AMERICAN UNIVERSITY OF BEIRUT

STUDIES ON A NEWLY EMERGING H9N2-AVIAN INFLUENZA OUTBREAK IN POULTRY OF LEBANON

by
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AN ABSTRACT OF THE THESIS OF

Samar Kamal Dankar for Master of Science
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Title: Studies on a Newly Emerging H9N2-Avian Influenza Outbreak in Poultry of Lebanon

Twenty-four poultry farms were investigated during the summer of 2004 for a first emergence of Avian Influenza (AI) outbreak in Lebanon. Commercial layers and meat breeder farms showed a significant drop in egg production greater than free-range layer farms. In addition, the HI test confirmed that most of AI-ELISA positive chicken flocks had H9 antibodies. The isolated and propagated AI virus from brains of broilers was confirmed as H9N2 subtype.

Another experiment was done in October 2004; in order to evaluate an Intervet killed vaccine against H9N2 virus in poultry layers. The vaccine was administered two times in layers of a free-range farm, and three times in the commercial intensive farm. Results showed that killed H9N2 vaccine needs to be boosted 2 or 3 times in order to have a successful seroconversion.

The objective of the third study is to evaluate the impact of essential oils of *Eucalyptus* and peppermint (Mentofin®) on homogeneity of immune responses to live vaccines and on performance of MG/ H9N2-infected broilers. Mentofin treatment was allowed for three days, starting at 21 days of age. The pattern of improvement of coefficient of variation (CV) value was observed for titers against Infectious Bronchitis Virus and Newcastle disease virus. Broilers of the Mentofin treatment showed better performance, less signs and lesions, better feed conversion, and lower cumulative mortality, when compared to the control untreated group. Overall, a net profit equivalent to $ 491.20 (after subtraction of Mentofin cost) was saved per 10,000 Mentofin-treated birds.

Diluted *Origanum ehrenberjii* and *syriacum* essential oils were tested *in vitro* for their antibacterial effect and *in vivo* for their antiviral effect against H9N2-AI intracerebral challenge in day-old chicks. The diluted essential oils were compared *in vivo* to the prevalent drugs against AI-H9N2. Amantadine sulfate reduced mortality to 30 while *O. syriacum* reduced mortality to 60% in comparison to challenged and untreated control group (90%).

Key words: Avian Influenza, H9N2, Mentofin, *Eucalyptus*, Peppermint, *Origanum*, Essential oil, Killed vaccine

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ABBREVIATIONS

%	Percent
/	Per
µl	Microliter
AI	Avian influenza
AIV	Avina influenza virus
AREC	Agricultural Research and Education Center
AUB	American University of Beirut
C	Celsius
CRD	Completely Randomized Design
cRNA	Complementary Ribonucleic acid
d	days
ELISA	Enzyme-Linked Immunosorbent Assay
et al.	Et allii (and others)
HA	Haemagglutinin
HI	Haemagglutination-inhibition
HPAI	Highly pathogenic avian influenza
IgG	Immunoglobulin G
Kg	Kilograms
LPAI	Low pathogenic avian influenza
ml	Milliliters
mm	millimeter

mRNAs	Messenger Ribonucleic acid
MPAI	Moderate pathogenic avian influenza
RNA	Ribonucleic acid
Vs.	Versus

CHAPTER I

INTRODUCTION

Avian influenza virus belongs to the family of Orthomyxoviridae. It is classified into influenza type A, B and C. Influenza B and C infect only humans, while influenza A infects a wide range of animals, including avian species and mammalians (swine, humans, whales, seals…). Recently Influenza virus type A was isolated from marten, cats, dogs and leopards.

Avian influenza virus type A is subdivided into 16 H subtypes and 9 N subtypes, according to differences in HA (haemagglutinin) and NA (neuraminidase) glycoproteins, resulting in 144 combinations.

Highly pathogenic AI (H5 and H7) probably arising from low pathogenic AI (Ito *et al*., 2001), is causing severe problems and high mortalities in poultry, and is infecting humans since 1995 (Menno and Hien, 2005). HPAI virus is establishing in humans and causing severe disease, and killing around 60% of the infected subjects.

Low pathogenic avian influenza causes a wide range of clinical signs ranging from mild to severe signs, and a severe drop in egg production in chicken layers. In 1999, LPAI H9N2 crossed the host barrier and infected humans, causing mild disease (Peiris *et al*. 1999). Therefore, the virus is able to circulate freely, between different host species, causing concern of antigenic drift and shift.

Several studies tried to develop and evaluate antiviral drugs against AIV, like M2 inhibitors (Amantadine and Rimantadine), and Neuraminidase inhibitors (Oseltamivir and Neuraminidse). However, recently, the AI virus developed resistance to these drugs (Nicholson *et al*., 2003; Jong *et al*. 2006).

In our study, we aim, to serologically screen the Lebanese poultry farms for AIV presence, and to isolate and type the virus. We evaluated a new Intervet killed vaccine and the number of vaccine doses required to enhance humoral immunity in chicken layers. Moreover, we evaluated the efficacy of Mentofin, a combination of *Eucalyptus* and peppermint based drug for its efficacy against respiratory diseases. We also, intended to develop a new *Origanum* essential oil based drug, against AI-H9N2 in poultry.

CHAPTER II

LITERATURE REVIEW

A. Pathogenicity and Nature of Avian Influenza Virus

1. Description of AIV

Influenza virus belongs to the family of Orthomyxoviridae. It is grouped into types A, B and C according to differences in NP and M1 proteins.

Influenza B and C infect only humans while Influenza A infects a wide range of animals in addition to humans, horses, sea mammals (whales and seals), pigs, and many avian species. Recently, the H5N1 avian influenza circulating in Asia has expanded its host range to include cats, tigers and leopards, which generally have not been considered susceptible to influenza A (Keawchareon et al. 2004; Kuiken et al, 2004). Influenza virus is an enveloped negative- sense RNA with a segmented genome comprising 8 gene segments encoding for 10 proteins, namely 3 surface proteins and 7 internal proteins (Hatta and Kawaoka, 2002). In 2006, H5N1 influenza virus was discovered in Germany in Stone Marten, another mammalian species. The animal was found alive but showing signs of severe illness (WHO, 2006).

The surface proteins are haemaglutinin (HA), Neuraminidase (NA) glycoproteins, and M2 while the internal proteins are polymerases complex proteins (PB1, PB2 and PA), matrix 1 (M1), nucleoprotein (NP) and nonstructural proteins 1 and 2 (Fig 1).

According to the nature of HA and NA surface proteins, influenza viruses type A are divided into 16 distinct H subtypes (1 – 16) and 9 different N subtypes (1- 9)

resulting in 144 combinations (Wright and Webster, 2000). Recently, a new subtype (H16) was isolated from black- headed gulls caught in Sweden and the Netherlands in 1999 and reported in the literature in 2005. The samples collected from animals were identified by RT- PCR and HI tests (Fouchier et al. 2005).

Virus strains are classified according to host species of origin, geographic site and year of isolation, and serial number; however, for influenza A, serological properties of subtypes of haemagglutinin and neuraminidase are used for subtype classification.

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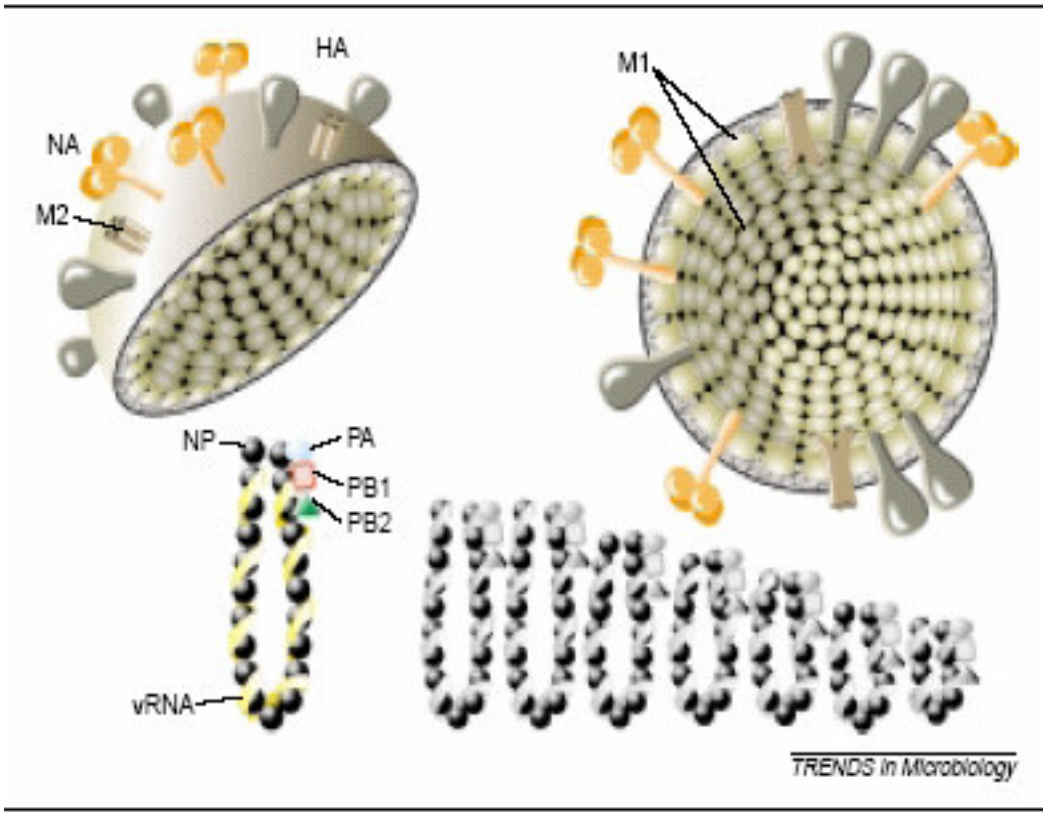

Fig.1. The structure of the influenza A virus

The virion consists of nine proteins. Of these, the two glycoproteins haemaglutinin (HA) and neuraminidase (NA) and the M2 protein are located on the virion surface. The Matrix protein (M1) resides within the lipid envelope. Inside the viral particule, eight segmented genomic RNAs (vRNAs) are associated with the RNA-dependent RNA polymerase subunits (PB1, PB2, and PA) and the nucleoprotein (NP). A non-structural protein (NS cleaved into NS1 and NS2) is not shown (Hatta and Kowaoka, 2002).

2. Highly Pathogenic Avian Influenza (HPAI) versus Low Pathogenic Avian Influenza (LPAI)

Most researchers agreed that the cleavage site in the HA glycoprotein is responsible for pathogenicity determination. Cleavage site (HA0) in haemaglutinin surface glycoprotein is either formed by 1 amino acid or a sequence of amino acids. Cleavage of HA is a prerequisite for infectivity resulting in free HA1 and HA2, where

the latter one is involved in virus-cell fusion, because it exposes the membrane fusion peptide located at the amino terminus of the HA2 subunit (Lazarowitz *et al*. 1975).

a. HPAI, LPAI, and MPAI

Low pathogenicity AI have one amino acid (Arginine) or two basic amino acids at the position -1 and -3 (H7 viruses) and position -1 and -4 (H5 viruses) of their cleavage site (HA0) (Wood *et al*. 1993) thus are cleaved by proteases enzymes such as trypsin- like enzyme present in the intestinal and respiratory tracts, hence restricting their replications to these organs, and never become systemic (Garten and Klenk, 1999; Horimoto and Kawaoka, 2001).

While, highly pathogenic AI have a multiple sequence of amino acids (Arginine and lysine) at their cleavage site (HA0) (R- X- R/ K- R) (where R represents the Arginine basic amino acid, K represents the lysine basic amino acid, while the X represents an acidic amino acid) and thus are cleaved by ubiquitous proteases such as furins and PC 6 Proteases (Horimoto *et al*., 1994) which are present in the Golgi apparatus of all body cells, thus leading to systemic infections.

The cleavage site is represented by (PENPKTR/GLF) amino acids in LPAI, while in HPAI are represented by (PENPK (7 a. a.) TR/GLF) on their cleavage (where 7 a. a. are inserted), (Pasick *et al*., 2005).

Hulse *et al*. (2004) added that when lysine (basic amino acid) was replaced by glutamic acid (acidic amino acid) at position 338 of cleavage site MP virus became more virulent, and the opposite was also true. This allows us to conclude that a change in one amino acid in the cleavage site could modulate virus's pathogenicity.

Tollis and Trani, 2002 added that HPAI is known to be less transmissible than LPAI, this may be due to the fact that the birds affected with the virulent strain die very quickly.

Some viruses exist that are not highly pathogenic despite possessing the known characteristics of high pathogenicity. Their HA contain multiple basic amino acids at the cleavage site and has glycosylation patterns similar to that of the highly pathogenic H5 viruses. These viruses cause disease of intermediate severity with systemic infection but relatively low mortality. Currently, little is known about these viruses. Although it is established, as mentioned above, that the nature of the HA cleavage site plays an important role in determining pathogenicity, there are viruses with multiple basic amino acids at the HA cleavage site that are not always HP (Hulse *et al*. 2004)

An experiment showed that intravenous injection of an HP virus into chickens resulted in an IVPI (intravenous pathogenicity index) of 3.00, (IVPI is explained in details page 27) while an MP virus had a much lower IVPI of 1.50, both viruses had the same HA1 domains with the same polybasic amino acids at the HA cleavage site.

Therefore, other determinants of pathogenicity are likely to exist in viruses of intermediate pathogenicity. For example, both the virulent and avirulent H5N2 viruses isolated during the Pennsylvanian outbreak in 1983 had identical amino acid sequences at the HA cleavage site and two other amino acids within HA were associated with the acquisition of virulence (Webster *et al*. 1986).

b. Role of HA in Pathogenicity

Recent investigations of the specific amino acids within HA showed that they were also, in addition to the cleavage site, involved in virus's pathogenicity.

Hulse *et al*. (2004) found that, a substitution in amino acids at residues 97, 108, 126, 138, 212, and 217 of HA affect pathogenicity (Table 1 and Fig. 2).

Table 1. Substitution of amino acids according to different residues

Residues in HA Pthogenicity	97	108	126	138	212	217
MP*	--	Threonine	--	Polar histidine	Lysine	Serine
HP**	Aspartic acid	Isoleucine	Aspartic acid	Hydrophobic leucine	Glutamic acid	Proline

* MP= Moderate pathogenicity
** HP= High pathogenicity

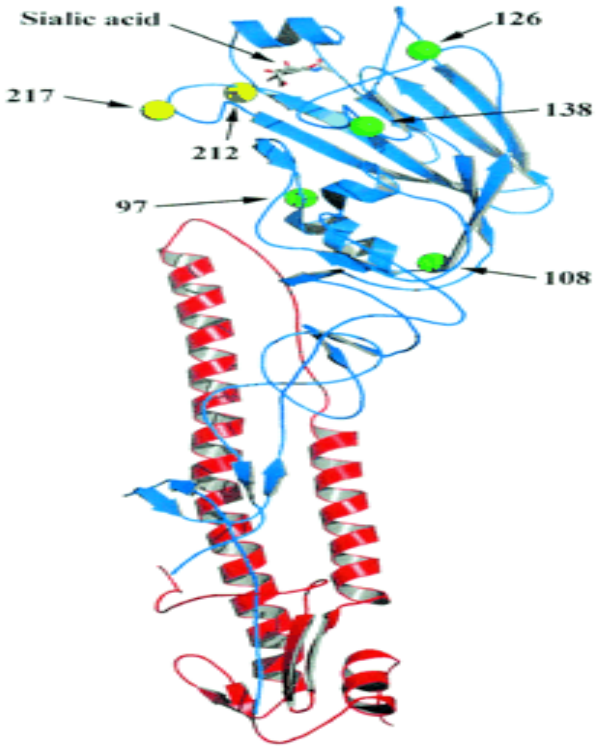

Fig.2**.** Location of amino acid differences between the HP and MP viruses shown in the three-dimensional structure of A/Duck/Singapore/3/97 H5 HA. The blue portion of the molecule is HA1, and the red portion is HA2 (Hulse *et al*. 2004).

c. Role of NA in Pathogenicity

Several studies indicate that NA plays some role in pathogenicity (Rott *et al*. 1976, Webster and Bean 1978). The NA has the role in facilitating mobility of the virus by removing sialic acid residues from HA after binding and release of virions from cells (Els *et al*. 1989).

Viral particles with low NA activity can't be released efficiently from infected cells (Mitnaul *et al*. 1996). A balance in HA and NA activities is crucial; there must be enough HA activity to facilitate virus binding and enough NA activity to allow release of virus progeny (Mitnaul *et al*. 2000). Greater NA activity results in higher HA cleavage in multiple organs.

A new study reported that an additional glycolysation sites in NA enhanced virulence but the mechanism by which glycolysation affects pathogenicity is still unknown. This might be because the highly glycosylated NA of the HP virus may facilitate increased activation of the host proteases required to cleave the virus's HA, a mechanism suggested by Schulman (Schulman 1983). Although this additional glycosylation site was not present in the highly pathogenic viruses isolated from chickens and humans in 1997, it was present in the NA of the human influenza virus A/ HK/ 213/ 03 (Mitnaul *et al*. 1996). Indeed, highly balanced actions of HA and NA have been shown necessary to achieve a productive infection.

d. Role of NS and PB Proteins in Pathogenicity

Observations showed that highly pathogenic avian H5N1 and H7N7 viruses transmitted to humans possessed multiple basic amino acids at their cleavage site. HP viruses are capable of human transmission and of systemic replication, reaching the

brain. However, H5N1 isolated from HK 1997 had a lysine amino acid at position 627 of PB2. This determines the efficiency of the viral replication in mouse cells but does not determine viral tropism toward different organs in mouse (Shinya *et al*. 2004). However, this was not always consistent, since other genotypes of H5N1 that emerged in 2001 did not possess a lys627 at PB2 protein and still were neurotropic in mice (Alexander et al. 2004).

Recent findings showed that the non- structural (NS) gene of HK H5N1/ 97 viruses has a role in the determination of high pathogenicity in mammals. Specifically, NS1 (one of the two proteins encoded by NS gene) contributes to viral pathogenesis by allowing the virus to disarm or resist the host's defense (INF defense) in many ways (Seo and Webster, 2002; Seo *et al*. 2002).

Experiments showed that an influenza virus possessing the NS gene of the 1918 pandemic blocks the expression of INF- regulated genes in lung human cells. An insertion of the NS gene to H5N1/ 97 viruses supports this theory in a model (mice) but not in another (pigs) (Seo *et al*. 2002).

Many findings showed that multiple gene constellations are involved in influenza virus pathogenicity but the outcome of infection is significantly host dependent.

e. Potential of Viruses to Become More Virulent or Pathogenic

Recent studies have demonstrated that avirulent strains have the potential to become virulent while replicating in chickens when a multiple basic amino acid is inserted at the cleavage site (Ito *et al*., 2001) due to a transcription fault (Pasick *et al*. 2005) but the cleavage site should be physically capable to accept the addition of a basic amino acid, thus maintaining the ability to cleave the HA (Tollis and Trani, 2002).

Wagner *et al*., 2000, confirmed that usually efficient virus propagation depends on a highly balanced activity of HA and NA, so the increase in receptor- binding activity mediated by HA should be accompanied by a concomitant increase in the receptor-binding activity of the NA.

Studies on H5 and H7 demonstrated that a deletion of 22 amino acids in the NA stalk decreased its ability to release the virus from the cells, while a carbohydrate at the HA head decreased the affinity of the virus for cell receptors (Mastrosovich et al., 1999).

