After molecular characterization and phylogenetic analysis of the HA and NA genes, the strain EGY06 was closely related to the 2006 predecessor Egyptian viruses of 2.2.1 clade, whereas EGY10 clustered within the classic 2.2.1/c group that commonly isolated from small-scale commercial farms and human since 2009.

The efficacy of four chemical disinfectants to inactivate both isolates was carried out in accordance to the guidelines of the German Veterinary Medical Society (Deutsche Veterinärmedizinische Gesellschaft, DVG) for testing of disinfection procedures and chemical disinfectants. The experiments were performed using suspension tests without and with protein load (40% Bovine Calf Serum "BCS") as well as wood and gauze as a carriers (also loaded with BCS), at room temperature and incubation times of 15 to 120 min. The obtained results showed that the use of Glutaraldehyde, Formalin or TH4® 0.5% without protein load led to complete inactivation of the virus after 15, 30, 60 or 120 min contact time. Use of

Virkon®S 0.5% with and without protein load led to survival of the virus even after 60 min. In contrast, using Formalin and TH4® (1% and 2%) with and without protein load led to complete inactivation of the virus even at the shortest contact time of 15 min. Similar results were obtained after using Glutaraldehyde 1%, while treatment of H5N1 with Glutaraldehyde 2% led to gel formation.

After treatment of contaminated carriers (poplar wood and gauze) with Formalin, Glutaraldehyde and TH4® 0.5%, the virus was inactivated after 30 min. Concentration of 1% of the three disinfectants was sufficient to inactivate the two isolates within 15 min contact time, except in case of Virkon®S which required higher concentrations to give similar results.

The study indicated that the four chemical disinfectants could efficiently inactivate the two tested H5N1 viruses when used at higher concentration than the manufacturers recommended. The results of the present thesis highlight the sensitivity of HPAIV H5N1 to different disinfectants, which may improve biosecurity measures on the farms and reduce the economic losses caused by HPAIV H5N1.

KAPITEL 7: ZUSAMMENFASSUNG

Molekulare Aspekte und chemische Inaktivierung von Influenza H5N1-Viren ausägyptischen Hühnerbeständen von Ausbrüchen der Jahre 2006 bis 2010

Das primäre Ziel der aktuellen Studie war es, hoch pathogene aviäre Influenza-Viren (HPAIV) des Subtyps H5N1 genotypisch durch eine einschrittige Reverse Transkriptase-Polymerase-Kettenreaktion (RT-PCR) zu identifizieren und anschließend molekularbiologisch zu charakterisieren. Ein weiteres Ziel war, die Wirksamkeit von verschiedenen Konzentrationen und Einwirkungszeiten von vier chemischen Desinfektionsmitteln (Formalin, Glutaraldehyd, TH4® und Virkon®S) auf zwei Stämme (A/chicken/Egypt/0626/2006 "EGY06" und A/chicken/Egypt/1094/2010 "EGY10") des aviären Influenzavirus (AIV) des Subtyps H5N1 in vitro zu prüfen. Die beiden Isolate des AIV-Subtyps H5N1 wurden aus Kloaken- und Trachealtupfern von infizierten Masthühnerherden während der Ausbrüche aviärer Influenza (AI) 2006 und 2010 isoliert. Während der erste Stamm EGY06 aus einer nicht geimpften Herde im Februar 2006 im Gouvernement Alexandria isoliert wurde, wurde der zweite Stamm, EGY10, aus einer geimpften Herde im November 2010 im Gouvernement Marsa Matrouh isoliert.

Die klassischen Methoden zur Identifizierung der beiden Isolate wurden in der Abteilung für Geflügel und Hygiene, Veterinärmedizinische Fakultät, Universität Alexandria, Ägypten durchgeführt. Die molekulare Identifizierung und genetische Analyse erfolgten in der Gen-Analyse-Einheit des Nationalen Labors zur Qualitätskontrolle der Geflügelproduktion (NLQP), Ägypten. Mittels RT-PCR unter Verwendung spezifischer Primersets für die H5 und N1 Gene konnte bestätigt werden, dass es sich bei beiden Isolaten um AIV des Subtyps H5N1 handelt. Der molekularen Charakterisierung und der phylogenetischen Analyse der HA und NA zufolge war der Stamm EGY06 sehr eng verwandt mit dem früher im Jahr 2006 isolierten klassischen Stamm und wurde dem Clade 2.2.1 zugeordnet. Im Gegensatz dazu wurde der Stamm EGY10 im klassischen 2.2.1/c Gruppe zugeordnet, welcher häufig von kleinen kommerziellen Farmen und menschlichen seit 2009 isoliert.

Die Empfindlichkeit der Viren gegen verschiedene Desinfektionsmittel wurde auf Grundlage der Richtlinien der Deutschen Veterinärmedizinischen Gesellschaft (DVG) für die Prüfung von Desinfektionsverfahren und chemischen Desinfektionsmitteln geprüft. Die Experimente wurden mittels Suspensions-Test ohne und mit Proteinbelastung (40% Bovines Calf Serum (BCS)) sowie auf Keimträgern aus Holz und Gaze, die mit BCS belastet waren, bei

Raumtemperatur und Einwirkzeiten von 15 bis 120 Min durchgeführt. Die Verwendung von Glutaraldehyd, Formalin oder TH4® in einer Konzentration von 0,5% führte ohne Proteinbelastung zu einer Inaktivierung der Viren nach allen Einwirkzeiten (15, 30, 60 und 120 Min). Die Verwendung von Virkon®S 0,5% mit und ohne Proteinbelastung führte zum Überleben des Virus sogar nach 60 Min. Demgegenüber führte die Verwendung von Formalin und TH4® in einer Konzentration von 1% und 2% mit und ohne Proteinbelastung zu einer vollständigen Inaktivierung des Virus sogar bei der kürzesten Einwirkungszeit von 15 Min. Ähnliche Ergebnisse wurden nach Verwendung von Glutaraldehyd in einer Konzentration von 1% beobachtet. Die Behandlung von H5N1 mit Glutaraldehyd in einer Konzentration von 2% führte zu einer Gelbildung. Nach der Behandlung von kontaminierten Keimträgern (Pappelholz und Gaze) mit Formalin, Glutaraldehyd und TH4® in Konzentrationen von 0,5% wurde das Virus nach 30 Min inaktiviert. Während eine Konzentration von 1% der drei Desinfektionsmittel ausreichend war, um die beiden Isolate in 15 Min Einwirkzeit zu inaktivieren, konnte dieses Ergebnis im Fall von Virkon®S nicht erreicht werden, und eine höhere Konzentration war erforderlich um ähnliche Ergebnisse zu erzielen. Die Studie zeigte, dass die vier chemischen Desinfektionsmittel, wenn die verwendeten Konzentrationen höher als die vom Hersteller empfohlenen Konzentrationen sind, beide getesteten H5N1 Viren effektiv inaktivieren. Die Ergebnisse dieser Studie bieten einen neuen Ansatz zur Verbesserung der Biosicherheitsmaßnahmen in Geflügelbeständen und können zur Reduzierung der wirtschaftlichen Verluste beitragen

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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 in Anspruch genommen habe.

Berlin, den 07. Oktober 2013

Eman Marzouk