

AMERICAN UNIVERSITY OF BEIRUT

DEVELOPMENT OF A VACCINE AGAINST NEWCASTLE
DISEASE AND ANTIMICROBIALS FOR *SALMONELLA*
SEROVARs

by
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AN ABSTRACT OF THE THESIS OF

Mohammad Rashid Murtada

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Major: Poultry Science

Title: Development of a vaccine against Newcastle Disease and antimicrobials for *Salmonella* serovars

The thesis is divided into two parts namely, studies A and B. The objective of study A was to conduct a phylogenetic analysis on the predominant v-NDV strain in Lebanon, to develop an autogenous Newcastle Disease Virus (NDV) vaccine aiming at the control of v-NDV, and to evaluate its degree of protection and immunity in *Eimeria*-infected birds. A blend of eucalyptus and peppermint essential oils was also incorporated in this study to explore if essential oils have any immunopotentiating effect when administered to birds subjected to v-NDV and *Eimeria* challenges. A controlled challenge trial was conducted. Eighty day-old chicks were divided evenly into 8 different experimental treatments. Treatments 1, 2 and 3 were vaccinated with the developed NDV vaccine at d1 and d14, and received *Eimeria* challenge at d21 and v-NDV challenge at d28. The positive control Treatments 4 and 5 did not receive any NDV vaccination, and received both challenges. Treatments 6, 7, and 8 received the developed NDV vaccine, however they did not receive any challenge, acting as negative control for NDV. Evaluation of protection included an assessment of performance parameters namely, the % weight gain, feed conversion, mortality, and specific acquired immunity to the hemagglutinin and fusion proteins of the v-NDV. The priming and boosting with the developed autogenous vaccine conferred a 100 % survival in *Eimeria*-infected and uninfected birds that were challenged with v-NDV compared to 0.0% and 30.0% survivals in unvaccinated-challenged controls ($P < 0.05$). The acquired HI titers to hemagglutinin and fusion proteins of genotype VIc Lebanese v-NDV isolate were significantly higher in the vaccinated birds compared to unvaccinated-challenged controls at 34 days of age ($P < 0.05$). This study uncovered the high protection by killed autogenous ND vaccine against a controlled bivalent challenge with homologous v-NDV of genotype VIc and *Eimeria* spp.

Study B aimed at the identification of the *Salmonella* serovars involved in outbreaks that led to hospitalization of Saudi Arabian patients due to typhoid and non-typhoid ailments. In addition, this work investigated the susceptibilities of the identified human serovars to 23 active ingredients of the most common commercial drugs; including control isolates with known multiple drug-resistances. The Minimum Inhibitory Concentration (MIC) of a chemically characterized essential oil blend against isolates of all recovered serovars was determined, attempting to correlate their values to the frequency of resistance to the 23 antibiotics. The safety of the essential oil blend on epithelial membrane

of the conjunctiva and its mutagenic potential were assessed by Rabbit-Draize Eye and Ames tests, respectively. *Salmonella* susceptibility to active ingredients of 23 drugs was accomplished by single disk diffusion assay. The MIC was determined by a standard protocol. The distribution of the 16 separately admitted Saudi patients, due to their infection with different *Salmonella* serovars were: 4 (*S. Enteritidis*), 4 (*S. Typhimurium*), 3 (*S. Kentucky*), 3 (*S. Anatum*), and 2 (*S. Typhi*). The three *in vitro* effective drugs against all 14 non-typhoid isolates were Cefepime, Chloramphenicol, and Norfloxacin, while 10 drugs were effective against *S. Typhi* isolates. A positive correlation ($R=+0.46$) between the frequency of drugs that the *Salmonella* isolates were resistant to and the MIC of the essential oil-blend was observed ($P<0.05$). Only three drugs out of 23 were effective *in vitro* against the Non-Typhoids, while 10 drugs were fortunately effective against the Typhoids. The safety and MIC data related to the novel blend of the essential oils encourage pursuing its efficacy *in vivo* to help for future use against *Salmonella* infections.

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ABBREVIATIONS

| | |
|-------|--|
| Trt | Treatment |
| NRC | National Research Council |
| WHO | World Health Organization |
| OIE | World Organization for Animal Health |
| CDC | Centers for Disease Control and Prevention |
| NDV | Newcastle Disease Virus |
| v-NDV | Velogenic Newcastle Disease Virus |
| EO | Essential Oils |
| AGP | Antibiotic Growth Promoter |
| D | Day |
| AB | Antibiotics |
| SXT | Sulfamethoxazole-Trimethoprim |
| USDA | United States Department Of Agriculture |
| FDA | Food and Drug Administration |
| ICPI | Intracerebral Pathogenicity Index |
| FCR | Feed Conversion Ratio |
| BWG | Body Weight Gain |
| MIC | Minimum Inhibitory Concentration |
| EDS | Egg Drop Syndrome |
| IB | Infectious Bronchitis |

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To the memory of my father, Dr. Rashid Murtada

To my lovely mother, Bouthayna Yaghi

*To my family members, Shaza, Ali, Diana, Hussein,
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CHAPTER I

INTRODUCTION

Poultry Production is among the fastest growing livestock sectors in the world along with swine production. Globally, there is a continuous rise in the demand for poultry products with an average annual consumption growth of 2% (FAO 2015). According to FAO, poultry meat will reach 50% of the additional meat consumed in 2024. The main advantage of poultry products is that they are considered to be an affordable and healthy protein source, with few religious impediments, reflecting the fact that poultry will become the world's preferred meat. This rise in the poultry sector will mainly take place in developing countries that are aiming towards improving their food security status. These countries are known to have poor monitoring of poultry diseases and a lack of a National Poultry Improvement Plan (NPIP). Moreover, in such middle and low income countries, reporting of foodborne infectious diseases and the antibiotic resistant organisms is absent.