3. Replication of AI virus

a. Mechanism of AIV Replication

Inside the host HA (surface glycoprotein) attaches to cellular receptors to initiate virus penetration and thus promotes fusion of viral and cellular membranes. The NA (surface glycoprotein) is a tetramer composed of a cytoplasmic tail and a globular head. The active site lies in a large depression on the surface of the head (Gubareva *et al*. 2000)

Virus cell binding is mediated through the HA glycoprotein and the cell sialic acid receptors (lamb 1989). At this level, the sialidase of the neuraminidase targets the sialic acid receptors in the mucosecretions, to prevent HA binding to them, thus restricts binding of HA to cell receptors. The acidic environment (pH 5.5) created by M2 ion channel (surface protein) favors fusion and uncoating of the virus.

Specifically, acidification, and proteases host enzymes, cause the HA0 to undergo proteolytic processing into 2 subunits HA1 and HA2, exposing a fusion peptide responsible for viral cell fusion. Later, the virus is internalized through endocytosis into

endosomes (cytolasmic vesicles). The acidic environment allows the dissociation of exposed M1- vRNP and thus allows their release and entry into the nucleus of the host cell through the nuclear pores to undergo replication. In the nucleus, the production of messenger RNA (mRNA) and complementary RNA (cRNA) occurs. The viral RNA-RNA polymerase comprising the three subunits (PA, PB1 and PB2) serves as a catalyst responsible for mRNA synthesis. Viral mRNA is exported outside the nucleus by the mediation of NS1 protein to assist in protein synthesis. The cRNA remains in the nucleus and serves as a template for the production of negative- sense genomic RNA (vRNA). Some of the proteins re-enter the nucleus to assist in viral RNA replication (Whittaker *et al*. 2001).

The newly formed vRNP are exported outside the nucleus through the nuclear pores. Assembly and budding are the last step of replication where all the viral components are trafficked to the correct location called lipid rafts, in the cell, and are correctly processed. The final release of the viruses from the host cell relies on the neuraminidase action. Sialidase in the NA acts as a sialic acid receptor destruction from host cells; this step is essential or else the newly formed glycoproteins will rebind to these receptors, and thus aren't released outside the host cell (Whittaker *et al.* 2001). Thus, NA is essential for the new viral particles release from the cell. Once the final assembly step is accomplished, new virus particles bud from the plasma membrane, and thus the newly released viral particles restart a new infection cycle (Perez, 2004).

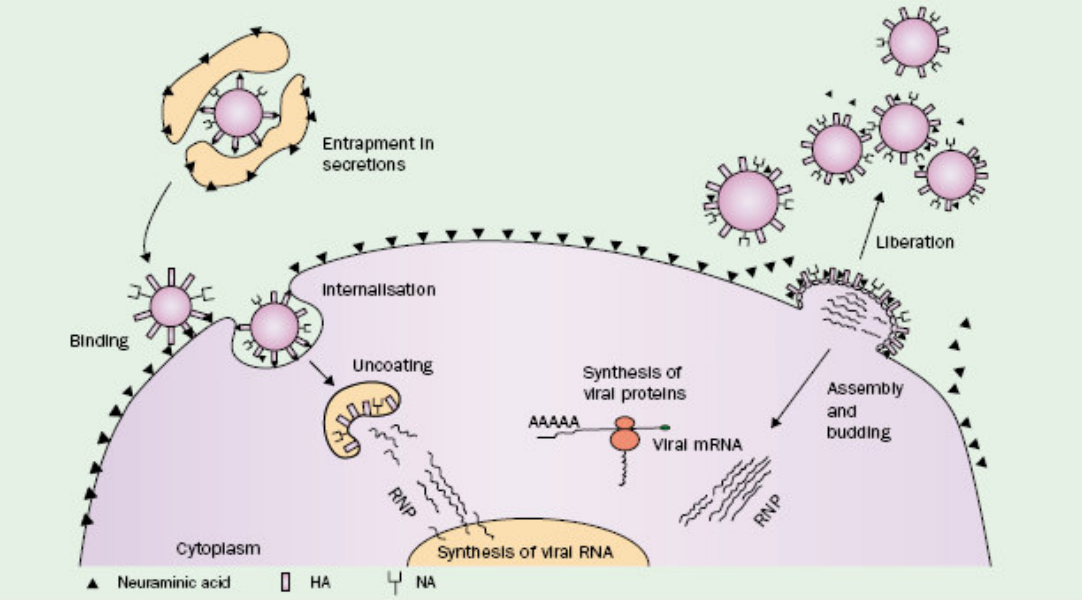

Fig. 3. Simplified influenza virus replication cycle

Virus attachment through HA to receptors containing terminal neuraminic acid residues and penetration into host cell, internalization of the virus into the endosome, fusion and uncoating, release of viral genome as vRNP into the cytoplasm, replication of viral RNA in the nucleus, Viral mRNA exported outside the nucleus for translation of viral proteins; assembly of virion inside the nucleus, budding, and subsequent release from host cell (Gubareva *et al*. 2000).

b. Antigenic shift, Antigenic drift, and Genetic Reassortment of AI Virus.

Observing how viruses adapt to restricted conditions in the host can provide important information on the requirements for efficient viral replication. In most instances, influenza viruses overcome barriers to replication by one of three mechanisms:

Genetic drift: is the accumulation of point mutations mainly in the glycoproteins (HA and NA). Antigenic drift leads to new virus strains replacing the existing strains; this feature enables the virus to evade immune recognition, thus leading to repeated outbreaks during the past years.

Antigenic drift occurs due to errors introduced by viral polymerase during replication since influenza viruses lack 'proof reading' mechanisms and are therefore

unable to repair errors that occur during replication. Or else, antigenic drift is caused by immune pressure, thus selection is antibody- mediated. Thus, vaccination can potentially contribute to increasing the rate of antigenic drift (Lee *et al*. 2004). It is mostly pronounced in humans, less in swine and equine.

Recent examples are observed in H9 and H5 by the observation of cross-reactivity determined by haemagglutination- inhibition of H9N2 viruses isolated between 1997 and 2003 and H5N1 viruses isolated between 1997 and 2004 with post infection serum of H9N2 and H5N1 respectively (Webster and Hulse, 2004). Antigenic drift was significantly pronounced in the HA gene of the H5N1 strains, post- infection sera collected from earlier strains (between 1997 and 2003) no longer react with the new 2004 viruses. Interestingly, the new 2004 virus post- infection sera react more strongly with the older viruses than with the homologous 2004 virus (Webster and Hulse, 2004).

Genetic shift or reassortment is the exchange of RNA (ribonucleic acid) segments between two genotypically different viruses, antigenic shift occurs whenever two or more viruses infect the same cell at the same time. The gene, being segmented might lead to the emergence of the new strains of influenza viruses (Nicholson *et al*. 2003). Genetic reassortment can result in the generation of a novel strain and/ or subtype (Webster and Hulse, 2004). Thus, a pair of influenza viruses each with 8 gene segments can result in 256 different combinations (Webster and Hulse, 2004). Pigs possessing both receptors for humans and for avian in their tracheas provide ideal conditions for reassortment (Brown, 2000). While quail allow the replication of all subtypes of influenza found in aquatic birds and may be involved in transmitting these viruses to chickens (Perez *et al*., 2003). If reassortment occurs, the likehood that the H5N1 virus can be more readily transmitted from person to person will increase

(Hammel and Chiang, 2005). Examples of antigenic shifts are the H9 virus (G1 lineage) is a reassortant of H9 virus with H5 / 97 viruses. G1 acquired the 7 internal genes from the H5/ 97 viruses, and thus became capable of human infection. While H9 virus (Y280 lineage) circulating in Asia is also known to be a reassortant with G9 viruses. The H9N2 virus thus acquired the ability to infect humans. The 1957 asian pandemic and the 1968 HK pandemic are examples of antigenic shift. Each arose from reassortment between humans and avian influenza virus. The acquisition of novel HA, NA and PB1 genes allow the virus to escape humoral immunity, in addition the viruses gained the ability to spread between humans (Webster and Hulse, 2004).

RNA-RNA recombination (exchange of genetic information between RNA segments). It is rarely observed in nature. (Li *et al*., 2003) (Fig. 4). In 2004, in British Columbia, the H7N3 HPAI outbreak emerged suddenly from a low pathogenic precursor. Analysis of the haemagglutinin (HA) genes showed a difference in the cleavage sites amino acids between the two viruses. The difference was due to a non-homologous RNA recombination between the HA gene and the M1 genes of the same virus, leading to a HPAI virus capable of human infections. Another recombination was observed in Chile in 2002, where the non- homologous recombination between the HA and the nucleoprotein gene lead to a virulence shift of H7N3 virus (Pasick *et al*. 2005).

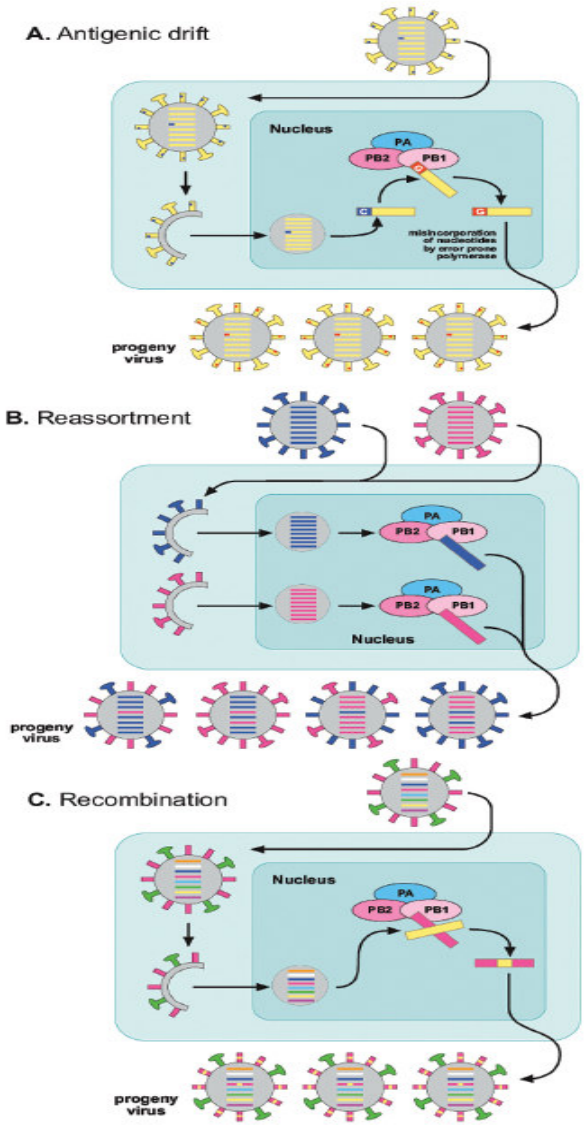

Fig. 4. The three mechanisms used by influenza viruses to adapt to their host ranges.
A. Antigenic drift occurs through errors during the replication of influenza viruses, which are unable to be repaired. These mutations accumulate amino acid changes within the viral genome, resulting in the existing strains being replaced by a new antigenic variant
Reassortment or antigenic shift is the exchange of ribonucleic acid (RNA) segments between two genotypically different influenza viruses infecting a single cell, which can result in the generation of a novel strain and/or subtype
Recombination occurs when two different sources of RNA contribute to a single influenza RNA segment (Pasick *et al*. 2005).

B. Pathogenicity of Avian Influenza in Poultry and Humans

1. HPAI and LPAI in Poultry (layers and broilers)

Avian influenza is causing damages in poultry ranging from subclinical, mild respiratory disease, decreased egg production, to acute fatal disease and this depending on: the sex, age of the bird, species affected, pathogenicity of the virus (HPAI vs. LPAI), concurrent situation of the flock and the management practices (Laudert et al., 1993).

Clinical signs of HPAI include various combinations of decreased activity and feed consumption, emaciation, decreased egg production, coughing, sneezing, rales, sinusitis, cyanosis of unfeathered skin, central nervous system disorders, and finally, severe disease with high morbidity and mortality that can be as high as 100% (Easterday et al., 1997). The animal might escape symptoms and dies very quickly (Easterday and Beard, 1994). HPAI viruses appear to be pantropic (high affinity to many kinds of tissues) in respect to their systemic replication and their ability to produce gross lesions, the most severe may result in severe inflammation, hemorrhage with necrosis and cellular death of many organs in the body, skin, brain, heart, lungs, pancreas, adrenal glands and visceral organs (Mo et al., 1997). While LPAI can be aymptomatic or cause a wide range of clinical signs varying from mild to severe signs of disease affecting respiratory, urogenital and/ or enteric systems (Easterday and Beard, 1994). The H9N2 influenza virus usually causes mild disease signs; however, some outbreaks are more severe, especially in mature birds, causing high morbidity rates, diarrhea, depression and reduced egg production in layers and breeders (Mc-capes

et al., 1986) it is also associated with coughing and respiratory distress in 75% of the birds with a mortality rate ranging from 10- 40 % (Naeem et al. 1999).

2. Zoonotic effect of avian influenza virus

Wild aquatic birds, notably members of the orders *Anseriformes* (ducks and geese) and *Charadriiformes* (gulls and shorebirds), are carriers of the full variety of influenza virus A subtypes, and thus, most probably constitute the natural reservoir of all influenza A viruses (Fouchier *et al*., 2003, Krauss *et al*., 2004). While all bird species are thought to be susceptible, some domestic poultry species - chickens, turkey, guinea fowl, quail and pheasants - are known to be especially vulnerable to the sequelae of infection. Avian influenza A viruses generally do not cause disease in their natural hosts. Instead, the viruses remain in an evolutionary stasis.

The haemagglutinin glycoprotein binds to sialic acid receptors in host cells. Human HA binds preferentially to sialic acid containing α2, 6- galactose linkages, while avian HA binds to sialic acid containing α2, 3- galactose linkages (Ito *et al*., 1997).

Knowing that pigs have both receptors in their trachea, thus they can support replication of influenza viruses of both avian and human origin, this fact makes pigs the "mixing vessel", where gene reassortment of swine, human and avian influenza viruses can occur resulting in the evolution of human pandemic strains (Scholtrisrek *et al*., 1998). In addition, epidemiological behavior of AI in people is related to the two types of antigenic variation: antigenic drift and antigenic shift.

Only a few haemagglutinin (H1, H2 and H3) and neuraminidase subtypes (N1 and N2) have established in human beings and have caused widespread respiratory

disease since 1918 (Stephenson *et al*., 2004). In addition, in Hong Kong in 1999 and again in 2003, H9N2 AI viruses were isolated from 3 children with mild respiratory symptoms, malaise, fever, anorexia, abdominal pain, coryza, vomiting and poor appetite (Peiris *et al*. 1999) but non human-to-human transmission occurred (Uyeki *et al*., 2003). The 1999 incidents were caused by antigenically different H9N2 viruses. The human infections in Hong Kong were caused by a virus that was related to A/ Quail/ HK/ G1/ 97 (H9N2) and possessed a genotype related to the H5N1/ 97 virus in all of its six internal genes (except for PB1 and PB2). A virus that was antigenically related to A/ duck/ HK/ Y280/ 97 (H9N2) and to A/ chicken/ HK/ G9/ 97 (H9N2) (resembling H5/ 97 virus by its PB1 and PB2 internal genes) and had a distinct avian- like genotype caused the infections in the Mainland China, again with mild illnesses (Guo *et al*., 2000). The child infected in 2003, was infected by an Y280 (H9N2) lineage (G9- like). Y280 is the most predominant lineage isolated from chickens and ducks in Asia. Both lineages have acquired receptor- binding to a 2, 6Gal linkage found on human cells, while only the Y280 lineage was isolated from swine in Southern china (Li *et al.,* 2003).

Highly pathogenic AI can also cause human infections. H5 and H7 caused many human infections, since 1995 in UK and lately in 9 different countries in Asia in 2005 (Menno and Hien, 2005). H5 infections are characterized by severe, usually bilateral, pneumonia complicated by acute respiratory distress syndrome clinically indistinguishable from severe human influenza. A large proportion of patients also complained of gastrointestinal symptoms, which are mostly common in children but not in adults. Evidence that the clinical spectrum of human H5N1 infections is not restricted to pulmonary symptoms was provided by a reported case of possible central nervous system followed by coma and death (Menno and Hien, 2005). While H7 caused mostly

conjunctivitis described as red tearful, painful, burning, and itching eyes accompanied by purulent fluid in the eyes and sensitivity to light or photophobia (Adam *et al*., 2004). Koopmans *et al*. (2004) was the only one to report cases showing influenza- like illness and confirmed human-to-human transmission to 3 household contacts in 2003 in the Netherlands infected with H7N7 (Koopmans *et al*. 2004). Two human cases of H7N3 infections have been reported by OIE in British Columbian outbreak in Canada showing conjunctivitis but no death occurred (Webster and Hulse, 2004)

Recently Avian H10N7 LPAI caused infections in 2 infants in Egypt causing fever and coughing (http://www.promedmail.org).

Avian influenza in people causes a broad range of illness, from symptomless infections through various respiratory syndromes, disorders affecting the lung, brain, liver, kidneys, and muscles, to fulminant primary viral and secondary bacterial pneumonia (Nicholson *et al*., 2003).

C. H9N2 Around the World

Avian influenza outbreaks caused by H9N2 viruses in poultry have been reported in Europe (Germany, Italy, and Ireland), Middle East (Iran, Pakistan, and Saudi Arabia), South Africa, Hong Kong and USA during the last 10 years (Naeem *et al*., 1999).

Specifically in the Middle East, avian influenza H9N2 was detected in northern Pakistan in 1998; infections resulted in sudden drops in egg production, chicken mortality of 2 to 3% per day and respiratory lesions (Naeem *et al*., 1999) while in southern Pakistan, in 2003 all flocks showing a decrease in egg production with or without respiratory signs were H9N2 positive (Naeem *et al*., 2003). Naeem mentioned

that the northern area has a mild temperature during the summer (up to15°C) whereas in the southern region it ranges between 25-45 °C.

The pathogenicity indexes of the H9N2 isolates in poultry are usually low, resulting in low mortalities and significant losses in egg production in chicken layers, but with high mortalities in certain instances in broilers.

In Iran, an epidemic of avian influenza AI (H9N2) occurred in broiler chicken farms during 1998- 2001. Mortality between 20% and 60% was commonly observed on the affected farms but, mixed infections of the influenza virus with other respiratory pathogens, particularly IBV and MG, were thought to be responsible for such high mortality, which resulted in great economic losses (Nili and Assi, 2003).

D. Potential of Human Pandemic by H9N2

H9N2 viruses have been circulating in Asia regularly in ducks before the 1990s. However, H9N2 have been detected in chickens after the 1990s. H9N2 subtype viruses have been detected in pigs later in 1998 and subsequently were isolated from humans in Hong Kong and Mainland China in 1999. Subbaro et al. (1998) reported that amino acid at position 627 of the PB2 internal protein plays and important role in the host range of the influenza A viruses. While Li et al. (2003) demonstrated that one amino acid substitution of aspartic acid to aspargine at position 701 of PB2 protein enables a duck infected with H5N1 to cross the host barrier to infect and replicate in mice.

Antigenic and genetic analyses of the H9N2 influenza viruses isolated from domestic ducks in southern China during 2000-2001 provide convincing evidence that the H9N2 influenza virus lineages established since the mid-1990s in chicken and quail

(Guan et al., 1999) have been transmitted back to domestic ducks, generating double or triple reassortants with influenza viruses already resident in ducks. Rather than indicating a one-way flow of H9N2 viruses and their genes from aquatic to terrestrial poultry as previously recognized (Guan et al. 2000; Guo et al. 2001), these findings indicated reverse transmission back to duck, i.e., there is a two-way transmission between terrestrial and aquatic birds. This resulted in the generation of multiple genotypes of H9N2 viruses containing internal genes of aquatic avian origin (Li et al., 2003).

The findings of the recent investigations suggest that these H9N2 reassortant viruses may have pandemic potential since they contain a receptor-binding profile (Matrosovich et al., 2001) that favors infection of humans and that some (e.g., the Qa/HK/G1/97-like lineage) have gene segments previously associated with human disease (Peiris et al., 1999). As H9N2 influenza viruses are not highly pathogenic for poultry, it makes them more, rather than less, likely to be of pandemic relevance. In fact, viruses that are less pathogenic for poultry have a greater opportunity to become widespread since they do not raise concern and permit their hosts to survive unhindered. Thus, they are free to continue to reassort and are more likely to have the opportunity to find the best gene constellation (Webster et al., 1992) that permits infection of humans and facilitates further person-to-person transmission. The connecting peptides of the HA of the 1968 H3N2 and 1957 H2N2 pandemic viruses indicate that they are unlikely to have been highly pathogenic for poultry. Two-way transmissions between different types of poultry in southern China increase the opportunity to generate influenza viruses with pandemic potential. These findings demonstrate that such viruses may be directly generated from ducks (Li et al. 2003).

E. Recommended methodology in surveillance of LPAI and HPAI

OIE and FAO (2004) have set rules and diagnostic procedures for avian influenza detection.

Step 1: Initial screening tests

Rapid direct antigen detection tests or immunofluorescence tests can be used for screening tests. Samples can be taken from live or dead birds. These tests detect group antigens. These are relatively cheap and rapid test to perform.

Step 2: Confirmatory tests

Confirmatory tests are done through virus isolation. Samples are taken from tracheal or cloacal swabs (live birds); spleen and lung specimens (live birds). If transported, samples should be kept in special transport medium (PBS or TPB).

Isolated virus is inoculated in SPF embryonated eggs. Two passages are required before declaration of negative results.

PCR (Polymerase chain Reaction) either RT- PCR (reverse transcriptase PCR) or RRT-PCR (Real Time PCR) are used in order to detect the gene sequence of the isolate. The PCR method is known to be as sensitive as the embryonated eggs inoculation, but is less sensitive in the case of bacterial contamination.

Step 3: Procedures for characterization of isolates

Once isolated, and replicated, it is time for agent characterization. This can be done by several methods, either by haemagglutinin typing and neuraminidase typing, or by RT- PCR and RRT- PCR that detect and analyze the gene sequence requiring specific primers for H and N subtypes.

Step 4: Pathogenicity determination

Pathogenicity determination can be done either by PCR that can detect the multibasic amino acids at the cleavage site, or by live bird challenges which enables us to determine the intravenous pathogenicity index (IVPI) or intracerebrally pathogenicity index (ICPI) and thus the classification of the virus as HPAI or LPAI.

Step 5: Serological assays or subtype- specific test

Several tests are used for subtype determination. Haemagglutination inhibition test (HI) is useful, or agar gel immunodiffusion test (AGID), and competitive ELISA (enzyme linked immunosorbant assay). These tests are useful for subtyping using monoclonal antibodies (Aymard *et al.,* 2003) an ELISA format for the detection of H7-specific antibodies has been reported (Sala *et al.,* 2003), but there is no such assay presently available for the detection of H5-specific antibodies in avian sera. These tests are also used for AI monitoring in a flock. AI monitoring through serology are useful for LPAI viruses but not for HPAI viruses due to high and rapid mortalities.

Note the definition of HPAI (OIE Manual of Standards for Diagnostic Tests and Vaccines):

Any influenza virus that is lethal for six or more of eight 46-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of 1:10 dilution of a bacteria-free infective allantoic fluid, or that has an IVPI of greater than 1.2.

After intravenous injections of 0.1 ml (100 µl) of diluted infective allontoic fluid (1:10) with an HA titer > 1: 16 (or > 2^4 or $\log_2$ 4) to six- week- old SPF chickens. The chickens were examined for clinical signs of viral disease every 24 h over a 10-day period. The birds received a score of 0 if they appeared normal, 0 if they were normal, 1 if they were sick, 2 if they were severely sick, and 3 if they were dead. Chickens were

classified as being sick if they displayed at least one clinical sign, such as depression; reluctance to move, etc…Chickens were classified as severely sick if they displayed more than one clinical sign. The index was then calculated as the mean score per bird per observation.

For ICPI, each of the ten day- old- chicks were infected by the intracerebral route with 50 µl fresh infective allontoic fluid diluted 1: 10 in isotonic saline. Pathogenicity indices were calculated by scoring birds: 0, normal; 1, sick and 2 if dead, at a daily observation for 8 days and calculating the mean score per bird per observation (Reference).

Any H5 or H7 virus that does not meet the criteria in item 1 above, but has an amino acid sequence at the haemagglutinin cleavage site that is compatible with HPAI viruses.

Any influenza virus that is not an H5 or H7 subtype that kills one to five chickens in the test described above and grows in cell culture in the absence of trypsin.

F. Vaccines

1. Factors Required for Vaccines Effectivity

Avian influenza virus has become endemic in a number of species including humans, horses, swine and poultry. Vaccination has been widely practiced not only to reduce the incidence of clinical disease but in some cases a part of an eradication strategy, aiming to clear a region or segment of a population of the infectious disease (Suarez 2005). For a vaccine to be a valuable control tool, it should have 3 specific qualities:

First it should reduce or prevent clinical disease; second it should reduce or eliminate virus shedding into the environment from infected birds to prevent the virus from spreading to uninfected flocks. Third, vaccination strategy step 1 and 2 can help breaking the infection cycle (Lee and Suarez, 2005).

The ideal vaccine would fully prevent virus infection, a situation often called sterilizing immunity. However, sterilizing immunity is rarely if ever obtained with a commercial vaccination program, and is extremely unlikely to be achieved with a mucosal infection like avian influenza virus. However, a good vaccine for AI can reduce the amount of virus being shed by several $\log_{10}$ of virus. Reduction in virus shedding means fewer viral particles shed in the environment reducing spreading of the virus to other flocks (Suarez 2005).

2. H9 Vaccines in Humans

Although H9 viruses do not have a multibasic haemagluttinin, and they show a low pathogenicity for avian species, 2 lineages G1 A/ HK/ 1073/ 99 (the lineage that infected 2 infants in HK, showing related genes to H5N1 /97) and Y280 A/ HK/ 2018/ 03(lineage infecting humans in the Mainland China and HK in 2003, showing genes related to G9 lineage) (G9- like) are capable of human infections (Peiris *et al*., 1999). Whole virus G1 H9 vaccine produced cross-reactive antibody responses against both G1 and G9 viruses and protected mice against challenge with either virus. By contrast, whole G9 H9 vaccine induced homologous antibodies and was able to protect only against H9 G9 challenge. A single dose of H9 vaccine induced adequate immune responses in mice (Rimmelzwann *et al*., 1999; Lu *et al*., 2001). Since Y280 do not grow

well in eggs, another reassortant vaccine has been produced G9/PR8 (PR8 a lineage not known to infect humans) having the HA and NA genes from the H9N2 G9 lineage and the 6 other proteins from the PR8 lineage. The formalin-inactivated G9/PR8 vaccine protected mice against G9 challenge after 1 vaccine dose, and protected mice against G1 and G9 H9 challenge after 2 vaccine doses and increased as well antibody titers (Chen *et al.,* 2003; Hualan *et al*., 2003).

3. AI Vaccines in Equine

Despite widespread vaccination using inactivated, whole-virus vaccines in the United

States (sometimes as often as every 4±6 weeks in settings with frequent relocation of horses), influenza continues to be a major problem for the equine industry. Influenza virus is the most common primary respiratory pathogen in North America, and respiratory disease ranks second only to colic (pain in the abdomen) as the most common disorder requiring veterinary attention (Traub-Dargatz *et al*., 1991; Mumford, 1992). In particular, influenza can have a substantial impact on athletic horses, because of the need to withdraw them from training for prolonged periods of time to allow their respiratory epithelium to heal. An effective influenza virus vaccine would be of benefit to both reduce disease and discomfort in horses and to reduce the financial impact of influenza on the equine industry. Mumford has clearly demonstrated that an immune-stimulating complex (ISCOM) (Iscoms are cage-like structures, typically 40 nm in diameter, that are comprised of antigen, cholesterol, phospholipid and saponin) equine influenza virus vaccine induces superior immune responses and protection against disease and infection, compared to inactivated-virus vaccines.

However, even this vaccine is not completely protective against infection in all animals, and it is not commercially available in the United States (Mumford *et al*., 1994a, b). DNA vaccines are a novel alternative to conventional vaccine strategies; they offer many of the potential benefits of live virus vaccines without their risks. In particular, because immunogens are synthesized *de novo* within DNA transfected cells, antigen can be presented by MHC class I and II molecules, resulting in stimulation of both humoral and cellular immune responses. Administration of plasmids encoding hemagglutinin genes from influenza viruses is an effective method for priming and/or inducing virus-specific immune responses, and for providing partial to complete protection from challenge infection in mice, horses and pigs (Olsen 2000).

4. AI Vaccines in Pigs

Beyond the impact of influenza for the swine industry, pigs, being a" mixing vessel", are also very important in the global ecology of avian influenza in humans. Beyond their role in genetic reassortment, pigs also pose a threat as sources for zoonotic transmission of swine influenza viruses (Wentworth *et al*., 1997), sometimes resulting in the death of the people infected (Wentworth *et al*., 1994).

One mechanism for control measures is vaccination, but as in horses, the inactivated whole-virus vaccine that is commercially available in the United States does not consistently provide complete protection from challenge infection (Macklin *et al*., 1998). DNA vaccine technology offers a novel approach to the development of effective immunization strategies for influenza in both horses and pigs, and an immunologist's tool to better understand the nature of protective immunity against influenza (Olsen 2000).

5. Vaccines in Poultry (against HPAI and LPAI)

a. Conventional Vaccines

Conventionally, vaccines used against LPAI have been prepared from infective allantoic fluid and inactivated by formalin and emulsified with mineral oil.

The existence of a large number of virus subtypes, together with the known variation of different strains within a subtype, pose serious problems when selecting strains to produce influenza vaccines, especially for LPAI. In addition, some isolates do not grow to a sufficiently high titer to produce adequately potent vaccines without costly prior concentration. While some vaccination strategies have been to produce autogenous vaccines (homologous), i.e. prepared from isolates specifically involved in an epizootic, others have been to use vaccines prepared from viruses possessing the same haemagglutinin subtype (heterologous) that yield high concentrations of antigen. In the USA, some standardization of the latter has been carried out in that the Center for Veterinary Biologics have propagated and hold influenza viruses of several subtypes for use as seed virus in the preparation of inactivated vaccines (Bankowski 1985).

Since the 1970s in the USA, there has been some use of inactivated vaccines produced under special license on a commercial basis (Halvrson 1998). These vaccines have been used primarily in turkeys against viruses that are not highly pathogenic. Conventional vaccination against the prevailing strain of LPAI has also been used in Italy for a number of years (Daprile 1986). Vaccination against H9N2 infections has been used in Pakistan (Naeem *et al.* 1999), Iran (Vasfi Marandi *et al.* 2002), the People's Republic of China (Liu *et al.*, 2002) and in Lebanon since the summer of 2004 (Barbour, 2006 personal communication).

Vaccination against HPAI of H5N2 subtype was used in Mexico following outbreaks in 1994–1995, and against H7N3 subtype in Pakistan (Lee *et al*. 2004; Garcia *et al*., 1998) following outbreaks in 1995. In Mexico, the HPAI virus appears to have been eradicated, but LPAI virus of H5N2 has continued to circulate, while in Pakistan highly pathogenic AI viruses genetically close to the original highly pathogenic AI virus were still being isolated in 2001 (Swayne and Suarez 2001) and 2004. Following the outbreaks of HPAI caused by H5N1 virus in Hong Kong in 2002 (Sims 2003) a vaccination policy was adopted there using an H5N2 vaccine. In 2004 the widespread outbreaks of highly pathogenic AI H5N1 in some countries of South-East Asia resulted in prophylactic vaccination being used in the People's Republic of China and Indonesia. Prophylactic vaccination has also been used in limited areas in Italy to aid the control of H5 and H7 LPAI viruses.

b. Recombinant Vaccines

Recombinant vaccines for AI viruses have been produced by inserting the gene coding for the influenza virus haemagglutinin into a live virus vector and using this recombinant virus to immunize poultry against AI. Recombinant live vector vaccines have several advantages:

They are live vaccines able to induce both humoral and cellular immunity,

They can be administered to young birds and induce an early protection, e.g. the fowl poxvirus can be administered at 1 day of age, is compatible with the Marek's disease vaccine, and provides significant protection 1 week later,

They enable differentiation between infected and vaccinated birds, since, for example, they do not induce the production of antibodies against the nucleoprotein or matrix antigens that are common to all AI viruses. Therefore, only field-infected birds

will exhibit antibodies in the AGID test or ELISA tests directed towards the detection of influenza group A (nucleoprotein and/or matrix) antibodies.

However, these vaccines have limitations in that they will replicate poorly and induce only partial protective immunity in birds that have had field exposure to or vaccination with the vector virus, i.e. fowl poxvirus or infectious laryngotracheitis viruses for currently available recombinant vaccines (Lyschow *et al*., 2001; Swayne *et al*. 2000), which restricts their use. If used in day-old or young birds the effect of maternal antibodies to the vector virus on vaccine efficacy may vary with the vector type. In the case of fowl poxvirus recombinant vaccine, it has been reported that effective immunization was achieved when given to 1-day-old chicks with varying levels of maternal immunity (Swayne *et al*. 2000). However, when very high levels of maternal antibodies are anticipated due to previous infection or vaccination, the efficacy of the fowl pox vector vaccine in such day-old chicks should be confirmed. In addition, because the vectors are live viruses that may have a restricted host range (for example infectious laryngotracheitis virus does not replicate in turkeys) the use of these vaccines must be restricted to species in which efficacy has been demonstrated.

The use of recombinant vaccines is restricted to countries in which they are licensed and are legally available. The recombinant fowlpox-AI-H5 vaccine is licensed in El Salvador, Guatemala, Mexico and the USA (Swayne 2003). Recombinant fowl poxvirus vaccines containing H5 HA have been prepared and evaluated in field trials (Beard *et al*., 1991; Gracia- Gracia *et al*., 1998; Quiao *et al*., 2003), but the only field experience with this vaccine has been in Mexico, El Salvador and Guatemala where it has been used in the vaccination campaign against the H5N2 virus. Between 1995 and

2001, Mexico used more than 1.423 billion doses of inactivated H5N2 vaccine in their H5N2 control program (Villareal-Chavez C. & Rivera Cruz, 2003). In addition, Mexico, Guatemala and El Salvador have used over 1 billion doses of the recombinant fowlpox-AI-H5 vaccine for control of H5N2 LPAI from 1997 to 2003.

6. Adequate Flock Immunity

No studies have conducted to determine the level of herd immunity that is protective for poultry against HPAI or LPAI (Ellis *et al*., 2004); however, some factors affect adequacy of immune level to protect against field infections. In his study, Ellis *et al*, showed that there is a difference in immune responses between young vs old chickens. Prime vaccination for old chickens (20 to 50 days of age) showed less antibody titer than young ones, since immunosupression caused mostly by some diseases (IBDV, NDV) that probably occurred in older chickens might lead to inadequate immune system priming, and consequently to inappropriate antibody response following the second vaccination too.

7. Drawback of Vaccination

The inappropriate use of vaccination against HPAI in poultry has become a part of the problem spread, and has serious implications on human health, the WHO is now warning that poultry vaccination for HPAI viruses can lead to silent transmission. China announced lately her decision to vaccinate all its 4 billion chickens to avoid the problem, especially after human infections. China's announcement caused heightened state of threat and alert especially to all its neighboring countries.

Experiments showed that vaccination, with a killed oil adjuvented H5N1 HPAI, gave a protection of chickens after challenge; knowing that challenge was given 3 weeks after the vaccine delivery, the peak of antibody production. This vaccine seems to assure protection against challenge at the peak of the antibodies level, but unfortunately wasn't tested for protection against later or earlier challenges. In addition, Ellis warned of the use of this vaccine in Hong Kong, due to the close proximity of the farms due to limited land availability. Ellis was aware of the close proximity with respect to humans and poultry, in addition that the use of vaccination may accelerate antigenic drift of the virus, thus leading to frequent changes of vaccine composition, and also, there might be prolonged but undetectable virus shedding in vaccinated chickens, thus vaccination against HPAI may undermine the push for biosecurity.

Inactivated H9N2 LPAI vaccines have been used in chickens since 1998 but the antigenic drift of the H9N2 viruses that emerged in China may be due to the incomplete protection induced by the vaccination makes the use of these vaccines inefficient. Thus, it is necessary to evaluate how well the prevailing isolates match the present vaccine and update the vaccine formulation accordingly (Lee *et al*., 2004).

8. Vaccination as a Step in AI Control and Eradication

An eradication program must include strict flock quarantines, increased biosecurity, increased passive and active surveillance and education (Swayne *et al*., 1999; 2003).

Vaccines used nowadays are killed oil adjuvanted vaccines or the fowl pox recombinant vectored vaccine. Killed vaccine is more widely used, injected subcutaneously; however, they have many drawbacks; the cost of vaccination, the need

for serological surveillance, the fear of antigenic drift due to immune selection and the differentiation between infected vs. vaccinated infected birds (Suarez 2005). Differentiating between vaccinated and infected animals can be done by the following 4 strategies: First using unvaccinated sentinels in the farm. Sentinels need to be tagged and continuous serological surveillance is needed to test for AI exposure. However, the presence of sentinels in the farm may increase the risk of infection in the flock, in addition to the hard management and handling of the sentinel birds (Capua and Marangon 2004; Suarez 2005). Subunit vaccines can be used consisting of haemagluttinin protein oil-adjuvanted. Third, heterologous vaccine, using a killed oil adjuvanted vaccine containing the same haemagluttinin (H) subtype as the field strain, but a different Neuraminidase (N) subtype. Antibodies to the N of the field strain virus are a marker for field strain infection (Capua *et al*., 2003; Capua and Marangon 2004, Suarez 2005). Fourth strategy consists of the use of differential immune response to the influenza non-structural protein 1 (NS1). The NS1 is the only protein that is not packaged in the virus but it is produced in large quantities in the infected cells when the virus infects cells naturally. Since killed AIV vaccine does not replicate in the cell, NS1 protein is not produced, thus no antibodies are formed against NS1 protein. This DIVA approach was demonstrated previously with equine influenza virus in horses using ELISA format (Birch-Machin *et al*., 1997). However when the AI virus of killed vaccine is previously grown in allontoic fluid of chicken embryos, some NS1 proteins are detected. The small amount of NS1 detected is considered as a drawback of this strategy; however, some can count on the level of antibodies to differentiate not only vaccinated from infected animals, but also to identify vaccinated and then infected birds (Suarez 2005).

G. Antiviral Drugs

1. Mode ofAaction

To date two classes of antiviral drugs are approved for treating influenza A: The M2 inhibitors, adamantanes (Amantadine and Rimantadine), and the Neuraminidase (NA) inhibitors (Oseltamivir and Zanamivir) (Wutzler *et al*., 2004).

a. Adamantanes Mode of Action

The M2 inhibitors are approved for treatment and prophylaxis of influenza A for individuals 1 year and older. Amantadine inhibits the virus replication by interfering with M2 protein ion channel during the early stages of infection (Wang *et al*., 1993).

Although amantadine has been recommended for use 30 years ago, it is scarcely been used because of high frequencies of central nervous system and headache side effects especially in the elderly and those with decreased renal function (Wutzler *et al*., 2004)

b. Neuraminidase Inhibitors Mode of Action

Oseltamivir and Zanamivir belong to the same class of antiviral drugs consisting of neuraminidase (NA) inhibitors. Both drugs have been introduced into clinical practice between 1999 and 2002 (Wutzler *et al*., 2004). This class is approved for treatment of influenza A and B in adults and in children older than 1 year for oseltamivir and 13 years old for zanamivir. The neuraminidase inhibitor drugs block the viral replication at nanomolar concentrations by targeting a region of the neuraminidase surface glycoprotein of influenza viruses that is highly conserved from strain to strain (Von Itzstein *et al*., 1993).