In developing countries poultry diseases pose two major threats. The first threat is that of the devastating species-specific poultry diseases like Newcastle Disease Virus (NDV), Infectious Bursal Disease Virus (IBDV), and *Mycoplasma* spp. that increase the economic burden and decrease the growth rate, expansion and efficiency of the poultry industry. The second, and the most significant threat imposed by poultry diseases, is the threat of zoonotic poultry diseases known to infect both humans and animals such as Salmonellosis, Campylobacteriosis, Botulism, and Avian Influenza infections. Moreover, it has been shown that poultry farm animals are reservoirs of such diseases, and play a major

role in the rise of multidrug resistant pathogens that is attributed to the overuse of antibiotics in poultry production leading to the selection for multidrug resistant organisms, or what is commonly referred to nowadays as “Superbugs”.

Newcastle disease virus (NDV), a disease that is known to infect more than 240 bird species, is considered to be one of the major poultry diseases if not the major one until the rise of Highly Pathogenic Avian Influenza (HPAI). The endemicity of the v-NDV is still reported during the last 5 years from many parts of the world, resulting in devastating disease outbreaks in poultry, including breeders, commercial layers, and broilers (Ababneh, Dalab et al. 2012; Radwan, Darwish et al. 2013; Umali, Ito et al. 2015; Barbour, Shaib et al. 2013). Despite the inclusion of different classical and vectored commercial vaccines for the control of different forms of the NDV, the disease is still endemic in poultry in many countries, located in the five continents of our planet, except that of Antarctica (Alexander and Senne 2008).

In spite of all the efforts of introducing new commercial vaccines aiming at protection against a vast range of v-NDV genotypes, many outbreaks of v-NDV in vaccinated birds reflect the failure of commercial NDV vaccines in offering acceptable protection and reduced viral shedding (Kapczynski, Afonso et al. 2013; Roohani, Tan et al. 2015). This is the main reason behind the regulations in most developed countries to eradicate the poultry that are infected by v-NDV (OIE Manual 2012), aiming at keeping their poultry sector free of this List A diseases (Alexander and Senne 2008). Unfortunately, and due to the absence of National Poultry Improvement Plans (NPIP) from most developing countries, and the unavailability of compensation to the farmers for

eradication, due mostly to poor economy, most of the poultry managers in these countries still introduce vaccines and other biologic enhancers (Barbour, Shaib et al. 2013), hoping to protect against the endemic v-NDV outbreaks in their vicinities.

Salmonella is considered to be one of the major foodborne infectious diseases of great public health significance. Most developed countries have a system of annual reporting of the frequency of recovered *Salmonella* serovars from human and other animal hosts (CDC, 2015); however, most developing countries rely on sporadic reporting, based on targeted surveillances of *Salmonella* outbreaks (Barbour, Ayyash et al. 2015).

The most common drugs experimented on against gram-negative bacteria were 13 cell wall inhibitors namely, Amoxicillin/Clavulanic acid, Ampicillin, Aztreonam, Cefamandole, Cefepime, Cefixime, Cefotaxime, Cefoxitin, Ceftazidime, Cefuroxime Sodium, Cephalothin, Imipenem, and Piperacillin/Tazobactam (Wilke, Lovering et al. 2005). In addition, the most searched six drugs inhibiting the protein synthesis in bacteria were Amikacin, Chloramphenicol, Gentamicin, Kanamycin, Tetracycline, and Tobramycin (Schlünzen, Zarivach et al. 2001), while those inhibiting the nucleic acid synthesis were three quinolones namely, Ciprofloxacin, Nitrofurantoin, and Norfloxacin (Kohanski, DePristo et al. 2010), and the one folate pathway-inhibitor was the bivalent synergist of Sulfamethoxazole/Trimethoprim (EFSA 2014).

Due to the frequent occurrence of *Salmonella* serovars with resistance to multiple synthetic drugs, international organizations (WHO, OIE, and EFSA) and individual researchers (EFSA 2014) are continuously investigating the presence of antimicrobial

activities in herbs (Weerakkody, Caffin et al. 2010), biofilms on sea algae, and various metabolic products (Cox, Abu-Ghannam et al. 2010). This trend in research emerged as a result of documented surveillances, presenting data on multiple drug resistance in different *Salmonella* serovars (WHO 2014). A blend of chemically-characterized eucalyptus and peppermint essential oils (1/1, v/v ratio) has been subjected to various investigations, including the evaluation of its activity against viral (Barbour, Shaib et al. 2013), protozoal (Barbour, Ayyash et al. 2015), and bacterial (Kalemba and Kunicka 2003) etiologies of economic diseases, with an absence of data related to safety.

The objective of study A was to conduct a phylogenetic analysis of the predominant v-NDV strain circulating in Lebanon, to develop an autogenous poultry vaccine that aims at the control of v-NDV, and to evaluate its degree of protection and immunity in challenged birds by *Eimeria* spp. A blend of eucalyptus and peppermint essential oils was included in this study to find out if the use of essential oils can alleviate the injury caused by v-NDV.

Study B aimed at the identification of the *Salmonella* serovars involved in outbreaks that led to hospitalization of Saudi Arabian patients due to typhoid and non-typhoid conditions. In addition, this work investigated the susceptibilities of the identified human serovars to 23 active ingredients of the most common commercial drugs; including control isolates with known multiple drug-resistances. Moreover, the MIC of the essential oil blend against isolates of all recovered serovars was determined, attempting to correlate their values to the frequency of resistance to the 23 antibiotics. The safety of the essential oil blend on mucosal membrane of the conjunctiva and its mutagenic potential were

assessed by Rabbit-Draize Eye and Ames tests, respectively.

CHAPTER II

LITERATURE REVIEW

A. Newcastle Disease Virus

Avian Paramyxovirus serotype 1 (APMV 1), commonly known as Newcastle Disease Virus (NDV), is a virus belonging to the genus *Avulavirus* and family *Paramyxoviridae* (OIE Manual 2012). NDV is known to infect more than 240 bird species. However, the severity of NDV depends on both the host and the viral strain (Alexander, Aldous et al. 2012).

Strains of NDV virus are grouped according to the signs they induce in infected birds (Alexander 2000) NDV strains can be grouped into five pathotypes (Alexander and Senne 2008):

1. Viscerotropic Velogenic (VVND): a highly pathogenic form that causes gut infection demonstrated by hemorrhagic intestinal lesions.
2. Neurotropic Velogenic (NVND): a form causing respiratory and neurological signs leading to high mortality.
3. Mesogenic: a form of intermediate virulence causing low mortality, respiratory signs and nervous signs in some birds.
4. Lentogenic: a mild form that causes subclinical respiratory infection of low virulence.

5. Asymptomatic: a form that causes avirulent infections and subclinical enteritis.

NDV infection with virulent forms namely viscerotropic velogenic and neurotropic velogenic viruses requires immediate notification to animal health authorities since NDV is an OIE listed notifiable disease (OIE Manual 2012).

B. NDV viral structure

The APMV-1 is a rounded pleomorphic virus having a diameter ranging between 100-500 nm (Kolakofsky, de la Tour et al. 1974). The APMV-1 genome consists of a single molecule of single-stranded negative sense RNA virus (Kolakofsky, de la Tour et al. 1974). NDV nucleotide sequencing showed that the virus consist of about 15,186 nucleotides (Phillips, Samson et al. 1998; Lamb, Paterson et al. 2006) with six genes coding for 7 proteins. L protein, RNA polymerase associated with the nucleocapsid; HN protein responsible for hemagglutinin and neuraminidase activities and representing the larger projection on the surface of the paramyxovirus. F or the fusion protein appears as the smaller viral surface projection. NP, nucleocapsid protein; P, phosphorylated, nucleocapsid associated that codes for both V protein and M, Matrix protein due to its overlapping reading frame. Figure 1 shows the structure of NDV virus and Figure 2 represents the order of genes of NDV proteins in the viral genome.

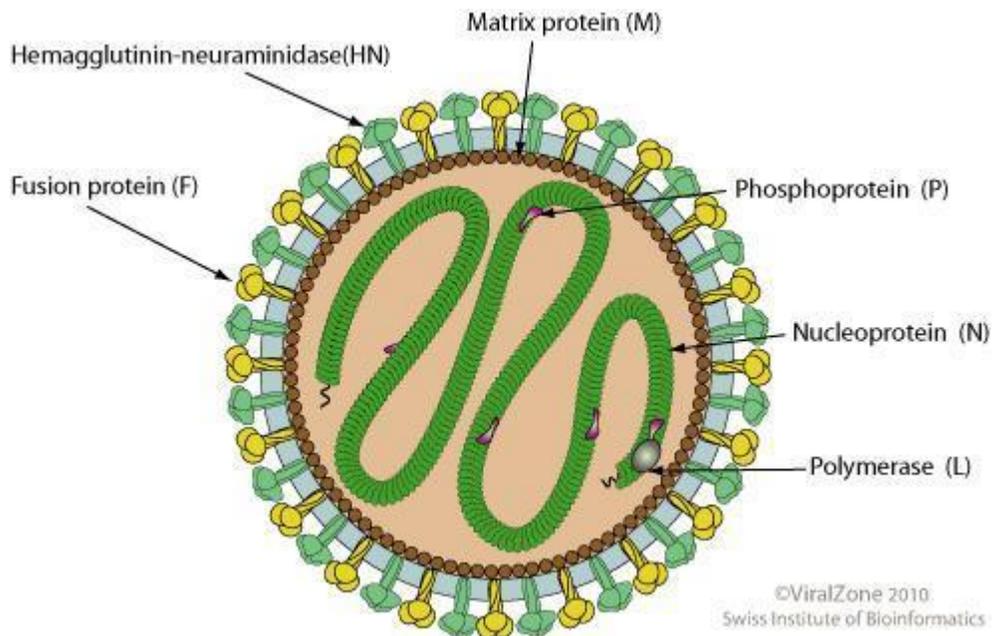


Figure 1. NDV viral structure (ViralZone, 2010).