The action of the neuraminidase inhibitors leads to the prevention of the release of newly formed viruses from the cell surface of infected cells. These inhibitors possess antiviral activity *in vitro* to all nine neuraminidase subtypes of influenza A virus and to the neuraminidase of influenza B (Wutzler *et al*., 2004).

Zanamivir use is restricted since it is taken using an inhalator. Zanamivir and Oseltamivir are recommended for the treatment of influenza in adults but only Oseltamivir is used for children, and unless they are at risk; therapy can be started 48 hours after onset of symptoms (Nicholson *et al*., 2003).

2. Distribution and Half-Lives of Commercial Drugs

a. Adamantanes Family

After the oral administration of a single 100 mg amantadine hydrochloride capsule, several studies showed mean maximum plasma concentrations of 0.2 to 0.4 mcg/ml and mean times to peak concentration of 2.5 to 4 hours. Mean half-lives ranged from 10 to 14 hours. Across other studies, amantadine plasma half-life has averaged 16 ± 6 hours (range 9 to 31 hours) in 19 healthy volunteers.

67% of amantadine binds to plasma proteins in vitro. A large proportion of the substance binds to red blood cells. Amantadine concentrations in the erythrocytes are 2.66 times higher than plasma concentrations in healthy volunteers. The apparent volume of distribution is 5-10 l/kg, which suggests extensive tissue binding. The volume of distribution falls as the dose increases. Concentrations of amantadine in the lungs, heart, kidneys, liver, and spleen are higher than blood concentrations. The drug accumulates in the nasal mucosa after several hours. Amantadine crosses the blood

brain barrier, but it is not possible to measure to what extent this occurs. It also passes into the breast milk and crosses the placenta (Leaflet Symmetrel, Novartis Company).

b. Neuraminidase Inhibitors Family

Tamiflu (Oseltamivir phosphate) is available in a capsule containing 75 mg for oral use. The chemical formula of Oseltamivir phosphate is $C_{16}H_{28}N_2O_4$. Oseltamivir is readily absorbed from the gastrointestinal tract, with approximately 80% bioavailability after oral administration of Oseltamivir phosphate. It is extensively converted by hepatic esterases to Oseltamivir carboxylate, while less than 5% of the dose is eliminated unchanged. A lipophilic side chain of the active drug binds to the virus enzyme, blocking its ability to cleave sialic acid residues on the surface of the infected cell and resulting in an inability to release progeny virions. Oseltamivir is well distributed to the nasal mucosa, the tracheal lining, and the tissues of the middle ear. Neither the prodrug nor the active form is highly protein bound. Following intravenous administration, the binding of Oseltamivir carboxylate to human plasma protein is low (3%). The binding of Oseltamivir to human plasma protein is 42%, which is insufficient to cause significant displacement-based drug interactions. While, at least 75% of an oral dose reaches the systemic circulation as Oseltamivir carboxylate. Oseltamivir carboxylate is eliminated by glomerular filtration and renal tubular excretion without further metabolism. The average half-life of elimination in adults is 6-10 hours (www. Rocheusa.com).

3. Resistance toAantiviral Drugs:

a. Resistance to Adamantanes

Drug resistance emerges rapidly during treatment. The genetic basis of resistance is a single nucleotide change resulting in amino acid substitution at position 26, 27, 30, 31, or 34 in the membrane- spanning region of M2 (Nicholson *et al*., 2003). In addition, amino acids substitution by amantadine within the M2 protein lead frequently to resistant mutants which are genetically stabile transmissible and virulent (Hayden *et al*., 1996). In a recent study, Ilyushina *et al*., proved that H5, H7 and H9 subtypes showed resistance to amantadine showing amino acid substitutions in the M2 protein at three positions: V27A, A30S and S31N.

b. Resistance to Neuraminidase Inhibitors

Resistance to Neuraminidase inhibitors involves either a mutation in the active site of the neuraminidase altering its sensitivity to inhibition, or a mutation in the haemaglutinin. Mutation in the HA confers to drug resistance thus, decreasing the affinity of the protein for the cellular receptor, thus enabling virus to escape from infected cells without the need for viral neuraminidase (Nicholson *et al*., 2003). Lately, De Jong *et al*. (2006) reported a high- level resistance of H5N1 virus to oseltamivir, due to a substitution in Neuraminidase isolated from 2 patients in Vietnam.

4. Use of antiviral drugs in poultry

Rinaldi used amantadine for treatment of infection in a large flock of Japanese quail in Italy. The mortality rate was reduced approximately by 50% but the infection rate was unaffected. In another study (Beard *et al*., 1984; Webster *et al*., 1985)

amantadine and rimantadine administered in the drinking water reduced the mortality; however the birds were still infected and shed virus. Additionally, there was a rapid emergence of amantadine-resistant viruses that killed hens receiving the drug; amantadine was present in different organs after autopsy, muscle, liver and the serum of treated birds. After withdrawal of the drug, the levels in serum and tissues fell to virtually zero within 24 hours; however, amantadine was maintained in the albumen and yolk of eggs for a period of at least 3 days post the end of treatment. To date this drug is not approved for use in birds need for human consumption (Easterday *et al*., 1997). The problem lies in selection for resistant strains to the drug; poultry farmers in Asia are adding amantadine to chicken feed as protective measures during H5 influenza outbreaks (Ilyushina *et al*., 2005), which will result sooner or later in emergence of H5-AI virus that is resistant to administered drug.

H. Essential Oil Against Viruses

Viruses require living cells in which to replicate. Consequently, most of their steps in replication involve normal cellular metabolic pathways, and this makes it difficult to design a treatment to attack the virion directly, or its replication, without accompanying adverse effects on the infected cells (Wagner and Hewlett, 1999). The development of viral resistance towards antiviral agents enhances the need for new effective compounds against viral infections. Medicinal plants have a variety of chemical constituents, which have the ability to inhibit the replication cycle of various types of DNA or RNA viruses. Compounds from natural sources are of interest as possible sources to control viral infection (Jassim and Naji, 2003).

1. Antibacterial and Antifungal Activity

The antibacterial and fungicidal activity of oregano has been reported in many works; its oil has a bactericidal effect against *Erwinia amylovora* (Scortichini and Rossi 1993) and many other microorganisms such as *Bacillus subtilis, Escherichia coli, Hafnia alvei, Micrococcus luteus, Proteus vulgaris, Staphylococcus aureus* and *Streptococcus faecalis* (Biondi *et al*. 1993). Research findings also report inhibiting effects on the growth of some plant and animal pathogens, as well as organisms causing food spoilage (Deans and Svoboda 1990; Deans *et al*. 1992). On honeybee pathogens, the bactericidal and fungicidal effects of oregano extracts are reported for *Bacillus larvae* (causing American foulbrood), *Ascosphaera apis* (causing chalkbrood) and *Bacillus alvei* (a secondary pathogen involved in European foulbrood). These findings, confirmed by Calderone *et al*. (1994), highlight the important role that oregano can also play in the management of honeybee diseases.

Essential oils present in oregano are active against the growth of fungi *Aspergillus niger, Aspergillus flavus* and *Aspergillus ochraceus*, as well as against the bacteria *Campylobacter jejuni* and *Clostridium sporogenes* (Paster *et al*. 1990, 1995; Shaaya *et al*. 1991) and *Clostridium botulinim* (Ismaiel 1988). The same property was found against *Phytophthora citrophthora*, *Phoma tracheiphita* and *Pseudomonas syringae* (Arras 1988). With regard of this latter activity, the high carvacrol content of oregano was found to play a major role. *Origanum syriacum* was found to be effective in controlling a number of pathogens such as *Fusarium oxysporum, Macrophomina phasealina, Botrytis cinerea and Exserohilum turcicium (Setosphaeria tucica)* (Shimoni *et al*. 1993), whereas *O. compactum* is active against spore germination, mycelial

elongation and sporulation of *Aspergillus niger, Penicillium italicum* and *Zygonhynchus* sp. (Tantaoui-Elaraki *et al*. 1993).

The antibacterial/fungicidal activity of oregano oil is used today in a number of practical applications. An interesting example is provided by the following case: an aerosol formulation of its extracts (containing 0.4% mixture of essential oils) was sprayed into three enclosed areas (school hall, analytical laboratory and library) and a drastic reduction of the microorganisms present in the air was registered (a drop of 74-93%) (Panizzi and Pinzuti 1989). *Origanum vulgare* ssp vulgare had great potential of antimicrobial activity against 10 different bacteria, and 15 fungi and yeast species tested (Sahin *et al*. 2004).

2. NematicidalAactivity

In this area, interacting and promising results have been recorded with leaf extract of *O. vulgare*. The toxicity of the preparation was dependent on concentration and duration of treatment (Ramraj *et al*. 1991). Abd-Elgawad and Omer (1995) made similar findings. The main compounds involved in these activities are p-cymene, terpinen-4-ol and carvone.

3. Anti-oxidant Aactivity

The anti-oxidant activity of natural sources, including Origanum species, has been investigated (Sawabe and Okamota 1994; Takacsova *et al*. 1995). Lagouri *et al*., (1993) have demonstrated the anti-oxidant activity of *O. vulgare subsp. hirtum* (Greek oregano), *O. onites* (Turkish oregano), *Coridothymus capitatus* (Spanish oregano) and *Satureja thymbra*. Results of these findings indicate that the anti-oxidant effect may be

related to the presence of carvacrol and thymol in the essential oils. Other chemical compounds such as terpenoids may be responsible for the anti-oxidant activity; this is the case of glucosides (Nakatani and Kikuzaki 1987) or flavonoids (Vekiari *et al*. 1993), both extracted from the leaves of oregano. Five phenolic acids with anti-oxidant properties were isolated by Kikuzaki and Nakatani (1989), their activity being greater than the one shown by alpha-tocopherol. In several reports thymol and carvacrol were found to be the main antioxidant components from Origanum oils, while Sahin *et al.* (2004) showed that *Origanum vulgare ssp vulgare* showed a low anti- oxidant activity maybe, since the carvacrol and thymol percentages in the oil were remarkably low (0.84 and 0.57%, respectively).

4. Analgesic and Anti-inflammatory Effect

Menthae piperita L. peppermint oil is widely used in traditional as well as in complementary medicine, especially in phytotherapy for the external treatment of human diseases e. g. various pain conditions, like headaches syndromes (Gobel *et al.* 1994) in addition to its effect against a wide range of gram negative and gram positive bacteria. Peppermint and its essential oils have been used and effective in the treatment of nervous disorders and mental fatigue (Tisserand, 1996) suggesting that they might exert some psychoactive actions; however, no scientific evidence can claim this.

However, any of these essential oils used for medicinal purposes might produce side effects like on brain functions, depending on the dose used.

Eucalyptus citriodora (EC), *Eucalyptus tereticornis* (ET) and *Eucalyptus globules* (EG) possess central and peripheral analgesic effects as well as neutrophil-

dependent and independent anti- inflammatory activities. Silva *et al*. (2003) suggests a further investigation for use of these Brazilian herbs as medicines.

5. Antiviral Activity

Ethanolic extracts of *O. vulgare* proved to be active against ECH09 Hill virus, in culture of monkey kidney cells, by inducing the formation of a substance with interferon-like activity (Skwarek *et al*. 1994).

Eucalyptus oil showed a virucidal activity against HSV-1 and HSV- 2 viruses *in vitro*. Virus titers were reduced significantly. Pretreatment of the virus by *Eucalyptus* essential oil prior to viral infection or during adsorption or after penetration into the host were tested. Results showed that *Eucalyptus* essential oil affect the virus before or during adsorption but not after penetration into the host cell (Schnitzler *et al*. 2001). Peppermint oil is known to reduce viral titers for both HSV-1 and HSV- 2 viruses. Pretreatment of both viruses with peppermint oil prior to infection resulted in a concentration – dependent reduction assays. Same was shown by Tang *et al*. (1990) when hypericin (Polycyclic anthrone isolated from St. Johnswort plant) was incubated with influenza virus or herpes simplex virus before infecting cells (Tang *et al*. 1990).

A steam distillate from *Hottuynia cordata* showed direct inhibition against HSV-1, influenza virus and HIV- 1 without showing toxicity. The same steam distillate was not effective against poliovirus. Three major components: methyl n- nonyl ketone, lauryl aldehyde, and capryl aldehyde are the responsible components for the viral inactivation *in vitro*, by interfering with the function of the viral envelope (Hayashi *et al*. 1995). Influenza virus A/ PR/ 8/ 34 growth was completely inhibited by a water

soluble extract of *Sanicula europaea*. It was suggested that *sanicula europaea* extract contains an anti- influenza virus substance (Turana *et al*. 1996).

6. Mechanism of killing Viruses by EssentialOils

Peppermint oil and Eucalyptus oil were tested for their antiviral activity against HSV- 1 and 2 viruses. Both showed a virucidal activity for the viruses prior to infection. The essential oils were able to interfere with the viruses' envelopes or glycoproteins before or during adsorption but not after penetration of the virus into the cell (Schuhmacher *et al*. 2003 and Schnitzler *et al*. 2001).

The antiviral effect of tea oil against tobacco mosaic virus, a non-enveloped plant virus, has been reported previously (Bishop, 1995). However the mechanism of this antiviral action was not analyzed.

Isoborneol, a monoterpenoid alcohol (monoterpene), a component of several plant essential oils, showed virucidal activity against HSV-1 virus.

Isoborneol inactivated the virus (inhibited viral replication) without affecting viral adsorption. Isoborneol inhibited the glycolysation of HSV-1; since the cellular membranes of infected cells become more fragile and permeable than those of uninfected cells, and it is likely that isoborneol penetrates them more easily, affecting the enzymes responsible for protein glycolysation located in the endocellular membranic system (Golgi, endoplasmic reticulum). Data showed that the mature fully glycolysated forms of two viral glycoproteins gB and gD were not detected when the virus was replicated in the presence of isoborneol while isoborneol did not affect the glycolysation of gB produced from a copy of the gB gene resident in the cellular genome (Armaka *et al*. 1999).

Another experiment showed that TMF (5, 6, 7- trimenthoxyflavone, C18 H 16 O5) isolated from many plants inactivates HSV-1 particles.

TMF was able to inactivate both enveloped (HSV-1) and non-enveloped (Poliovirus) virus without affecting the protein adsorption or penetration since the mechanism of action showed irreversible inactivation of virus particles (Hayaki *et al*. 1997).

CHAPTER III

MATERIALS AND METHODS

A. Experiment 1-Avian Influenza A Virus Outbreak in Different Poultry Types of Lebanon

1. Production losses on Poultry Farms

Twenty-four poultry farms distributed in the north, south, east and middle mountains of Lebanon were surveyed during the Avian Influenza virus (AIV) outbreak, between June to August of 2004. The farms included meat chicken breeders, commercial layers, free-range layers and broilers. The percent drop in egg production at the time of the visit in comparison to that before the outbreak was recorded in the egg-producing farms. The percent mortality during the AI outbreak was also included in this study.

2. Signs and Lesions

The signs and lesions in each chicken type were recorded in order to help us in differentiating between low pathogenicity AI (LPAI) and high pathogenicity AI (HPAI).

3. Quantitating the AIV-specific Antibodies

Blood samples for determination of AIV-specific antibodies were collected from brachial veins of individual birds in each of the 24 investigated farms. The number of randomly collected blood samples was equivalent to about 0.4% of the total flock size of breeders, commercial layers and broilers; however, the number of collected blood in free-range layers was about 4 % of the flock size. Serum was collected from

each blood sample, and was kept at -20°C for further analyses of AIV-specific antibodies.

4. ELISA for AIV-specific antibodies

The Synbiotics Profloc® Avian Influenza Virus ELISA kit (San Diego, USA) was used to detect the presence of AIV-specific antibodies. This kit demonstrates broad reactivity to all subtypes of AIV type A (Easterday and Hinshaw, 1991). This commercial ELISA kit categorizes serum samples with positive antibodies to AIV when serum to positive (S/P) ratio is ≥ 0.500, while samples are defined as suspects when the S/P ratio is between 0.300-0.499, and negatives when the S/P values are <0.300.

5. Hemagglutination-Inhibition test

The haemagglutination-Inhibition (HI) test (Beard, 1980) was used to confirm the presence of H9 (AIV)-specific antibodies in serum samples that proved positive by the ELISA (S/P ratio ≥0.500). Any ELISA AIV-positive sera showing negative reaction to H9 component of H9N2 subtype in the HI test were sent to Intervet, Holland for defining their specificities to other AIV subtypes. It is worth noting that the H9N2 antigen, positive and negative control sera for H9N2, used in the HI test were kindly provided by Dr. Hans Vervuurt, R&D Service Laboratory, Intervet International, Boxmeer, The Netherlands. Future investigations should use reagents from OIE. The H9N2 working antigen was used in the HI test at 8 hemagglutination units/50µl, and each serum sample was subjected to 2-fold serial dilution, including the positive and

negative control sera. The chicken red blood cells were used in the test at 1% (V/V) in a phosphate buffered saline diluent.

6. Direct AIV Detection and Identification

Direct immunofluoresence test (Wang *et al*., 1991) was applied on cryostat sections of 4 brains and 4 individual tracheas of broilers during an AIV outbreak while manifesting tracheitis, cerebral congestion, and nervous disorders. Another 4 brains and 4 individual tracheas of healthy broilers were included as negative controls. Briefly, H9-AIV-specific sera provided by Dr. Vervuurt were applied on acetone fixed cryostat sections. A conjugate made in rabbits against H&L chains of chicken IgG and labeled with fluorescin was used in the test. The tissues were viewed under a fluorescent microscope for the presence of AIV. A homogenate of 3-pooled brains of these broilers was also injected through the allontoic membrane of 10-day-old chick embryos, free from specific pathogens including NewCastle Disease virus and Avian Influenza virus, in an attempt to propagate the AIV (Bano *et al*., 2002).

The harvested allontoic fluid virus was subjected to hemagglutination (HA) activity test against 1.0% chicken RBC (Beard, 1980).

Based on presence of HA activity, the unknown virus antigen was used in 8 HA units in HI test against each of H9- and NewCastle Disease Virus specific antibodies.

Following these steps, the propagated virus in allontoic fluid was sent abroad to Dr. Ruth Manvell at the Central Veterinary laboratory, in Weybridge, U.K. for final identification of its AIV-subtype based on the haemagglutinin and neuraminidase identity

B. Experiment-2- Evaluation of Avian Influenza H9N2 Intervet killed vaccine in chicken layers

1. Birds and design

Two experiments were conducted, in order to evaluate the H9N2-AIV Intervet International boxmer Holland killed vaccine.

The first one was conducted in Jezzine where around 210 day –old chicks, Lohman brown classic, free range- layers, were moved to a backyard in Jezzine Lebanon on September 25, 2004.

The flock was housed in a cement barn that had access to a backyard, covered with wire mesh. The second experiment was conducted in Bekaa, Ryak, where around 6500 day- old Hi- Line chicks, commercial layers, were moved to a classical commercial layer farm on September 13, 2004.

Each chicken was vaccinated subcutaneously in the neck with 0.5 ml of Intervet International Boxmer Holland vaccine, during the night according to a schedule shown in Table 2 and 3.

2. Serum Collection for vaccine evaluation

Serological profiling is a technology that is followed in many countries of the world to evaluate the efficiency of a vaccine, by detecting and quantitating the antibodies in the serum acquired to antigens of the vaccine.