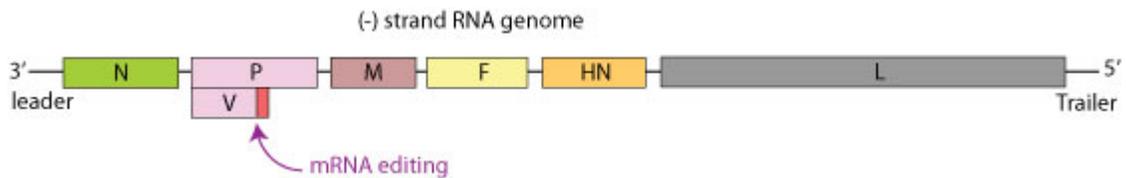


Figure 2. Order of genes of NDV proteins in viral genome (ViralZone, 2010).

1. *The role of F and HN proteins in virus replication*

The first step in NDV replication is the attachment of the virus to the host cell receptors done by HN protein. The next step is the fusion of the virus and cell membrane that is done by the action of the fusion protein (F) allowing the nucleocapsid complex to enter the cell. Host proteases are responsible for the cleavage of the nonfunctional fusion

protein F0 into F1 and F2. This cleavage plays a major role in determining the pathogenicity of NDV strains (de Leeuw and Peeters 1999).

C. NDV Pathogenicity

The impact of NDV outbreak mainly depends on the virulence of the isolated strain. Conventionally, *in vivo* tests have been used to identify the virulence of NDV strains. Intracerebral Pathogenicity Index (ICPI) has been the most reliable and widely adopted test by World Organization for Animal Health (OIE). However, advancement in molecular diagnosis explained the variation in pathogenicity of NDV strains. The latter method is currently being adopted instead of conventional *in vivo* pathogenicity tests like ICPI. Table 1 compiles the ICPI and cleavage site sequencing of NDV strains of different pathogenicity.

1. Intracerebral Pathogenicity Index (ICPI) Test

The ICPI test is a widely adopted test used to determine the pathogenicity of NDV isolated viruses (OIE Manual 2012). According to OIE the test procedure is as follow:

1. Infected allantoic fluid with HA titer >1:16 is diluted in sterile saline at a concentration of 1/10.
2. A volume of 0.05 ml of diluted virus is injected intracerebrally into 10 Specific Pathogen Free (SPF) hatched chicks.

3. Chicks are offered water and feed, and monitored over 24 hours for 8 days.
4. Chicks are scored 0 if normal, 1 if sick and 2 if dead or unable to eat and drink.
5. ICPI is the average mean score per bird per observation over 8-day period.
6. Highly virulent viruses will have an ICPI near 2.0, whereas low virulent viruses (Lentogenic and Asymptomatic viruses) will give ICPI close to 0.0.

2. Molecular basis for pathogenicity

The NDV fusion protein F0 cleavage into F1 and F2 is vital for NDV replication in the host, otherwise non-infectious viral particles are produced. *In vitro* studies indicated that trypsin could cleave F0 for all NDV strains (Nagai, Klenk et al. 1976). Low virulent strains could replicate only if trypsin is added, unlike virulent strains that could replicate in a wide range of cell types with or without trypsin (Rott 1985). Advancement in molecular diagnosis enabled the comparison of the F gene nucleotide sequence of various NDV strains (Chambers, Millar et al. 1986; McGinnes and Morrison 1986). Nucleotide sequencing of NDV strains showed that all strains had arginine, a basic amino acid (AA), at the cleavage site 116. Viruses of low virulence had leucine at residue 117 and another basic AA at residue 113. On the contrary, virulent strains had phenylalanine at residue 117 and multi-basic AAs at residues 115 and 112 in addition to those present at 113 and 116. The presence of multi-basic AAs at the cleavage site of virulent NDV strains (Glickman, Syddall et al. 1988) indicates that F0 protein of these strains could be cleaved by proteases present in a wide range of cell types, resulting in a fatal systemic infection. For low

virulence lentogenic viruses, F0 cleavage can be activated in cells having trypsin-like proteases such as respiratory or intestinal cells.

In conclusion, molecular studies clarified the variation in NDV strains tropism, and the different lesions they can induce in infected chicken.

Table 1. Cleavage site at specific AA sequence & ICPI of NDV strains of various virulence.

| NDV Strain | Pathotype for Chicken | Cleavage Site AA 111 to 117 | ICPI | References |
|-------------|--------------------------|--------------------------------|------|------------------------------------|
| Herts 33 | Velogenic | -G-R-R-Q-R-R*F- | 2.0 | (Toyoda, Sakaguchi et al. 1989) |
| Beaudette C | Mesogenic | -G-R-R-Q-K-R*F- | 1.6 | (Collins, Bashiruddin et al. 1993) |
| La Sota | Lentogenic | -G-G-R-Q-G-R*L- | 0.4 | (Collins, Bashiruddin et al. 1993) |

D. NDV Economic Significance

Newcastle disease virus has a great global economic impact being one of the major poultry diseases if not the major poultry disease in economic value until the emergence of highly pathogenic avian influenza H5N1 (Alexander and Senne 2008). In developing countries the economic impact of NDV includes direct losses from bird mortality and the cost of control measures including vaccination. Even in NDV free countries, additional

costs are imposed by frequent routine testing that is required for maintaining status and supporting trade (Leslie 2000) and the cost of eradication of any velogenic NDV (v-NDV) outbreak. Some examples on the high cost of v-NDV irradiation are the two outbreaks in California, USA in 1971 and 2002 that costed \$ 52 million and more than \$200 million, respectively (Miller, King et al. 2007). Velogenic NDV is endemic in many countries causing 100% mortality in commercial chicken and trade embargos that negatively affect the commercial poultry industry. In many developing countries backyard chickens represent an important source of nutritional support since poultry products are considered to be the cheapest source of animal protein. In such countries, outbreaks of NDV pose a major threat towards maintaining healthy diets (Alexander and Senne 2008).