Ten to twenty individual blood samples were collected from the brachial vein of individual birds on each farm. The samples were centrifuged at 720 xg (International Equipment Company, Needham Heights, Mass., USA) for 10 minutes and the sera were

collected and kept frozen at - 20ºC for later serological analysis. Serum collection schedule is explained in details in Tables 2 and 3, for both farms.

Table2. Schedule of HI-LINE flock (Ryak) vaccination and blood collection

Age (days)	Vaccine	Blood samples
Day old		25
Day 7	1st vaccine	
Day 29		20
Day 69		20
Day 70	2nd vaccine	
Day 91		20
Day 105		10
Day 106	3rd vaccine	
Day 127		22

Table 3**.** Schedule of Lohman Brown Classic flock (Jizzine) vaccination and blood collection

Age (days)	Vaccine	Blood samples
Day 27	1st vaccine	11
Day 69		20
Day 77	2nd vaccine	
Day 119		19

3. ELISA for Convalescent Evaluation of AIV Titers

Serum samples collected after administering the killed vaccine and those collected at vaccination were assessed in the same day for their antibody level specific to AIV antigens by the indirect ELISA method. The ELISA test procedures used are described below.

4. ELISA Procedure for Screening of AIV Vaccinated Chickens

Commercial ELISA systems designed by ProFLOCK® Synbiotic Corporation,

San Diego, USA, was made to quantitate the relative levels of antibodies in chicken serum that are specific to AIV.

The ProFLOCK® IBV ELISA Kit, San Diego, USA, is a rapid serologic test for the detection of AIV antibody in chicken serum samples (Indirect ELISA)**.** It was developed primarily to aid in the detection of pre and post-vaccination AIV antibody levels in chickens, and for studying the indirect effect to field exposure in chickens by serological profiling.

5. Statistical Analysis

Analysis of variance of vaccine titers (at vaccination and after vaccination) was carried out by the One Way Analysis of Variance (ANOVA-1) using the SPSS version 14.0. Means were then separated by Duncan's Multiple Range test ($\alpha = 0.05$).

C. Experiment -3- Essential oils of *Eucalyptus* and peppermint improve the homogeneity of immune responses and performance in MG/ H9N2- infected broilers

1. Broilers

About 20,000 day-old chicks of meat type produced by *Mycoplasma gallisepticum* (MG) and Avian Influenza (AI) (H9N2)-infected breeders were allocated for this study in a farm with a recurrent history of MG and AI (H9N2) outbreaks. The birds were distributed into four similarly constructed open-system houses located at 10 meters apart from each other. The management in the 4 houses was the same, including the use of the same starter and grower feed formulation (NRC, 1984) and the same water source.

2. Vaccination and MentofinTtreatment

The vaccination program was similar in the 4 houses and was applied between 1- 19 days of age. The vaccine types, mode of administration, and vaccination age are summarized in the following Table 4:

Table 4. Vaccine types, mode of administration and vaccination age

Vaccine	Mode of administration	Age (days)
1- live vaccine containing Mass strain of IBV (MA5)+ Clone 30 of NDV	Spray	1
2- Killed NDV+ AIV	Subcutaneous	1
3- Live IBDV (L)	Drinking water	12
4- Live IBV 4/91	Spray	15
5- Live Lasota NDV	Spray	17
6- Live IBDV (L)	Drinking water	19

The Mentofin treatment (EWABO, Wietmarschen, Germany) was given in drinking water to birds in two out of the 4 houses, in the morning and evening and at each of the following ages namely, 21, 22, and 23 days of age. The concentration and amount recommended by the manufacturer for 10,000 birds is 104 ml of Mentofin in 500 liters of drinking water. The total Mentofin used in 3 days, with 2 administrations / day is equal to 104 ml * 6 = 624 ml. The price of the 624 ml for treatment of the 10,000 birds is 28.80$.

3. Blood collection and Analysis

Blood was collected from randomly chosen birds in each of the 4 houses and at 1, 17, 31 and 45 days of age. At each age, ten individual birds from each house were randomly bled from the brachial vein. Since two houses are included in each treatment, then the number of blood samples collected at each age per treatment is 20.

Blood samples were analyzed by ELISA (Symbiotics Corporation, College Park, Maryland, USA) for titers specific to MG, AI, NDV, IBV, and IBDV.

4. Signs of Respiratory Reactions

Signs and lesions of respiratory reactions to vaccines start to appear usually after the completion of the vaccination program in the period between 20-35 days of age. The observations were made at different ages, namely, 20 (2 days before Mentofin treatment), 25 (2 days after Mentofin treatment), and 32 (9 days after Mentofin treatment) days of age to record the frequency of randomly selected birds (10 birds/treatment) with presence of mucous in trachea and thoracic airsacculitis.

5. Performance in Mentofin and Control Groups

The evaluated parameters of performance in each house included cumulative percent mortality of broilers, average body weight at market age, and feed conversion. The mean of each performance parameter was deduced from the two houses of each treatment, knowing that broilers in two houses were treated with Mentofin, while broilers in the other two houses were the control- untreated group. Feed conversion was

computed from the record of feed consumption in each house and the total live body weight at market age.

6. Economic Study

An economic study is concluded extrapolating for an additional net saving in US dollars due to Mentofin treatment in 10,000 birds based on improvement in feed conversion. The feed cost is included for Mentofin versus control – untreated birds. Saving in feed cost is calculated by subtracting the cost of feed consumed by Mentofin group from the cost of feed consumed by controls, knowing that the average live weight of broilers at the market age was the same in both treatments (2.10 kg/ broiler).

The net saving in US dollars was then calculated by subtracting the cost of Mentofin treatment from the saving in feed cost by the Mentofin group.

7. Statistical Analysis

One-way Analysis of Variance was used to compare the mean titers to NDV, IBV and IBDV between the two treatments at each specific time of sampling. This method compared also the titers within the same treatment per time to deduce the significance of seroconversion caused by the vaccines at P=0.05. The % Mortality was compared among the two treatments by the Chi-square method.

D. Experiment -4- Antiviral and antibacterial effect of *Origanum syriacum* and *ehrenberjii* essential oils against avian influenza H9N2

1. Preparation of AI H9N2 Virus Stock (inoculum)

H9N2 virus was isolated at the Animal Science Laboratory of the AUB from a brain of a broiler. It was typed at the Central Veterinary Labortaory, Webridge, England. The virus was first propagated in day-old chicks' brains. In the following *in vivo* experiment, 20 day- old chicks were subdivided equally into 2 groups. Group I was injected intracerebrally in the left hemisphere with 50 µl of sterile saline using 1 ml tuberculin needles (27 gauge). This group is the control group. While group II was injected with 50 µl of the virus AI H9N2 intracerebrally containing 64 HA units. Both groups were observed for mortalities.

The dead chicks of the challenge group were dipped in soaped water, to avoid dander contamination before dissection. The head was completely dipped in an iodine solution. Aseptically, the head was deskinned using sterile scissors and forceps.

The deskinned surface of the head was soaked in an iodine solution, prior to brain extraction to prevent any contamination. Extracted brains were stored at -20 º C.

The 7 brains were taken off the freezer, soaked with sterile saline, and then homogenized by sterile manual tissue homogenizer, with 4 ml of sterile saline to facilitate homogenization.

Moreover, the homogenate is centrifuged in an Ependorff centrifuge for 10 min at 11.5 xg. The supernatant was collected. Antimicrobials were added in the following concentrations, in order to prevent bacterial contamination namely, Penicillin (2µg/ml), gentamycin (10,000 IU/ml) and Fungiozone (25µl/ ml).

a. H9N2 Avian Influenza Virus Propagation in Embryonated Eggs

Thirty 10 day-old embryonated eggs were used for viral inoculation (Swayne *et al*., 1998).

Twenty- five eggs were each inoculated with 0.1ml of the inoculum prepared in the previous experiment. Five eggs were left uninoculated as a control group. All the eggs were put in the incubator at 100 º F and 85% relative humidity where eggs were turned automatically several times daily to promote proper embryo development.

After 2 days of incubation, the 30 eggs were candled to report any death cases. The dead eggs during the first 2 days were discarded. The third day post inoculation, all the remaining eggs (29 eggs) where put in the fridge for 2 hours to ensure their death and to avoid hemorrhages when collecting the allontoic fluid. Hemagglutination test was done to each allontoic fluid separately using 1% chicken RBCs diluted in Phosphate Buffer Saline diluent (Beard, 1980). The positive allontoic fluids were pooled and centrifuged in Ependorff centrifuge for 20 min at 11.5 xg.

Later, the HA test was done for the pooled allontoic fluid, using serial dilution (dilution factor 1/2) in order to determine the HA unit of the virus. The allontoic fluid was of 256 HA units.

The pooled allontoic fluid was centrifuged in Ependorff centrifuge for 20 min. at 11.5 xg. The supernatant containing the virus was collected and mixed in equal amount (1:1) with sterile Tryptose phosphate (TP) broth (HiMedia Laboratories India). The mixture was stored in sterile aliquots at –80°C.

The control allontoic fluid with HA = 0, was subjected to the same procedure, and was stored at -80°C.

2. Preparation of the Essential Oil

The essential oil was extracted from the *Origanum sp.* plant by steam distillation method (Craveiro *et al*. 1976) done by IBSAR AUB.

The Origanum plant was cut 10 cm above the ground, and allowed to dry at room temperature for 10 days. The essential oil was extracted by steam distillation method. The distillation took 2 hours for the water to boil, and almost 2 other hours to yield the oil. After 4 hours the distillation was stopped.

The yield of the different species was computed by the following equation.

$$\text{Yield} = \frac{\text{Volume of extracted oil (liter)}}{\text{Mass of the plant used in the distillation (Kg)}} * 100$$

The chemical profile of the different *Origanum spp.* essential oil was done using GC-MS method (Craveiro *et al*. 1984) in the Core Laboratory, Faculty of Medicine, AUB.

3. In vitro Susceptibility of Bacteria to Different Origanum spp. Essential Oils

Different *Origanum sp*. were tested *in vitro* for their antibacterial effect against different types of bacteria by using the Kirby Bauer test (Bauer *et al*. 1966).

These bacteria were transferred from the Triple sugar iron (TSI) agar (Hi media laboratories, India) to the Brain Heart Infusion (BHI) broth (Hi media Laboratories, India). After 5 hours (log phase), the turbidity of broth culture was adjusted to match the turbidity of barium chloride (BaCl2) prepared by mixing 99.5ml of 0.36 N H2SO4 with 0.5 ml of 1% BaCl2 (Mc Ferland turbidity standard). The turbidity adjustment was made by dilution with BHI medium.

A volume of 0.1 ml of each bacterial culture was seeded on Muller Hinton (MH) Agar (Mast group Ltd, Merseyside, U.K.) plates, using sterile L-glass rod. A volume of 20 µl of the essential oil was loaded on each blank sterile disk of 6.5 mm diameter (Mast group Ltd, Merseyside, U.K.). The diameter of the area of inhibition around the disk was measured in cm, after the Muller-Hinton Agar cultures were incubated for 24 hours at 37°C (Bauer *et al*., 1966).

a. Antibacterial Activity of Non-diluted *Origanum syriacum* (wild)

Non-diluted *Origanum syriacum* was tested for its antibacterial activity against the following bacteria using the Kirby Bauer test (Bauer *et al*., 1966):

Salmonella typhimirium

Salmonella enteritidis

Proteus spp.

Streptococcus faecalis

Klebsiella spp.

Escherichia coli

b. Antibacterial Activity of Diluted *Origanum syriacum* (wild)

Origanum syriacum (wild) was then tested for its antimicrobial activity (Bauer *et al*., 1966) against 2 bacteria, a gram negative *Salmonella typhimirium*, and a gram positive *Streptococcus feacalis*, using different dilutions of this essential oil (1:10, 1:20, 1:40, 1:80 and 1:100).

Origanum syriacum essential oil was diluted in 40% DMSO (Halawi, 2005) in sterile distilled water.

Table 5. The essential oil dilution in 40% DMSO in sterile distilled water

Dilution	Essential Oil (µl)	DMSO (µl)	Sterile DW (µl)	Total volume(µl)
1:10	20	72	108	200
1:20	10	76	114	200
1:40	10	156	234	400
1:80	10	316	474	800
1:100	10	396	594	1000

c. *In vitro* Testing of the Synergistic Effect of Anise and *Origanum syriacum* (wild)

The same *in vitro* experiment described above was repeated substituting the water used as diluent for the essential oil, by Anise diluted in distilled water.

Anise preparation: 100 g of Anise were boiled in 500 ml of distilled water (dilution of Anise in water is 1:5). The mixture was filtered twice using a filter paper (Watmen filter number 1). The mixture was centrifuged in Ependorff centrifuge at 11.5 xg for 10 min. Finally, the mixture was sterilized by passing it in Millipore filter of 0.22 mµ pore diameter.

Diluted Anise was mixed with the previously mentioned amount of essential oil and DMSO (Table 5) in order to study their inhibitory effect on the 2 bacteria mentioned above.

d. Antibacterial Activity of Diluted *Origanum ehrenberjii* (Wild and cultivated)

Diluted *Origanum ehrenberjii* (Wild and cultivated) was tested for its antimicrobial activity against 2 bacteria, a gram negative *Salmonella typhimirium*, and another gram positive *Streptococcus feacalis*, using different dilutions of this essential oil (1:100, 1:200, 1: 300, 1:400, 1:500, 1:600, 1:700, 1:800, 1:900, 1:1000, 1:1100, 1:1200, 1:1300, 1:1400 and 1:1500). The essential oil was diluted in 40% DMSO and sterile distilled water as shown in Table 6.

Table. 6. *Origanum ehrenberjii* essential oil dilutions in 40% DMSO.

Dilution	Oil (µl)	40% DMSO(µl)	Total volume
1:100	10	90	100µl
1:200	10	190	200µl
1:300	10	290	300µl
1:400	10	390	400µl
1:500	10	490	500µl
1:600	10	590	600µl
1:700	10	690	700µl
1:800	10	790	800µl
1:900	10	890	900µl
1:1000	10	990	1ml
1:1100	10	1090	1.1ml
1:1200	10	1190	1.2ml
1:1300	10	1290	1.3ml
1:1400	10	1390	1.4ml
1:1500	10	1490	1.5ml

A summary of the in vitro experiments is shown in Table 7.

Table 7. Summary of the *in vitro* experiments

Genus	Species	Cultivated or wild sp.	Dilution of the essential oil	Diluent	Bacteria tested
Origanum	syriacum	Wild sp.	None	None	8[1]
Origanum	syriacum	Wild sp.	1:10- 1:100	40% DMSO in SDW[3]	2[2]
Origanum	syriacum	Wild sp.	1:10- 1:100	40% DMSO in 5%Anise in SDW	2[2]
Origanum	ehrenberjii	Cultivated sp.	1:100- 1:1500	40% DMSO in SDW	2[2]
Origanum	ehrenberjii	Wild sp.	1:100- 1:1500	40% DMSO in SDW	2[2]

Salmonella typhimirium, Salmonella enteritidis, Proteus spp., Streptococcus faecalis, , Klebsiella spp.,and Esherichia coli
Salmonella typhimurium and Streptococcus faecalis
SDW: Sterile distilled water

4. In vivo Antiviral Effect of Different Origanum spp. Essential Oil on Day- old Chicks

a. Birds and Design

Six *in vivo* experiments were conducted on male egg-type day-old chicks. Chicks were obtained from a hatchery in Bekaa (Riyak) and transferred to the animal rooms at AUB campus.

Chicks were randomly assigned to the rooms according to each experimental design. Thus, the chicks of the different groups in the same room were given a specific body mark for differentiation, using a marker pen.

Wood shavings were spread over the floor of each room to a depth of around 5 cm, prior to chicks' entrance. Two feeders and two waterers were used in each animal room, and one height- adjustable 250 watts infrared lamp was used per room.

Feed and water were provided *ad libitum* throughout the course of the experiments.

The isolation rooms were cleaned and disinfected as well as the equipments by the end of each experiment. The dead chicks were disposed of according to the Safety and Sanitary Regulations and laws at AUB.

b. *In vivo* Antiviral Effect of *Origanum syriacum* (wild) Essential Oil Injected Intramuscularly in Day- old Chicks Challenged Intracerebrally With AI-H9N2

In March 2005, 120 day-old chicks were distributed equally in 12 groups, in 2 different animal rooms. The objective of this experiment was to study the antiviral effect of the *Origanum syriacum* (wild) essential oil injected intramuscularly against an intracerebral AI- H9N2 challenge, and to compare the efficacy of the essential oil to the present commercial drugs, used against AIV.

In the morning of the first day of the experiment, each chick was injected with 50 µl of the test substance in the pectoral muscle accordingly following a disinfection of the injection area with iodine.

Two hours following the morning treatment, each chick of the challenged group was injected 50 µl of the AIV (128 HA) intracerebrally following a disinfection of the injection area (the head) with iodine. In the afternoon, all the chicks were administered the test substance treatment. Chicks were checked daily for mortality.

Preparation of the test substances: each commercial drug was diluted according to the manufacturer's instructions, taking into consideration the modification of the dosage according to animal weight and blood volume. The essential oil was diluted 1:80, based on the *in vitro* tests findings against gram positive and negative bacteria.

Table 8. Experimental design of day- old chicks challenged with AI-H9N2 virus and treated with commercial drugs or essential oil of *Origanum syriacum*

Group	Test substance	H9N2challenge[4]	Room
A	PK-Merz (Amantadine sulfate) [1]	NO	1
B	Symmetrel (Amantadine hydrochloride) [1]	NO	1
C	Tamiflu (Oseltamivir)[2]	NO	1
F	NO	NO	1
G	40%DMSO	NO	1
B'	Symmetrel	Yes	2
C'	Tamiflu	Yes	2
D'	Essential oil+40% DMSO[3]	Yes	2
E	NO	Yes	2
H	40%DMSO	Yes	2

1 tablet from Pk-Merz (100 mg/tablet) (Amantadine sulfate) was crushed and added to 100 ml sterile saline, mixed well, and 50 µl was injected per chick intramuscularly in order to reach a dilution of 1 mg/ml in blood. The same was applied to Symmetrel.
2 tablets of Tamiflu (75 mg/ tablet) were mixed in 100 ml sterile saline and 50 µl were injected intramuscularly / bird in order to reach to a dilution of 1.5 mg/ml of blood.
The used essential oil was diluted 1:80 in 40%DMSO in sterile distilled water
The virus was removed from the freezer (-80 C) and thawed quickly. The virus was stored diluted 1:1 v/v (allontoic fluid: TPB) resulting in 128 HA/ 8 ml.

c. *In vivo* antiviral effect of *Origanum syriacum* essential oil injected subcutaneously in day- old chicks challenged intracerebrally with AI-H9N2

In May 2005, 160 day-old chicks were distributed equally into 4 groups (40 chicks/ group).

The objective of this experiment was to study the antiviral effect of the *Origanum syriacum* essential oil injected subcutaneously (in the peritoneal cavity) against H9N2-AI virus, and to compare the efficacy of the essential oil to commercial drugs used against AI.

In the afternoon of the first day, each chick was injected with 50 µl of the test substance subcutaneously and following a disinfection of the injection area (the peritoneal cavity) with iodine.

In the morning of the second day of the experiment, each chick was injected 50 µl of the test substance subcutaneously, and following a disinfection of the injection area (the peritoneal cavity) with iodine.

Two hours following the morning treatment, each chick of the challenged group was injected with 50 µl of the AIV containing 128 HA units, intracerebrally, following a disinfection of the injection area (the head). In the afternoon, all the chicks were administered to the test substance treatment. Chicks were injected with the test substance twice daily (morning and afternoon) during all the course of the experiment. Chicks were checked daily for mortality.

Preparation of the test substances: Amantadine sulfate (Pk- Merz) commercial drug was diluted according to the manufacturer's instructions, taking into consideration the modification of the dosage according to animal weight and blood volume. The essential oil was diluted 1:80, based on the *in vitro* tests findings.