E. Transmission and Spread

NDV infection can take place either through inhalation or ingestion of viral particles. NDV vertical transmission through embryonated eggs is controversial. The spread of the disease in commercial poultry farms is likely due to the following (Lancaster 1966; Alexander 1988):

1. Movement of live birds
2. Contact with other animals
3. Movement of people and equipment
4. Movement of poultry products
5. Airborne spread

6. Contaminated feed
7. Contaminated water
8. Vaccines

F. Emergence of Virulent Viruses

There are three different justifications for the rapid emergence of velogenic NDV (Hanson 1972, Hanson and Spalatin 1978):

1. NDV was always present in poultry, however unnoticed until the commercialization of poultry production.
2. Pathogenic strains were enzootic in other bird species, and have less severity in bird species other than chicken.
3. Virulent viruses arose by mutation from low pathogenic viruses.

The first theory was considered unlikely, but possible; since backyard and village chicken also show clinical signs, morbidity and mortality. The second explanation was considered to be the most likely, and is supported by NDV panzootic that took place from 1970-1973. During the 1970s panzootic, velogenic NDV was introduced by the movement of caged birds, mainly psittacine species, that are resistant to strains known to be highly pathogenic in chicken (Alexander 2000).

Cormorants and pigeons are also known to be reservoirs of virulent NDV. Other researchers showed experimental evidence that the rise of virulent NDV could be triggered

by mutation. A study showed that low virulent waterfowl NDV isolate with F0 cleavage site sequence of ERQER*L became highly virulent for chicken, and with cleavage site KRQKR*F upon nine passages in chicken air sacs, and five intracerebral passages in chicken (Shengqing, Kishida et al. 2002).

G. NDV Epidemiology

The first recorded NDV outbreak was in 1926, in Java, Indonesia and Newcastle-upon-Tyne, England (Alexander 2000). Initially, NDV spread rapidly in Asia and took 16 to 40 years to become a true panzootic. The second panzootic emerged in the late 1960s and had a global worldwide spread in 4 years. It is believed that the source of the second NDV panzootic was the trade of captive wild birds (Alexander 2001). The third panzootic has been attributed to v-NDV infection in pigeons and is believed to be continuing till now posing a great threat to domestic poultry (Alexander 2011).

Many countries in the Middle East are endemic for NDV. The first NDV outbreak in Lebanon was reported in 1968 by the Fanar Regional Poultry Laboratory. In this outbreak, virulent NDV was detected in chickens in Talamara, Lebanon. In addition, outbreaks of v-NDV genotype VIa were also reported in the late 1960s and 1970s in Lebanon, Iraq, Kuwait, and Israel making this genotype an “ancient” strain originating in Middle East (Aldous, Mynn et al. 2003). Genotypes VI viruses are still the predominant NDV strains circulating in the Middle East, and were isolated from domestic poultry, pigeons and falcons, in addition to the new rise of genotype VII field isolates (Aldous,

Mynn et al. 2003; Herczeg, Wehmann et al. 1999). Table 2 lists the predominant circulating NDV genotypes in the Middle East. Ministries of Agriculture in different countries are required to report any v-NDV outbreak to the OIE. The control of v-NDV strains detected in 2011 were documented in literature (Barbour, Shaib et al. 2013), while the outbreak reported by Lebanese Ministry of Agriculture in Hasbaya in 2016, were left without documentations.

Table 2. Circulating v-NDV genotypes in the Middle East (Aldous, Mynn et al. 2003).

| Genotype | Year | Pathotype | Main geographical origins | Main hosts |
|----------|--------------|-----------|-----------------------------|---------------------|
| VIa | 1968 to 1996 | Velogenic | Africa, Europe, Middle East | Chicken, Falcon |
| VIb | 1984-2000 | Velogenic | Europe, Middle East | Pigeon |
| VIc | 1989 to 1999 | Velogenic | Europe, Middle East | Chicken, Falcon |
| VIIId | 1997 to 2000 | Velogenic | Asia, Middle East | Chicken, Ostrich |

H. Poultry Vaccines

Vaccines are commonly used in commercial poultry production to control and/or reduce infection of viral, bacterial, or protozoal field challenges. In addition, vaccines are also used to immunize breeder hens aiming at maximizing maternal immunity passed to hatching chicks.

Vaccine selection and vaccination programs are mainly dependent on local field challenges, risk management, and cost efficiency (Zander, Bermudez et al. 1997).

1. NDV vaccination

The main goal of NDV vaccination is to result in protective immunity against viral infection and replication. However, in reality NDV vaccination protects birds against the fatal consequences of the virus and reduce viral shedding that might still occur (Miller, King et al. 2007).

NDV vaccines can be grouped into 3 categories: live, inactivated and recombinant vaccines.