Table 9. Experimental design of day- old chicks challenged with AI-H9N2 virus and treated with commercial drugs or essential oil

Group	Number of chicks	Test substance[1]	AI challenge[2]	Room
A	40	PK-Merz	Yes	1
B	40	Essential oil +DMSO (40%)	Yes	2
C	40	NO	Yes	3
D	40	NO	NO	4

[1.] The test substance administration was explained in details in the footnote of Table.6
[2.] The challenge was described in details in the footnote of Table.6

d. Adjustment of the Essential Oil Concentration and the Challenge

In vivo safety testing of Origanum ehrenberjii (cultivated sp.) injected intramuscularly in day-old male egg-type chicks

On June 27, 2005, 50 day- old chicks were divided equally into 5 groups. The chicks were assigned in the same room. The objective of the experiment was to test different concentrations in day-old male egg-type chicks of *Origanum ehrenberjii* (cultivated sp.) essential oil injected intramuscularly.

Table 10. Experimental design of day- old chicks administered different dilutions of *Origanum ehrenberjii* (cultivated sp.) essential oil

Group	Dilution
A	1:80
B	1:160
C	1:320
D	1:640
E	0 (Control)

[1] The essential oil was diluted in 40% DMSO in sterile distilled water with a serial dilution of a dilution factor of 1/2.

The computation of the 50% endpoint (LD50) of the AIV HA units by the method of Reed and Munech

On July 12, 05, 110 day-old-chicks were divided equally into 11 groups. Ten groups were kept in one room, while the 11th group was kept in a separate room.

The objective of this study is to evaluate the LD50 of the stock AI H9N2 virus *in vivo* based on Reed and Munech method (Turner 1965).

The experiment consists of injecting the chicks intracerebrally with 50µl of different dilutions of the stock virus, containing each different HA units. The experimental design is explained below in Table 11.

Table 11. Experimental design of day- old chicks injected intracerebrally with AI-H9N2 virus in different HA units

Group	HA units/ 50µl	Room
1	128	1
2	64	1
3	32	1
4	16	1
5	8	1
6	4	1
7	2	1
8	1	1
9	0.5	1
10	0.25	1
11 (Control)	0	2

e. Effect of Wild *Origanum ehrenberjii* Essential Oil Injected Intramuscularly in Day-old Chicks Challenged Intracerebrally With AI-H9N2

One hundred sixty day-old male egg-type chicks were distributed equally into 8 groups (20 chicks/ group). Five groups were put in one room, while the other 3 groups occupied the second room.

The objective of this experiment was to study the antiviral effect of the *Origanum ehrenberjii* (wild) essential oil injected intramuscularly against H9N2-AI virus, and to compare the efficacy of the essential oil to commercial drugs used against AIV.

In the afternoon of the first day, each chick was injected with 50 µl of the test substance intramuscularly in the pectoral muscle, following a disinfection of the injection area with iodine.

In the morning of the second day of the experiment, each chick was injected with 50 µl of the test substance intramuscularly, following a disinfection of the injection area with iodine.

Two hours following the morning treatment, each chick of the challenged group was injected with 50 µl of the AIV containing 4 HA units intracerebrally following a disinfection of the injection area (the head). In the afternoon, all the chicks were administered the test substance treatment. Thus, chicks were injected with the test substance twice daily (morning and afternoon) during all the course of the experiment. Chicks were checked daily for mortality. The experimental design is explained in Table 12.

Preparation of the test substances: Amantadine sulfate (Pk- Merz) commercial drug was diluted according to the manufacturer's instructions, taking into consideration the modification of the dosage according to animal weight and blood volume. The

essential oil was diluted 1:600 in 40% DMSO in sterile distilled water, based on the *in vitro* tests findings.

Table 12**.** Experimental design of day- old chicks injected with AI-H9N2 virus intracerebrally following treatment with *Origanum ehrenberjii* essential oil

Group	Treatment	AI challenge[1]
A	PK-Merz	Yes
B	Essential oil +DMSO (40%)	Yes
C	NO	Yes
A'	PK-Merz	NO
B'	Essential oil +DMSO (40%)	NO
C'	NO	NO
D	Sterile TPB	NO
E	Sterile Saline	NO

[1] AI- H9N2 challenge is with 4 HA units/bird, based on the findings of the LD50. The virus is diluted with sterile Tryptose Phosphate Buffer.

f. Effect of Cultivated *Origanum ehrenbenjii* Essential Oil Injected Intramuscularly) in Day-Old Chicks Challenged Intracerebrally With AI-H9N2

One hundred sixty day-old chicks were distributed equally into 8 groups (20 chicks/ group). The experimental design is the same as that used in the previous experiment shown in Table 12, but it differed this time by having cultivated instead of wild *Origanum ehrenberjii*.

The objective of this experiment was to study the antiviral effect of the cultivated *Origanum ehrenberjii* essential oil injected intramuscularly against H9N2-AI virus, and to compare the efficacy of this essential oil to commercial drugs used against AI.

In the afternoon of the first day, each chick was injected with 50 µl of the test substance intramuscularly, following a disinfection of the injection area with iodine.

In the morning of the second day of the experiment, each chick was injected with 50 µl of the test substance intramuscularly following a disinfection of the injection area with iodine.

Two hours following the morning treatment, each chick of the challenged group was injected with 50 µl of the AIV containing 4 HA units intracerebrally, following a disinfection of the injection area (the head). In the afternoon, all the chicks were administered the test substance treatment. Chicks were injected with the test substance twice daily (morning and afternoon) during all the course of the experiment. Chicks were checked daily for mortality.

Preparation of the test substances: Amantadine sulfate (Pk- Merz) commercial drug was diluted according to the manufacturer's instructions, taking into consideration the modification of the dosage according to animal weight and blood volume. The essential oil was diluted 1:600 in 40% DMSO in sterile distilled water, based on the *in vitro* tests findings.

A summary of the in vivo experiments is shown in Table 13.

Table13. Summary of the *in vivo* experiments

Experiment	Treatment			Treatment Route	Challenge Intracerebrally (HA unit)	Number of chicks/ group
	Commercial drugs	Essential oil	Essential oil dilution			
1	Amantadine sulfate Amantadine chloride Tamiflu	Origanum syriacum (wild sp.)	1:80 (oil is diluted in 40% DMSO in sterile distilled water)	Intramuscularly	128 HA	10
2	Amantadine sulfate	Origanum syriacum (wild sp.)	1:80 (oil is diluted in 40% DMSO in sterile distilled water)	Subcutaneously	128 HA	40
3	None	None	None	None	LD50[1]	10
4	None	Origanum ehrenberjii (cultivated sp.)	1:80- 1:640	Intramuscularly	None	10
5	Amantadine sulfate	Origanum ehrenberjii (wild sp.)	1:600 (oil is diluted in 40% DMSO in sterile distilled water)	Intramuscularly	4HA	20
6	Amantadine sulfate	Origanum ehrenberjii (cultivated sp.)	1:600 (oil is diluted in 40% DMSO in sterile distilled water)	Intramuscularly	4HA	20

The LD50 was administered by injecting in different groups (10 chicks/ group) the H9N2 in different HA units/ 50 µl, namely 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0

g. Statistical Analysis

Percentage mortalities in each experiment were compared using non-parametric tests chi-square, on the SPSS software 14.0.

CHAPTER IV

RESULTS AND DISCUSSION

A. Experiment 1-Avian Influenza A Virus Outbreak in Different Poultry Types of Lebanon

Table 14 shows a similar average drop in egg production at the time of investigation in both the meat breeders and commercial layers namely 46.0% and 47.3 %, respectively; however the average drop in egg production in the free-range layers was only 11.1%.

Table 14. Production losses [a] on 24 poultry farms in 4 provinces of Lebanon during influenza A virus outbreak

Lebanese Province	Investigated farm	Chicken type	Age (Weeks)	% Drop in egg production
North	1	Meat breeders	24	46
	2	Meat breeders	41	46
	3	Meat breeders	23	46
East (Bekaa Valley)	1	Meat breeders	NR [b]	NR
	2	Commercial layers[©]	52	40
	3	Commercial layers	40	70
	4	Commercial layers	40	72
	5	Commercial layers	36	63
	6	Commercial layers	25	48
	7	Commercial layers	104	41
	8	Commercial layers	25	46
	9	Commercial layers	56	46
	10	Commercial layers	NR	NR
	11	Broilers	5	NA [d]
Middle Mountains (Mten)	1	Commercial layers	32	0
South (Jezzine)	1	Free-range layers	72	0
	2	Free-range layers	28	10
	3	Free-range layers	36	0
	4	Free-range layers	36	10
	5	Free-range layers	72	0
	6	Free-range layers	28	45
	7	Free-range layers	28	15
	8	Free-range layers	32	15
	9	Free-range layers	36	5

Average mortality in either commercial layers or meat breeders during 9 days of the outbreak is 2.0% while that in free range was 1.0%. The broiler farm had a mortality of 35%.
Not recorded
Intensive system
Not applicable

This difference in loss of egg production could be mainly due to difference in farm management, where the free-range layers spend most of the day-hours outdoor in the backyard where the bird density per meter square averages 0.2 layer, while in the intensive commercial layers and meat breeders, the birds stay indoor in a density of 3

layers/m^2, that is 15 times more birds/ m^2 than that of free-range layers. The indoor environment associated with more density of birds, results most likely in more stress and a higher exposure to AIV particles during the outbreak, thus meeting the requirements for initiation of more severe outbreak (Pomeroy, 1986). Moreover, Table 15 confirms that only six out of nine (66.7%) of free-range layer farms were confirmed by ELISA to have AIV infection, while 9 out of 10 (90%) of commercial layer farms and three out of four (75%) meat breeder farms were positive for AIV.

Table 15. Seroprevalence of antibodies specific to avian influenza (AI) on 19 out of 24 investigated poultry farms

Lebanese Province	Investigated farm	Chicken type	Percent of serum samples [(a)] with AI antibodies [(b)]		
			Positive	Suspects	Negative
North (Akkar)	1	Meat breeders	95.8	0.0	4.2
	2	Meat breeders	100.0	0.0	0.0
	3	Meat breeders	10.0	15.0	75.0
East (Bekaa Valley)	1	Meat breeders	0.0	23.0	77.0
	2	Commercial layers	100.0	0.0	0.0
	3	Commercial layers	100.0	0.0	0.0
	4	Commercial layers	100.0	0.0	0.0
	5	Commercial layers	100.0	0.0	0.0
	6	Commercial layers	60.0	20.0	20.0
	7	Commercial layers	90.0	10.0	0.0
	8	Commercial layers	20.0	50.0	30.0
	9	Commercial layers	100.0	0.0	0.0
	10	Commercial layers	100.0	0.0	0.0
	11	Broilers	92.5	7.5	0.0
Middle mountains (Mten)	1	Commercial layers	0.0	0.0	100.0
South (Jezzine)	1	Free-range layers	12.5	25.0	62.5
	2	Free-range layers	12.5	0.0	87.5
	3	Free-range layers	33.3	11.1	55.5
	4	Free-range layers	33.3	0.0	66.6
	5	Free-range layers	50.0	12.5	37.5
	6	Free-range layers	100.0	0.0	0.0
	7	Free-range layers	0.0	0.0	100.0
	8	Free-range layers	0.0	0.0	100.0
	9	Free-range layers	0.0	0.0	100.0

[(a)] The number of randomly collected and analyzed serum samples was equivalent to about 0.4% of the total flock size of breeders, commercial layers and broilers, while it was around 4% on free-range farms.
[(b)] The commercial ELISA kit categories positive AI samples when S/P values≥0.5, suspects when S/P values are between 0.500-0.499 and negatives when S/P values are < 0.300.

In addition, Table 16 shows that all of the randomly selected ELISA positive samples for AI from meat breeders and commercial layers were confirmed by HI test to contain H9-specific antibodies; however, three out of six AIV-ELISA positive free-

range layer farms namely number 3, 4 and 5 had HI-antibodies to H1 (R&D service laboratory, Intervet, Holland). The pathogenicity of different subtypes of AIV differs in egg-type chicken (Mo *et al*., 1997).

Table 16. Results of Hemagglutination-Inhibition [(a)] (HI) with H9N2 antigen performed on randomly selected ELISA positive samples for AI

Lebanese Province	Investi-gated farm	Chicken type	No. of selected ELISA (positive) samples for AI	No. (%)(positive) by HI test	Mean HI titer
North (Akkar)	1	Meat Breeders	23	23(100)	1/1228.8
	2	Meat Breeders	24	24(100)	1/1126.4
	3	Meat Breeders	2	2(100)	1/1024.0
East (Bekaa Valley)	1	Meat Breeders	0	NA [(b)]	NA
	2	Commercial layers	20	20(100)	1/563.2
	3	Commercial layers	5	5(100)	1/563.2
	4	Commercial layers	10	10(100)	1/819.2
	5	Commercial layers	15	15(100)	1/780.8
	6	Commercial layers	12	12(100)	1/115.2
	7	Commercial layers	9	9(100)	1/1024.0
	8	Commercial layers	2	2(100)	1/96.0
	9	Commercial layers	10	10(100)	1/307.2
	10	Commercial layers	NES[(c)]	NA	NA
	11	Broilers	37	37(100)	1/147.2
Middle Mountains (Mten)	1	Commercial layers	0	NA	NA
South (Jezzine)	1	Free-range layers	NES	NA	NA
	2	Free-range layers	NES	NA	NA
	3	Free-range layers	3	0(0) [(d)]	<1/2
	4	Free-range layers	3	0(0) [(d)]	<1/2
	5	Free-range layers	4	0(0) [(d)]	<1/2
	6	Free-range layers	9	9(100)	1/486.4
	7	Free-range layers	0	NA	NA
	8	Free-range layers	0	NA	NA
	9	Free-range layers	0	NA	NA

[(a)] HI test specific for H9 antigen of the virus H9N2 used at 8HA units
[(b)] Not Applicable
[(c)] Not Enough Serum Volume left for HI test
[(d)] These AI-ELISA positive sera with negative reaction to H9N2 antigen were sent to Intervet, Holland for specificity study to other AI subtypes

The predominant signs and lesions in each differently managed chicken type were different (Table 17). Both the meat breeders and commercial layers had typical

signs of coughing, sneezing, rales, lacrimation and depression; in addition their lesions were typical in nature as indicated in Table 17.

Table 17. Predominant signs and lesions in each chicken type

Chicken type	Predominant	
	Signs	Lesions
Meat breeders & Commercial layers	Coughing, sneezing, rales, lacrimation, depression	Tracheal inflammation, conjunctivitis, hemorrhagic lesions associated with a complex of abnormalities [(a)]
Free range Layers	Sneezing and depression	Tracheal inflammation, conjunctivitis

[a)] Abnormalities included: Blochy red discoloration of the shanks, foci of necrosis in comb, pale combs, and pale internal organs such as in the pancreas and liver.

Both the signs and lesions in meat breeders and commercial layers suggest the presence of low pathogenicity Avian Influenza (LPAI) (Mo *et al*., 1997; Nili and Assi, 2002); regarding the less stressed free-range layers, the signs of lacrimation, rales and coughing were not noticed; in addition more important is the lack of presence of hemorrhagic lesions including blochy red discoloration of shanks, foci of necrosis in comb, pale combs and pale internal organs such in the pancreas and liver. This was confirmed in the free-range layer farm number '6' that showed infection titer to H9 component of the virus (Table 16) similar to that in commercial layer and breeder farms.

Regarding the broilers, an additional sign of nervous disorder was apparent in the AIV- out-breaking flock, associated with a cerebral congestion lesion, two observations that were not present in the other chicken types. Actually, previous research workers have indicated this difference in signs, lesions, and decline in

performance among layers and broilers (Bano *et al*., 2002; Nili and Assi, 2002). The broilers' susceptibility to H9N2 virus seems to be much higher than that reported for layers.

The seroprevalence of antibodies specific to AI on 19 out of 24 investigated poultry farms (Table 15) is indicative of the widespread nature of infection in different provinces of Lebanon. Some farms had 100% seroconversion to AI positive birds (breeder farm no.2; commercial layer farms 2, 3, 4, 5, 9, and 10; free-range layer farm no. 6); however, in other farms the infection titer was apparent, but not evenly distributed in the flock (breeder farm no. 3; commercial layer farm no. 8; and many of the free-range layer farms). In situations, where the farms are having high percentage of suspects, it is recommended to repeat the screening on such farms after 2-3 weeks to evaluate the degree of AIV infectivity in the flocks (Easterday *et al*., 1997).

The results of hemagglutination-inhibition test, using the H9N2 antigen, performed on randomly selected ELISA positive samples for AI (Table 17) proved the predominance of infectivity with AI virus that has the H9 component, except for the three free-range layer farms no. 3, 4, and 5 that had H1-specific antibodies. This indicates the high adaptability of the H9 virus to poultry. It is worth noting that the nearest country to Lebanon that reported the presence of an AI virus containing H9 component, specifically the H9N2, is Saudi Arabia (Herman, 2004).

Three major tests were included in this study (Table 18) to help in confirming the neuraminidase AIV infectivity. The direct immunofluorescence confirmed the presence of AI virus in the brains and trachea of broilers (Table 18).

Table 18. Results of direct immunofluorescence on poultry tissue using AIV group A specific serum, H9-specific HI on propagated AI virus from broiler brain, and Central Veterinary Laboratories analysis of propagated AI virus from chicken

Tests	Sample nature (number)	Results no. positive (%)
Direct Immunoflorescence	Brain [(a)] (4)	4(100) for AIV
	Trachea [(a)] (4)	4(100) positive for H9-AIV
	Brain (4) negative control	0(0)
	Trachea (4) negative control	0(0)
HI [(b)]	Allontoic fluid of propagated AI from brains (1)	1(100) positive for H9-AIV
Typing [(c)]	Allontoic fluid of propagated AI from brains (1)	1(100) H9N2

[(a)] Brain and Trachea of broilers outbreaking with AI
[(b)] Hemagglutination-Inhibition test. The HI test for NewCastle Disease Virus was negative.
[(c)] Typing performed in Central Veterinary Laboratories, Weybridge, U.K

The AIV propagated from brains of broilers reacted positively against the H9N2- specific antibody, and showed inhibition in the HI test, thus confirming the presence of H9 component in the isolated-propagated AI virus (Beard, 1980). The propagated virus present in allontoic fluid was confirmed in Central Veterinary Laboratory, Weybridge, U.K., by DR. Ruth Manvell, as H9N2-AIV, an indication of the possible involvement of this virus in most of the flocks that had serological presence of H9-specific antibodies.

It is worth noting that the isolation of H9N2 from the brains of broilers is an indication of the systemic nature of this infection, an observation not yet reported before for this subtype of AIV. Future sequencing of the haemaglutinin molecule of the H9N2 isolated from the brains should examine the possible presence of multiple basic amino acids in the H9 component which could have lead to systemic infection. This is a prerequisite before reaching to a conclusion of the systemic nature of infection by the

H9N2. Furthermore, the haemagglutinin protein present in the allontoic fluid containing the H9 component of the AIV didn't react in the HI test against antibodies specific to NewCastle Disease Virus, thus dropping out the possibility of NewCastle Disease involvement in central nervous system of broilers.

In brief, the H9N2 is most likely the predominant Influenza A virus involved in the poultry outbreaks in Lebanon, resulting in significant drop in egg production in meat breeders and commercial layers, followed by free-range layers, and with high mortality in broilers. Signs and lesions are indicative of a LPAI strain involvement. The HI test confirmed that most of AI-ELISA positive chicken flocks had H9-antibodies. The presence of AIV antigens in brains and tracheal tissues and the identification of H9 and N2 components in the propagated virus incriminate the H9N2 as the most likely virus involved in the first emergence of AIV outbreaks in Lebanese poultry.