2. NDV vaccination policies

Countries have different policies regarding quality control of NDV vaccines. For example, Sweden bans the use of any vaccine, while in the Netherlands, vaccination of all poultry is enforced by the government.

According to OIE, the seed virus of live NDV vaccines must have an ICPI of less than 0.4, and master seed of inactivated vaccines must have an ICPI value of less than 0.7, restricting the use of v-NDV as master seed for inactivated vaccines (OIE Manual 2012). On the other hand, in v-NDV endemic countries, like Mexico, homologous v-NDV vaccines are commonly produced to protect poultry against NDV field challenges (Afonso

and Miller 2013).

3. Live vaccines

Live NDV vaccine strains are grouped into Lentogenic viruses, viruses of low virulence, and Mesogenic viruses, viruses of intermediate virulence. Lentogenic NDV viruses are of low virulence, and are the mostly used vaccine strain candidates having a range of virulence (Borland and Allan 1980). For example, the widely used lentogenic vaccine strain Lasota, is of higher virulence than the other commonly used lentogenic strain Hitchner B1. Plaque selected clones of parent viruses are also commonly used vaccine strains like ND clone virus that is cloned from the Lasota strain.

Live Lentogenic NDV vaccines are sold as a lyophilized NDV-infected allantoic fluid stored at 4°C (Gallili and Ben-Nathan 1998).

Table 3 lists the eight NDV strains commercially used in the production of live NDV vaccines. Detailed production steps of live lentogenic NDV vaccines according to Gallili and Ben-Nathan are shown in Figure 3.

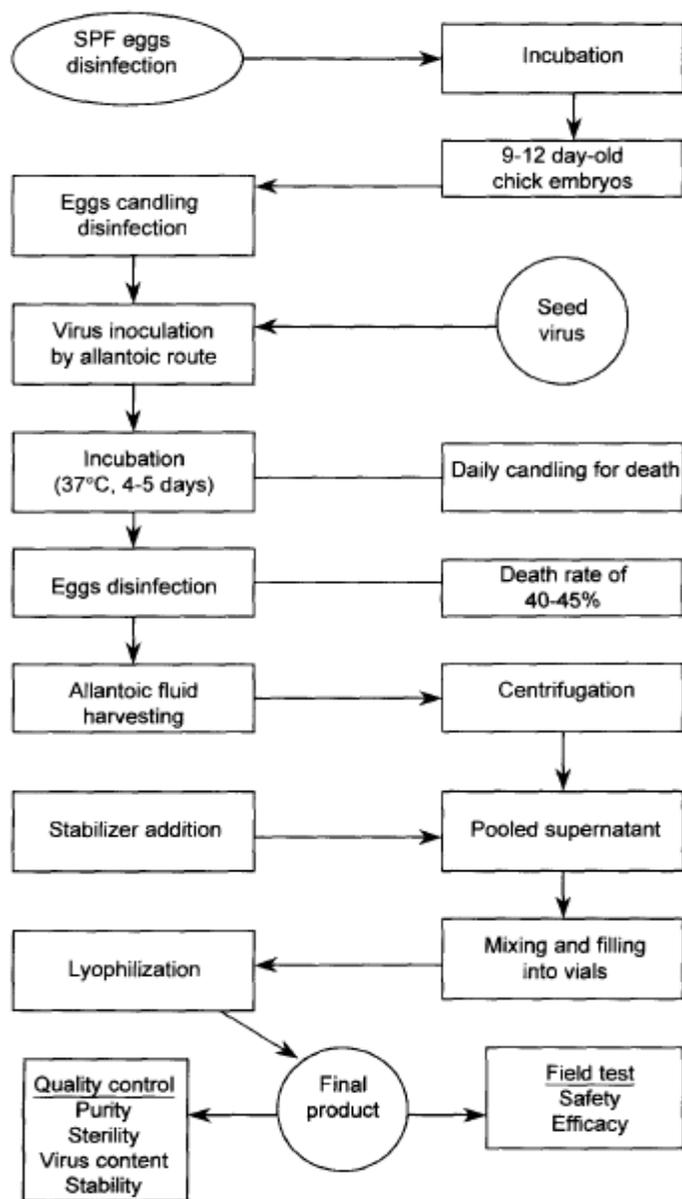


Figure 3. Schematic diagram of live lentogenic NDV vaccine production (Gallili and Ben-Nathan 1998).

Table 3. Live NDV vaccine strains (Grimes 2002).

| Strain | Description |
|-----------|--|
| F | Lentogenic. Usually used in young chickens but suitable for use as a vaccine in chickens of all ages. |
| B1 | Lentogenic. Slightly more virulent than F, used as a vaccine in chickens of all ages. |
| LaSota | Lentogenic. Often causes post vaccination respiratory signs, used as a booster vaccine in flocks vaccinated with F or B1. |
| V4 | Avirulent. Used in chickens of all ages. |
| V4-HR | Avirulent. Heat Resistant V4, thermostable, used in chickens of all ages. |
| I-2 | Avirulent. Thermostable, used in chickens of all ages. |
| Mukteswar | Mesogenic. An invasive strain, used as a booster vaccine. Can cause adverse reactions (respiratory distress, loss of weight or drop in egg production and even death) if used in partially immune chickens. Usually administered by injection. |
| Komarov | Mesogenic. Less pathogenic than Mukteswar, used as booster vaccine. Usually administered by injection. |