B. Experiment-2- Evaluation of H9N2 Avian Influenza Intervet killed vaccine in chicken layers

Serological profiling was used in evaluating a killed vaccine against H9N2-AI. The humoral antibody response for H9N2-AI virus was measured before and around 21 days post vaccination. Previous literature suggests that the immune response to killed vaccines will show its significance after 21 days of the administration, hence paired serum samples are recommended for serological profiling whereby the first sampling occurs at the time of vaccination and the convalescent sampling 3 weeks later (Gillingham 2003). Table 19 represents the immune response to H9N2-AI using ELISA, in commercial layers, in Ryak, while Table 20 shows the immune response to H9N2-AI using ELISA in free-range layers, in Jizzine.

Table 19. Mean titer of HI- line commercial layers flock (Ryak)

Age	Days post vaccination	Samples	Mean titer	CV (%)
Day-old	--	25	780 [c]	30.65
Day 29	22d post 1st vaccine	20	533[cd]	48.86
Day 69	62d post 1st vaccine	20	144 [d]	58.58
Day 91	21d post 2nd vaccine	20	2109 [a]	46.53
Day 105	Day before 3rd vaccine or 35d post 2nd vaccine	10	1546 [b]	38.05
Day 127	21d post 3rd vaccine	22	2339 [a]	24.77

[a, b, c, d] Mean titers in a column with different superscripts were significantly different (P<0.05)

Table 20. Mean titer of Lohman Brown Classic free-range layers flock (Jizzine)

Age	Days post vaccination	Samples	Mean titer	CV (%)
Day 27	--	11	0 [c]	--
Day 69	42d post 1st vaccine	20	1445 [b]	29.20
Day 119	42 d post 2nd vaccine	19	4442	23.28

[a, b, c,] Mean titers in a column with different superscripts were significantly different (P<0.05)

The ELISA titer specific to H9N2-AI showed mainly a significant difference in humoral antibody level before and after vaccination (Table 19 and 20).

With respect to Ryak farm (Table 19), humoral antibody titer was 780 in day-old chicks. The titer decreased 22 and 62 days post the first vaccination. Twenty-one days after the second vaccine administration, antibody titers increased and were significantly greater than the titers at day- old and post the 1st vaccination. However, this titer increase didn't last; it decreased significantly after 14 days.

Twenty-one days post the third vaccination, the antibody titer increased significantly to reach a titer of 2339 (Table 19). The significant increase in humoral antibody titers shows that the birds responded to the vaccine by acquiring more immunity levels.

With respect to Jizzine farm (Table 20), humoral antibody titers increased significantly 42 days post the first vaccine administration (from 0 to 1445 respectively) and increased almost 300% at 42 days post the second vaccine administration (from 1442 to 4442).

The killed vaccine was able to boost significantly the immune response in both free-range layers and commercial layers. However, 2 vaccine administrations are required for free-range layers and 3 vaccinations for commercial layers, to reach a high and lasting titer. The difference between the humoral antibody titers between the 2 farms is mainly due to the farm management. The free range-layers spend most of the day-hours outdoor in the backyard, while the commercial layers stay indoors all the time. Such indoor environment creates more stress on the animals, which is expressed in this experiment on response to vaccination, and in the previous experiment, in signs and lesions following AI infection.

On the other hand, unlike other vaccines, AIV requires 2 or more vaccine doses to enhance the humoral immunity in poultry. WHO suggests 2 vaccine doses of live AI H9N2 vaccine in humans in order to reach a peak titer 2 weeks after the second vaccine administration (WHO, Subbarao *et al.*). Cruz- Coy *et al*. (2004) proved that a single booster vaccination with live Fowl Pox H5 subtype vaccine with or without the adjuvant formulation, at either 21 or 42 d of age did not protect birds from morbidity or mortality after challenge (Cruz- Coy *et al*., 2004). Since AI virus (LPAI) replication is restricted

to the digestive and respiratory tract, it does not become systemic (Horimoto and Kawaoka, 2001), and will probably take longer time to develop humoral IgG antibody levels in the serum. Tumpey *et al*., have compared mucosal (intranasal) versus traditional parenteral (intramuscular) administration of an inactivated influenza H3N2 vaccine for the ability to produce cross- protection in mice. Mice that received mucosal immunizations of the vaccine were completely protective against the H5N1 challenge, while those that received the vaccine subcutaneously were not protected against the challenge. However, the route of vaccination didn't affect the serum IgG after 3 vaccine doses administrations (Tumpey *et al*., 2001).

However, the remaining question is whether the titers approached are protective antibody titers or not, thus further researches are required, to evaluate the level of protection provided by these antibodies against the field strain challenge in Lebanon.

C. Experiment 3- Essential oils of *Eucalyptus* and peppermint improve the homogeneity of immune responses and performance in MG/ H9N2- infected broilers

Results showed a proper seroconversion in both treatments at 31d and 45d of age following the NDV, IBV and IBDV vaccination (Tables 21-23). In addition, Mentofin was able to boost significantly ($P<0.05$) the immune response to IBV at 45d of age (Titer=8767.5) compared to that of the controls (Titer= 4836.0) (Table 22). It is worth noting that most of previous literature on IBV vaccination is pointing at low immunogenicity of IBV vaccinations, which is competed against by other live vaccine strains (Saif, 2003). The weakness in IBV immunogenicity is corrected significantly by this Mentofin treatment.

Tables 21 and 22 show the improvement in the homogeneity of broilers'titer specific to NDV and IBV respectively. The CV values are respectively reduced significantly by 58.8 % and by 53.02 % for NDV and IBV titers at 45 days of age, i.e., 22 days after the end of Mentofin treatment.

Table 21. Mentofin improvement of NDV titers homogeneity in broilers manifested by significant reduction in Coefficient of Variation (CV values) following treatment[†].

Treatment	Mean[‡] NDV titers/ Mean[§] CV values at different ages			
	1d	17d	31d	45d
Mentofin treated	$3267^{a,1}$/31.4	$378.5^{a,3}$/56.4	$866.0^{a,3}$/66.3	$1806.5^{a,2}$/40.6
Control untreated	$3267^{a,1}$/31.4	$503.5^{a,3}$/55.7	$976.5^{a,3}$/66.5	$2195.0^{a,2}$/69.0

[†] Treatment by Mentofin in the morning and evening at 21, 22 and 23 days of age, and according to manufacturer instructions.
[‡] Mean of titers of two houses per treatment. Vaccination by NDV vaccines occurred at 1 and 17 days of age.
[§] Mean of CV of two houses per treatment.
[a-b,1-3] Means in a column with different alphabetical superscripts are significantly ($P<0.05$) different; means in a row with different Arabic numbers-superscripts are significantly ($P<0.05$) different, a reflection of successful seroconversion.

Table 22. Mentofin improvement of IBV titers homogeneity in broilers manifested by significant reduction in Coefficient of Variation (CV values) following treatment[†].

Treatment	Mean[‡] IBV titers/ Mean[§] CV values at different ages			
	1d	17d	31d	45d
Mentofin treated	$5674^{a,2}$/45.1	$128.5^{a,3}$/32.0	$161.5^{a,3}$/66.7	$8767.5^{a,1}$/35.1
Control untreated	$5674^{a,1}$/45.1	$161.0^{a,2}$/25.4	$461.0^{a,2}$/76.5	$4836.0^{b,1}$/66.2

[†] Treatment by Mentofin in the morning and evening at 21, 22 and 23 days of age, and according to manufacturer instructions.
[‡] Mean of titers of two houses per treatment. Vaccination by IBV vaccines occurred at 1 and 15 days of age.
[§] Mean of CV of two houses per treatment.
[a-b,1-3] Means in a column with different alphabetical superscripts are significantly ($P<0.05$) different; means in a row with different Arabic numbers-superscripts are significantly ($P<0.05$) different, a reflection of successful seroconversion.

Regarding the IBDV titers, both the Mentofin and control groups, had very low CV values, not allowing for comparisons. In addition, the Mentofin helped in reducing the mean titer at 45 days of age in comparison to the controls, which could be due to the alleviation of the injurious effect of the vaccine strain of IBDV. It is worth noting that the reduced titer at 45 days of age is still more than enough in its offering of protection against Gumboro (Titer = 11062.5). The difference in the pathogenicity of the respiratory vaccine viruses from the immunosuppressive vaccine virus (Kadihalli *et al.*, 1994) demands more future investigation on Mentofin applications in flocks with high CV values for IBDV vaccines.

Table 23**.** Impact of Mentofin on IBDV titer homogeneity (CV) between treated[†] and control-untreated broilers.

Treatment	Mean[‡] IBDV titers/ Mean[§] CV values at different ages			
	1d	17d	31d	45d
Mentofin treated	$15205^{a,1}$/7.86	$7334.0^{a,3}$/38.76	$6357.0^{a,3}$/36.0	$11062.5^{b,2}$/4.5
Control untreated	$15205^{a,1}$/7.86	$7916.0^{a,3}$/35.6	$7153.5^{a,3}$/27.3	$12249.0^{a,2}$/7.5

[†] Treatment by Mentofin in the morning and evening at 21, 22 and 23 days of age, and according to manufacturer instructions.
[‡] Mean of titers of two houses per treatment. Vaccination by IBDV vaccines occurred at 12 and 19 days of age.
[§] Mean of CV of two houses per treatment.
[a-b,1-3] Means in a column with different alphabetical superscripts are significantly ($P<0.05$) different; means in a row with different Arabic numbers-superscripts are significantly ($P<0.05$) different, a reflection of successful seroconversion.

The recorded respiratory signs and lesions at 21, 25 and, 32 days of age i.e. 2 days before initiation of Mentofin treatment, 2 days after the end of Mentofin treatment, and 9 days after Mentofin treatment ended showed an improvement in the Mentofin

treated broilers compared to the control-untreated birds (Table 24). The signs of respiratory reactions in the period following the end of live vaccines administration were reduced in Mentofin treated birds as observed at 32 days of age, i.e., at 9 days following the end of treatment, in which the Mentofin-treated birds versus controls had 30% versus 100% occurrence of thoracic airsacculitis, and 50% vs. 60% occurrence of mucous in the trachea.

Table 24. Signs and lesions[1] of respiratory reactions in Mentofin treated versus control-untreated broiler flocks

Treatment	Broilers[2] with % thoracic airsacculitis / % tracheal mucous at different ages (days)		
	20	25	32
Mentofin treated	100% / 30%	100% / 30%	30% / 50%
Control untreated	90% / 30%	100% / 90%	100% / 60%

[1] Sign of tracheal mucous, and lesions of thoracic airsacculitis.
[2] Ten broilers were selected randomly from each treatment and at each of the following ages namely, 20 (2 days before treatment), 25 (2 days after treatment ended) and 32 (9 days after the end of treatment).

The results related to parameters of performance are illustrated in Table 25. The mean percent mortality in the Mentofin-treated broilers was reduced by 0.5% compared to that of the control-untreated; the average body weight at market age were typical (2.1 kg /broiler). Regarding the feed conversion, the Mentofin-treated broilers showed an improvement in feed conversion (1.96) compared to that obtained by the control- untreated birds (2.04).

Table 25. Performance in Mentofin-treated[1] versus control-untreated broiler flocks

Treatment	Mean mortality, body weight and feed conversion up to market age		
	Mortality %	Body Weight (Kg)	Feed Conversion
Mentofin treated	12.00[a]	2.10[a]	1.96[a]
Control untreated	12.50[a]	2.10[a]	2.04[a]

[1] Treatment by Mentofin in the morning and evening at 21, 22 and 23 days of age and according to manufacturer's instructions.
[a] Values in a column with the same alphabetic superscript are not different (P>0.05).

This is most likely due to the healing effect of Mentofin to the epithelial layers of the respiratory system that is suffering from the complexity of infections by MG (15 % of MG infection titers was present in sera at 31 days of age) and AI (5 % of AI vaccine or infection titers was present in sera at market age) in addition to infectivity by the administered live vaccine strains of NDV and IBV. This improvement by Mentofin led to better homogeneity of immune responses (Tables 21 and 22), reduced respiratory signs and lesions (Table 24), and improved performances (Table 25).

The healing and immunomodulation effects of herbs have been the objective of many researchers in the past, and is reviving in the recent years (Barbour *et al.*, 2002) due to the pressure put against the use of drugs in animal production (Gade, 2002).

The economic benefits of the use of Mentofin in the broiler industry could be deduced from the data presented in Table 26.

Table 26. Economic evaluation of Mentofin treatment[1] in relation to live body weight and improvement in feed conversion up to market age

Treatment	Values rounded per 10,000 produced broilers						
	Cost of Mentofin treatment	Feed consumption (Kg)/ Kg of bird	Feed consumption (Kg)/2.10 Kg average weight of broilers	Feed consumption (Kg) per 10,000 broilers	Feed Cost ($)/ 10,000 broilers	Saving in feed cost ($)	Net saving[2] ($)
Mentofin treated	$28.80	1.96	4.12 Kg	41200.0	13390.0	520.0	491.20
Control-untreated	--	2.04	4.28 Kg	42800.0	13910.0	--	--

[1] Treatment by Mentofin in the morning and evening at 21, 22 and 23 days of age.
[2] Net saving ($) = saving in feed cost – cost of Mentofin = $520.0 - $28.80 = $491.20.

The main effect of the Mentofin was an improvement in feed conversion, where feed in the Middle Eastern countries, usually imported from the USA, is responsible for around 70% of the cost of broiler production.

Table 26 shows that the feed cost in US dollars for 10,000 broilers administered Mentofin versus the same number of broilers deprived of medication is $ 13390.0 versus $ 13910.0, respectively. This saves $ 520.0 per 10,000 produced broilers (with average live body weight of 2.1 kg/ broiler). In subtracting the price of $28.80 of Mentofin substance from the saving on feed ($520.0), the net additional saving will be $491.20 per 10,000 broilers.

In conclusion, the essential oils of *Eucalyptus* and peppermint (Mentofin®) improved the homogeneity of immune responses to respiratory vaccine viruses of NDV and IBV. It reduced the respiratory signs and lesions in the period following challenges by live vaccine strains, and it resulted in slight reduction in mortality, associated with improvement in feed conversion that added up to an additional net saving of $491.20 per 10,000 birds after subtraction of the cost of Mentofin.

D. Experiment -4- Antiviral and antibacterial effect of *Origanum syriacum* and *ehrenberjii* essential oils

1. Essential oil yield and chemical profile

Dry weight (Kg), extracted volume (ml) and yield (%) of the different Origanum essential oils are presented in Table 27. Results showed no significant difference between *Origanum ehrenberjii* wild vs. cultivated yield (1.03 vs. 1.30 respectively).

Table 27. Dry weight, extracted volume and yield of the different *Origanum spp.*

Species	Extraction date	Dry weight (Kg)	Extracted volume (ml)	%Yield
Origanum ehrenberjii (cultivated sp.)	14/ 07/ 2005	2.0	20	1.00%
	21/ 07/ 2005	4.0	40	1.00%
	22/ 07/ 2005	3.6	40	1.10%
Mean		3.2	33.3	1.03[a]
Origanum ehrenberjii (wild sp.)	26/ 07/ 2005	4.0	50	1.25%
	27/ 07/ 2005	4.0	60	1.50%
	28/ 07/ 2005	6.5	90	1.38%
	29/ 07/ 2005	5.6	60	1.07%
Mean		5.0	65	1.30[a]

[a] Means with different superscripts (in the mean row) are significant ($\alpha < 0.05$)

The chemical profile of the different *Origanum* species essential oil was done at the Core Laboratory, Faculty of Medicine, AUB, using GC- MS method. The chemical profile of the different essential oils is shown in Tables 28, 29, 30, 31, and 32.

Table 28. Chemical profile of essential oil of wild *Origanum syriacum*

Compound	RI	RI ref	Area %
α-thujene	916	930	2.9
α-pinene	922	939	2.29
Camphene	937	954	0.49
Sabinene	962	975	0.07
β-pinene	965	979	0.62
1 Octen 3 OL	971	979	0.32
Myrcene	979	991	2.79
α-phellandrene	994	1003	0.67
δ - carene	997	1002	0.26
α-terpinene	1005	1017	4.83
para cymene	1014	1025	26.95
Limonene	1018	1029	2.01
γ-terpinene	1048	1060	20.17
α-terpinene	1079	1017	0.19
Cis-sabinenehydrate	1090	1070	0.07
Linalool	1095	1097	0.31
Borneol	1159	1169	0.29
terpinene - 4 - ol	1171	1177	0.74
Thymyl methyl ether	1230	1235	0.31
Thymol	1280	1290	18.21
Carvacrol	1288	1299	11.67
β - caryophyllene	1407	1409	2.69
Alloaromadendrene	1427	1441	0.06
α-Humulene	1443	1455	0.17
			99.08

Table 29. Chemical profile of essential oil of wild *Origanum ehrenberjii* (batch no. 1)

Compound	RI	RI ref	Area %
α-thujene	916	930	2.56
α-pinene	922	939	1.21
Camphene	937	954	0.16
Sabinene	962	975	0.13
β-pinene	965	979	0.27
Myrcene	980	991	2.13
α-phellandrene	995	1003	0.47
γ-terpinene	997	1060	0.25
α-terpinene	1005	1017	2.46
para cymene	1013	1025	11.83
β-phellandrene	1019	1030	1.16
γ-terpinene	1047	1060	10.6
α-terpinene	1080	1017	0.07
Borneol	1160	1169	0.12
terpinen - 4 - ol	1171	1177	0.51
Cis-Dihydrocarvone	1191	1193	0.05
Thymyl methyl ether	1228	1235	6.85
Thymol	1280	1290	21.29
Carvacrol	1290	1299	34.63
α - copaene	1364	1377	0.15
β - caryophyllene	1407	1409	1.52
α -Humulene	1443	1455	0.23
γ - elemene	1484	1437	0.15
β-Bisabolene	1501	1506	0.23
			99.03

Table 30. Chemical profile of essential oil of wild *Origanum ehrenberjii* (batch no. 2)

Compound	RI	RI ref	Area %
α-thujene	916	930	2.27
α-pinene	922	939	1.11
Camphene	937	954	0.14
Sabinene	962	975	0.11
β-pinene	965	979	0.25
Myrcene	980	991	1.57
α-phellandrene	995	1003	0.43
α-terpinene	1005	1017	2.24
para cymene	1013	1025	11.2
β-phellandrene	1019	1030	1.33
γ-terpinene	1048	1060	9.91
β-phellandrene	1080	1030	0.06
Borneol	1159	1169	0.12
terpinen - 4 - ol	1171	1177	0.5
Thymyl methyl ether	1228	1235	7.24
Thymol	1280	1290	21.84
Carvacrol	1290	1299	36.75
α - Ylangene	1364	1375	0.13
β - caryophyllene	1407	1409	1.52
α-Humulene	1443	1455	0.24
Germacrene B	1484	1561	0.19
β-Bisabolene	1504	1506	0.19
			99.69

Table 31**.** Chemical profile of essential oil of cultivated *Origanum ehrenberjii* (batch no. 1)

Compound	RI	RI ref	Area %
α-thujene	916	930	2.65
α-pinene	922	939	1.22
Camphene	963	954	0.14
Sabinene	962	975	0.08
β-pinene	965	979	0.28
Myrcene	979	991	2.33
α-phellandrene	995	1003	0.56
δ- carene	997	1031	0.21
α - terpinene	1005	1017	3.46
para cymene	1013	1025	12.9
β - phellandrene	1019	1030	1.3
γ-terpinene	1047	1060	17.05
γ-terpinene	1060	1060	0.28
cis sabinene hydrate	1091	1070	0.11
Borneol	1160	1169	0.1
terpinen - 4 - ol	1172	1177	0.34
Thymyl methyl ester	1229	1235	6.14
Thymol	1281	1290	38.41
Carvacrol	1288	1299	9.97
α - copaene	1364	1377	0.18
β - caryophyllene	1407	1409	1.42
α-Humulene	1443	1455	0.22
β-Bisabolene	1505	1506	0.12
γ-cadinene	1508	1514	0.11
			99.58

Table 32. Chemical profile of essential oil of cultivated *Origanum ehrenberjii* (batch no. 2)

Compound	RI	RI ref	Area %
α-thujene	916	930	2.69
α-pinene	922	939	1.24
Camphene	937	954	0.15
Sabinene	962	975	0.08
β-pinene	965	979	0.27
Myrcene	979	991	2.42
α-phellandrene	995	1003	0.56
δ- carene	997	1031	0.2
α - terpinene	1005	1017	3.53
para cymene	1013	1025	12.87
Limonene	1018	1029	1.38
γ-terpinene	1048	1060	16.64
γ-terpinene	1060	1070	0.4
Linalool	1091	1097	0.09
Borneol	1160	1169	0.12
terpinene - 4 - ol	1171	1177	0.32
Thymyl methyl ether	1220	1235	0.25
Thymyl methyl ether	1228	1235	5.91
Thymol	1282	1290	37.69
Carvacrol	1289	1299	10.83
α - copaene	1364	1377	0.18
β - caryophyllene	1407	1409	1.31
α-Humulene	1443	1455	0.2
Germacrene B	1484	1561	0.07
β-Bisabolene	1503	1506	0.2
β-Bisabolene	1507	1506	0.13
			99.73

2. In vitro Antibacterial Activity of Different Origanum spp.

The antibacterial effect of different *Origanum sp*. essential oil on different bacteria was determined. Many concentrations of the essential oils were used, and the mean diameter of inhibition zone of duplicates was measured in mm after 24 h of incubation at 37 ºC.

a. *Origanum syriacum*

Origanum syriacum essential oil was tested for its antibacterial activity, pure and diluted.