The objective of live vaccines is to establish infection in the flock, and benefit from herd immunity. Live lentogenic vaccines are administered orally via drinking water, nasally via spray, and ocular via eye drop. However, mesogenic live vaccines are usually inoculated intramuscularly, subcutaneously, or administered through wing-web injury. The main advantage of live vaccines is their efficient and inexpensive mass application techniques. Worldwide, the mostly used method of mass application of live lentogenic vaccines is by drinking water. Water is withheld from birds for a couple of hours, and then the vaccine is applied in fresh unchlorinated water. The addition of skim milk is proved to help stabilize the virus in drinking water, and neutralize free chlorine, thus preventing the inactivation of the live vaccine strains (Gentry and Braune 1972). The below Table 4

summarizes the advantages and disadvantages of live vaccines.

Table 4. Advantages and disadvantages of live NDV vaccines.

| Advantages of Live NDV Vaccines | Disadvantages of Live NDV Vaccines |
|--|---|
| Relatively inexpensive | Vaccine may cause disease (vaccine reaction) |
| Sold as freeze-dried allantoic fluid | Interference with maternally derived antibodies |
| Easy to administer (mass administration) | Need cold storage 4-5°C |
| Cell Mediated and Humoral Immunity | Killed easily by chemicals and heat |
| | Vaccine contamination |

4. Inactivated vaccines

Inactivated or killed vaccines are produced from infected allantoic fluid that is usually treated with β -propiolactone or formalin to kill the virus. The viral aqueous phase is then mixed with a carrier adjuvant. The first adjuvant used in the production of killed vaccines was aluminum hydroxide. After the advancement in the production of adjuvants, oil-emulsion based vaccines were adopted (Cross 1988). Advancement in the production and testing of adjuvants aims at increasing immunogenicity, and reduce the 42-days withdrawal period (Afonso and Miller 2013) that some countries have regarding oil-emulsion adjuvants.

Different NDV viral strains are used in the production of oil-emulsion vaccines like La Sota, Roakin, and several virulent viruses (Alexander and Senne 2008). The selection of seed virus is highly dependent on its ability to replicate in the allantoic fluid of embryonated eggs. Virulent viruses are known to kill embryonated eggs, and thus produce

low titers. On the other hand, low pathogenic viruses are known to replicate in embryonated eggs, and produce high titers. Killed vaccines can also be bivalent or trivalent, containing two or three killed viruses in the oil emulsion respectively. The most commonly used viruses with NDV oil-emulsion are Adenoviruses (Egg Drop Syndrome), Infectious Bronchitis, H9N2 avian influenza, and Infectious Bursal Disease virus (Meulemans, Letellier et al. 1988).

Inactivated viruses are administered by injection of individual birds intramuscularly or subcutaneously. The below Table 5 summarizes the advantages and disadvantages of inactivated NDV vaccines.

Table 5. Advantages and disadvantages of inactivated NDV vaccines.

| Advantages of inactivated NDV Vaccines | Disadvantages of inactivated NDV Vaccines |
|---|--|
| Easy to store (no need for cold chain) | Expensive to produce and apply |
| Not affected by maternal immunity | 42-day withdrawal period for oil-emulsion vaccines in some countries |
| Low vaccine reaction | Difficult quality control |
| Used if complicated pathogens are present | Minerals may cause serious problem if vaccinator was accidentally injected |
| High levels of humoral immunity and long period of protection | |

5. Recombinant NDV vaccines

Advancement in molecular biology enabled the cloning of key NDV genes that are

involved in pathogenicity. Fusion and HN genes are widely used genes in recombinant vaccines (Nakaya, Cros et al. 2001; Morgan, Gelb Jr et al. 1992). Vector viruses carrying the candidate gene(s) include mostly Turkey Herpesvirus (HVT) (Rauw, Gardin et al. 2010) and Fowlpox virus (Sharma, Zhang et al. 2002). Studies have shown that the use of fusion gene as a clone gene was more protective than the use of HN gene or the use of HN and F in combination (Morgan, Gelb Jr et al. 1992). The below Table 6 lists the advantages and disadvantages of recombinant NDV vaccines. Table 7 shows the list of licensed recombinant NDV vaccines in US (Armour and García 2014).

Table 6. Advantages and disadvantages of recombinant NDV vaccines.

| Advantages of recombinant NDV Vaccines | Disadvantages of recombinant NDV Vaccines |
|---|---|
| Protection against 2 viruses (rHVT-ND protects against ND and Marek's Disease) | Expensive to produce compared with live vaccines |
| Can be applied safely <i>in ovo</i> | Expensive individual application via injection to insure good viral replication (absence of mass application) |
| Safe vaccine that do not revert to virulence (contains only cloned genes in addition to vector virus) | Difficult to assess immunity by available methods such as HI and ELISA |
| Species-specific (replicate poorly in another host) | Protection upon challenge experiments is still controversial |
| Facilitate DIVA (Differentiation between Infected and Vaccinated Animals) | |

Table 7. Licensed recombinant NDV vaccines in US (Armour and García 2014).

| Vector | Gene Insert(s) | Vaccines (Licensed by) | Application |
|--------|----------------------------|----------------------------|-------------------------------|
| HVT | NDV F gene | Vectormune® HVT | In-ovo/Sc ¹ (1d) |
| | | NDV (Ceva) | |
| | | Innovax® ND-SB (Merck) | In-ovo |
| FPV | HN and F genes of NDV. | TROVAC® NDV (Merial) | Sc (1d) |
| | Unspecified gene(s) of NDV | Vectormune® FP N (Ceva) | Sc (1d)/WW ² (9wk) |
| MDV | NDV F gene | Experimental 3 | Sc |

¹ Sc=Subcutaneous

² WW=Wing-Web

³ Okamura et al., 2001; Sakaguchi et al., 1998; Sonoda et al., 2000.