Pure undiluted Origanum syriacum essential oil tested for its antimicrobial activity against Salmonella typhimurium, Salmonella enteritidis, Proteus spp., Streptococcus faecalis, Klebsiella spp. and Escherichia coli recorded an inhibition diameter of 52, 34, 30, 17, 30, and 40 mm, respectively.

Diluted *Origanum syriacum* essential oil was tested for its antibacterial activity against *Salmonella typhimurium* and *Streptococcus faecalis.* Recorded results are shown in Table 33.

Table 33. *In-vitro* inhibitory effect of different Origanum spp. against *Salmonella typhimurium* and *Streptococcus faecalis*

Origanum sp.	Dilution*	Salmonella typhimurium (mm)	Streptococcus faecalis(mm)
syriacum (wild)	Pure	52	17
syriacum (wild) in 40% DMSO in water	1:10	8.5	6.5
	1:20	9	6
	1:40	8.75	6.25
	1:80	7	6.75
	1:100	6.5	6.25
syriacum diluted in 40% DMSO in 5% Anise in water	1:10	10	8
	1:20	9	6.75
	1:40	8.5	6.75
	1:80	7.25	7
	1:100	6.75	6.5
ehrenberjii (cultivated) in 40% DMSO in water	1:100	10.5	12
	1:200	10	10.5
	1:300	9	12
	1:400	9	11
	1:500	8	10
	1:600	10	10
	1:700	8.5	10
	1:800	8.5	8
	1:900	8	8
	1:1000	9	11
	1:1100	8	8.5
	1:1200	7	11
	1:1300	7	7
	1:1400	7	9.5
	1:1500	7	6
ehrenberjii (wild) in 40% DMSO in water	1:100	11	9
	1:200	11	10
	1:300	10	10
	1:400	11	9.5
	1:500	10.5	9.5
	1:600	10	9
	1:700	10	8.5
	1:800	9	9.5
	1:900	9	9
	1:1000	9	9

*Diluent is 40% DMSO in water or 40% DMSO in 5% Anise in water

Diluted *Origanum syriacum* essential oil was diluted in 40% DMSO in water and in 40% DMSO in Anise. Anise (5%) was used as a diluent for the *Origanum*

syriacum essential oil, previously proven for its antimicrobial, antiviral, and antifungal activity against a wide spectrum of viruses, bacteria and fungi (De *et al*., 2002).

Diluted *O. syriacum* essential oil was tested for its antibacterial activity against *Salmonella typhimurium* and S*treptococcus faecalis*.

Results showed no major numerical difference between the antibacterial effects of *O. syriacum* diluted in water or in Anise. Thus, no synergistic effect was observed between *O. syriacum* and Anise.

As a result, *O. syriacum* diluted in DMSO and water was chosen for the *in vivo* further studies.

Although it is known that an inhibition zone of 10 mm is considered to be the limit zone of being an efficient antibacterial (Alma *et al*., 2003), we decided to use the dilution 1:80, since it was the maximum dilution that resulted in the minimum diameter needed.

b. *Origanum ehrenberjii*

Diluted *O. ehrenberjii* essential oil in 40% DMSO and water was tested for its antimicrobial activity against *Salmonella typhimurium* and *Streptococcus faecalis*. Recorded results are shown in Table 33.

As for *Origanum ehrenberjii* (wild and cultivated), we decided to use the 1:600 dilution in 40% DMSO and water in the *in vivo* studies, since it is the maximum dilution that still shows a 10 mm inhibition diameter as recommended by Alma *et al*., (2003) (Table 33).

Hence, *in vivo* analyses will follow the *in vitro* analyses to evaluate the effect of essential oil treatment on AI-H9N2 challenge in day- old chicks.

On the other hand, results showed a greater diameter of inhibition zone against Gram-negative bacteria compared to Gram-positive bacteria (Table 33). Origanum essential oil's main constituents "carvacrol" and "thymol" inhibit the growth of the bacteria by interfering with the bacterial membrane permeability, which further affects pH homeostasis and equilibrium of inorganic ions (Lambert *et al*. 2001). This activity is more pronounced in Gram-negative bacteria due to the hydrophobic constituents of essential oils. Thus the permeability of the cell membranes is dependent on the hydrophobicity of the solutes that have to cross the membrane and the composition of the membrane itself (Sikkema *et al*., 1995).

3. In vivo antiviral activity of Origanum syriacum

Diluted *Origanum syriacum* essential oil (1:80) in 40% DMSO and sterile distilled water, Pk- Merz (Amantadine sulfate), Symmetrel (Amantadine chloride) and Tamiflu (Oseltamivir) were tested for their antiviral activity intramuscularly in day-old chicks against AI-H9N2 containing 128 HA units.

Mortality was recorded 1- day post challenge in the morning (2^{nd} day of the experiment) and 2- days post challenge (3^{rd} day of the experiment). Mortality of each treatment of the challenged vs. non- challenged group, are shown in the Table 34.

Table 34. *In vivo* antiviral activity of diluted *Origanum syriacum* essential oil administered intramuscularly in day-old chicks challenged intracerebrally* with AI-H9N2 (128 HA)

Group		Mortality 1-day post challenge morning (%)	Total mortality 2 days post challenge morning (%)
Pk- Merz	Challenge	30[c]	70 [b]
	Unchallenged	0	0
Symmetrel	Challenge	60[b]	80[ab]
	Unchallenged	0	0
Tamiflu	Challenge	50[b]	70 [b]
	Unchallenged	0	0
Essential oil	Challenge	60[b]	100[a]
	Unchallenged	20	60
Control	Challenge	90[a]	100[a]
	Unchallenged	10	10

[a, b, c] Means of challenged groups in a column with different superscripts were significantly different (P<0.05)
* 50 µl/ bird

One-day post challenge, results showed a significant reduction in the mortalities of all the treatments compared to the control group. Pk- Merz was the best antiviral drug to inhibit the death of chicks. Pk-Merz reduced mortality from 90% to 30% compared to control group. Diluted *O. syriacum* reduced mortality from 90% to 60% 1-day post challenge and was comparable statistically to both Symmetrel and Tamiflu drugs (P< 0.05).

However, this result was not consistent two days post challenge, since only Pk-Merz and Tamiflu reduced the mortality significantly compared to control, Symmetrel, and to the tested essential oil.

It is worth noting that the administration of *O. syriacum* without challenge resulted in high mortality at 1 and 2 days post challenge, namely of 20 and 60 % respectively (Table 34), a sign of toxicity of the *O. syriacum* essential oil.

The previous experiment was repeated testing the same diluted *Origanum syriacum* essential oil (1:80) and Pk- Merz (Amantadine sulfate) administered subcutaneously, against intracerebral H9N2 (128 HA)-challenge.

Mortality was computed in the morning before the challenge (2nd day of the experiment) and 1-day post challenge (by the end of the experiment, 3rd day). Mortality of each treatment, challenged vs. non- challenged is shown in Table 35.

Table 35. *In vivo* antiviral activity of *Origanum syriacum (wild sp.)* essential oil injected subcutaneously in day-old chicks challenged intracerebrally with AI- H9N2 (128 HA)

Group		Mortality before challenge morning (%)	Total mortality 1-day post challenge morning (%)
Pk- Merz	Challenge	0 [a]	98 [a]
Essential oil	Challenge	8 [a]	98 [a]
Control	Challenge	0 [a]	100 [a]
	Unchallenged	0	0

[a] Means of challenged groups in a column with different superscripts were significantly different ($P<0.05$)

Once the test substance was administered subcutaneously, mortality wasn't reduced in any of the treatments compared to control group one-day post challenge (Table 35).

Amantadine and Tamiflu are recommended for use orally for humans (leaflet of Symmetrel, Pk-Merz and Tamiflu), and were given in previous studies in the drinking water for poultry (Beard *et al*., 1984; Webster *et al*., 1985). The drugs are readily absorbed from the intestine, and are well diffused in the lungs and nasal mucosa. No studies report the use of these drugs intramuscularly or subcutaneously. As for essential oil, it didn't show any significance or numerical decrease in mortality compared to control group. Siddiqui *et al*. (1996) found that Origanum essential oil destroyed the epithelial cells of Vero and Hep-2 cell culture in the first 12 h following administration when diluted 1:500. The same author also found that epithelial cells were

also more sensitive to toxicity than fibroblasts. The chick embryo fibroblasts showed relatively high tolerance than any other cell line used in his experiment (Siddiqui *et al*., 1996).

4. Lethal Dose 50 (LD50)

The computation of the 50% endpoint (LD_{50}) of the avian influenza virus (128 HA) by the method of Reed and Munech (1988) is illustrated in Table 36.

Table 36. The computation of the 50% endpoint (LD50) of the avian influenza virus HA units[1] by the method of Reed and Munech

AI HA units	Mort. Ratio[2]	Died[3]	Survived	Total Dead	Total survived	Mort. Ratio	Mort %[4]
128	8/10	8	2	63	2	0.9692	96.92
64	8/10	8	2	55	4	0.9322	93.92
32	8/10	8	2	47	6	0.8867	88.67
16	7/10	7	3	39	9	0.8125	81.25
8	7/10	7	3	32	12	0.7272	72.72
4	6/10	6	4	25	16	0.6097	60.97
2	7/10	7	3	19	19	0.5000	50.00
1	5/10	5	5	12	24	0.3333	33.33
0.5	3/10	3	7	7	31	0.1842	18.42
0.25	4/10	4	6	4	37	0.0976	9.76

[1] Every 10 one-day old chick was challenged with a different HA units of the main AIV stock (128 HA units)
[2] Mortality Ratio= Total number of dead chicks/Total number of treated chicks
[3] Death was recorded 24h post challenge
[4] Mortality %= (Total number of dead chicks/Total number of treated chicks)*100

Therefore the final LD50 endpoint titer equals to 2 HA units. Thus in the further *in vivo* testing, the birds were challenged intracerebrally with AI-H9N2 containing 4 HA units, the dose expected to kill 100% of the challenged birds.

5. In vivo Antiviral Activity of Origanum ehrenberjii

Diluted *Origanum ehrenberjii* (wild) essential oil (1:600) in 40% DMSO and sterile distilled water and Pk- Merz (Amantadine sulfate) were tested for their antiviral activity on day –old chicks against AI-H9N2 containing 4 HA units.

Mortality was computed 1- day before challenge (1st day of the experiment) and 1- day post challenge (3rd day of the experiment). Mortality for each treatment of the challenged vs. non- challenged group, is shown in the Table 37.

Table 37. *In vivo* antiviral activity of *Origanum ehrenberjii (wild sp.)* essential oil injected intramuscularly in day-old chicks challenged intracerebrally with AI- H9N2 (4 HA)

Group		Mortality 1- day before challenge (%)	Total mortality 1-day post challenge morning (%)
Pk- Merz	Challenge	0	60 [b]
	Unchallenged	0	0
Essential oil	Challenge	30	85 [b]
	Unchallenged	25	60
Control	Challenge	0	50 [a]
	Unchallenged	0	0

[a, b] Means of challenged groups in a column with different superscripts were significantly different (P<0.05)

Mortality computed 1day post challenge didn't show a significant reduction of Pk-Merz or the tested essential oil compared to the control group. However, when observing the % mortality (Table 37) of the non-challenged groups, we can conclude that the wild species of *Origanum ehrenberjii* showed toxicity *in vivo* on day-old birds (60% mortality) compared to control unchallenged group (0% mortality) (Table 37). Thus before the initiation of the following *in vivo* experiment, *Origanum ehrenberjii* (cultivated) was tested for its safety *in vivo*, and was shown to be safe after 4 essential oil's administrations over 2 days (Table 38).

Table 38. Results of the safety testing of essential oil of cultivated *O. ehrenberjii*

Group	E. Oil concentration	Mortality 1 day post injection*	Mortality 2 days post injection*
1	1:80	0	1
2	1:160	0	0
3	1:320	1	0
4	1:640	0	2
5	0 (control)	0	0

* The birds were injected the appropriate dilution twice daily.

Finally, the same previous experiment was repeated (Table 39), substituting the wild essential oil with a cultivated one. Results showed a significant reduction in mortality one-day post challenge of Pk-Merz medicine, compared to the group treated with essential oil and the control group.

Table 39. *In vivo* antiviral activity of *Origanum ehrenberjii* (cultivated) essential oil injected intramuscularly in day-old chicks challenged intracerebrally with AI- H9N2 (4 HA)

Group		Mortality 1- day before challenge (%)	Total mortality 1-day post challenge morning (%)
Pk- Merz	Challenge	0	30 [a]
	Unchallenged	0	0
Essential oil	Challenge	0	65 [b]
	Unchallenged	0	5
Control	Challenge	0	55 [b]
	Unchallenged	0	0

[a, b] Means of challenged groups in a column with different superscripts were significantly different ($P<0.05$)

Again, Pk-Merz showed a significant decrease in mortality (30%) compared to control challenged group (55%). PK-Merz is proven to be effective against AI-H9N2 challenge intracerebrally, since it is able to cross the blood brain barrier, but it's impossible to measure to which extent this occurs (Pk-Merz leaflet, Pharma, Frankfort, Germany).

Over all, we can conclude that the Pk-Merz medicine is effective against AI-H9N2 *in vivo*, since it reduced mortality from 90 % to 30% in day- old chicks (Table

34). In addition *Origanum syriacum* essential oil diluted 1:80 was also effective against H9N2 challenge, and reduced mortality significantly compared to control group (Table 34).

In addition, findings reflect toxicity of the wild species of *Origanum syriacum* and *ehrenberjii* essential oils, compared to cultivated species (Tables 28, 29, 30, 31, and 32). Observing the chemical profiling of the different essential oils used in our experiments (Tables 28, 29, 30, 31, and 32), we could notice the difference in carvacrol and thymol content with respect to wild and cultivated species. Carvacrol and thymol content of different *Origanum sp.* is summarized in Table 40.

Table 40. Carvacrol vs. thymol content in different *Origanum* essential oils*

Origanum	Component	Area %
ehrenberjii (wild sp.)	Carvacrol	34.63
	Thymol	21.29
ehrenberjii (cultivated sp.)	Carvacrol	9.97
	Thymol	38.41
syriacum (wild sp.)	Carvacrol	11.67
	Thymol	18.21

* Analysis carried at the Core Laboratory, Faculty of Medicine, AUB
Data is shown in Tables 28, 29 and 31

In the wild sp., carvacrol and thymol percentage are close, while in the cultivated sp. there is a large difference in carvacrol and thymol contents. Chiasson *et al.* suggested that the toxic effect of carvacrol and thymol did not correspond to their relative presence or to their levels in the oil; however, carvacrol toxicity is reduced in the presence of thymol, suggesting an antagonistic effect between the two phenolic compounds (Chiasson *et al*., 2001). Actually the thymol, in the cultivated *O. ehrenberjii* was high (38.41%) and the carvacrol was low (9.97%), while in the wild *O. ehrenberjii* and *O. syriacum* the thymol was lower namely 21.29 % and 18.21% respectively, and

the toxic compound (carvacrol) was high (34.63 % and 18.67%) in the wild *O. ehrenberjii* and *O. syriacum* respectively.

Future investigations should include slow release vehicle for the delivered essential oil in order to avoid the toxic effects of fast delivery. Example of slow release vehicle will be the liposomes (Sinicio *et al*., 2004).

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Four experiments were conducted in order to convey surveillance of AIV in Lebanon, evaluation of a killed AI-H9N2 vaccine, evaluation of Mentofin® against MG/H9N2 respiratory diseases in broilers and to evaluate the antiviral efficacy and safety of Origanum essential oils.

The first experiment describes the first emergence of H9N2 avian influenza virus outbreak in poultry farms of Lebanon, and its impact on poultry health and production. The H9N2 is most likely the predominant Influenza A virus involved in the poultry outbreaks in Lebanon, resulting in significant drop in egg production in meat breeders and commercial layers, followed by free-range layers, and with high mortality in broilers. Signs and lesions are indicative of a LPAI strain involvement. The HI test confirmed that most of AI-ELISA positive chicken flocks had H9-antibodies. The presence of AIV antigens in brains and tracheal tissues and the identification of H9 and N2 components in the propagated virus incriminates the H9N2 as the most likely virus involved in the first emergence of AIV outbreaks in Lebanese poultry. Therefore, screening of Lebanese poultry farms is essential to understand the course, spread and evolution of the virus, and to identify any new strain appearance in poultry in Lebanon.

The second experiment, was an evaluation of an Intervet killed vaccine in two different farms in Lebanon, commercial intensive farm, and another free-range layers farm. Three or two doses of vaccination are required to reach a high titer in chicken layers. However, further researches are required to evaluate the level of protection provided by these antibodies against AI-H9N2 challenge in Lebanon.

Third, Mentofin®, a *Eucalyptus* and peppermint oils based drug, administered in the drinking water was evaluated in broilers farm, in Chekka. The essential oils of *Eucalyptus* and peppermint (Mentofin®) improved the homogeneity of immune responses to respiratory vaccine viruses of NDV and IBV. It reduced the respiratory signs and lesions in the period following challenges by live vaccine strains, and it resulted in slight reduction in mortality, associated with improvement in feed conversion that added up to an additional net saving of $491.20 per 10,000 birds after subtraction of the cost of Mentofin®. Mentofin® was effective against respiratory poultry diseases; however, we recommend further research in order to understand the mechanism by which Mentofin reduced the stress on animals.

Finally, *Origanum syriacum* and *ehrenberjii* essential oils were tested for their antibacterial activity *in vitro*, and for their antiviral activity against H9N2-AI challenge in baby chicks. The essential oils were compared to the most common antiviral drugs against AIV. Amantadine sulfate is shown to be effective against H9N2-AI intracerebral challenge; however carvacrol and thymol in the wild species of the two essential oils seems to be responsible for the toxicity *in vivo*. Later studies could be done using a carrier to the essential oil that allows its slow release, thus avoiding toxicity.

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