I. Failure of Classical Vaccines, and The Rising Need for Autogenous Genotype-matched Vaccines

Developed countries have a systematic surveillance of poultry diseases. Any outbreak of a list “A” poultry disease, like velogenic Newcastle Disease, is subjected to direct reporting and eradicated. In 2002, v-NDV outbreak was reported in California and more than 3 million birds were eradicated at an estimated cost of more than \$200 million (Miller, King et al. 2007). On the other hand, in v-NDV endemic developing countries like

Lebanon, v-NDV outbreaks are left without reporting by the Ministry of Agriculture. Even if v-NDV outbreaks are reported, eradication of infected flocks is uncommon since, unlike highly pathogenic avian influenza, v-NDV is considered of minimal public health significance, and eradication poses a great economic burden on such low and middle income countries. In these v-NDV endemic countries, vaccination against NDV is the only option available for farmers.

Many countries around the world continue to have endemic v-NDV condition, even though billions of live, killed and recombinant vaccine doses are applied (Afonso and Miller 2013). The large number of outbreaks in NDV-endemic countries point out to the failure of commercial vaccines to induce protection and reduce viral shedding (Miller, Afonso et al. 2013). According to Afonso and Miller, NDV vaccine failure could be attributed to deficiencies in either vaccination methods, or the vaccine (Miller, Afonso et al. 2013). Regarding vaccination methods, the failure is mostly due to the absence of a “cold chain” that is needed for NDV live vaccine storage. The development of thermostable NDV vaccines such as I-2 NDV strain that aims at maintaining vaccine viability has been successful in maintaining vaccine viability in many middle-income countries (Grimes 2002).

NDV strains belong to single serotype and are divided into 2 classes. Class I viruses were mainly isolated from waterfowl, shore birds, and captive wild birds. Viruses belonging to class II are circulating in both wild birds and domestic poultry, and are subdivided into 16 genotypes (Miller, Decanini et al. 2010; Diel, da Silva et al. 2012). Even though all NDV strains are contained in the same serotype, antigenic and genetic diversity

is evident between NDV strains having different genotypes. There is increasing reports on classical commercial NDV vaccines failure in the protection against virulent forms of NDV (Miller, Afonso et al. 2013). For example, in Lebanon, most of the NDV commercial vaccines like La Sota and Clone 30, belong to genotype II and are not offering acceptable protection against the predominant v-NDV (Murtada et al. 2016). Moreover, researchers from the USDA reported the failure of classical vaccines in significantly lowering viral shedding and replication upon a controlled v-NDV challenge (Miller, Afonso et al. 2013). These failures were attributed to the antigenic difference between vaccinal strains and circulating field challenges, enhancing the escape and evolution of v-NDV strains and selecting for pathogenic variants (Afonso and Miller 2013). Miller, King et al. (2007) conducted an experiment aimed at comparing the protection of oil-emulsion inactivated vaccines prepared from five NDV strains belonging to five different genotypes. Four-week-old, SPF White Leghorn chickens were separated into 6 groups of 16 birds each. Groups were vaccinated subcutaneously with 0.5 ml after being acclimatized to the pens for 2 days. The control group received an allantoic fluid subcutaneous injection. CA02 group received CA02 vaccine belonging to Class II/genotype V, B1 group received the B1 genotype II vaccine, Ulster group received the Ulster class II/genotype I vaccine, Pigeon 84 group received the Pigeon 84 Class II/genotype VI vaccine, and Ak 196 group received the AK 196 Class I vaccine. Twenty-one days post-vaccination, all the groups were challenged with 105.7 EID₅₀ of CA02 v-NDV strain. In addition to standard observation of morbidity and mortality, assessment of viral shedding was conducted. Results showed that vaccinating with a strain homologous to the challenge virus induced high HI titers, and significantly reduced viral shedding, when compared to heterologous vaccines. The authors concluded

that the use of genotype-matched homologous vaccines not only protects against NDV clinical infection, but also significantly reduces viral shedding and dissemination (Miller, King et al. 2007). The study stressed on the importance of autogenous vaccines that are tailored vaccines produced from herd specific (homologous) antigens. In US, autogenous vaccines are licensed products produced in licensed facilities based on an approved outline of production. Good diagnostic laboratories and the choice of immunogenic adjuvants are important in ensuring the effectiveness of these products. Autogenous vaccines are used if commercial licensed products for a certain antigen are unavailable, or when commercially licensed products fail in providing adequate protection. In developed countries, there is a restriction on the development and use of autogenous vaccines for list “A” animal diseases like NDV, FMD and AI. However, in developing countries of middle and low income the use of vaccines aiming at inducing protection against animal list “A” diseases is common. For example, in Mexico, the use of autogenous NDV vaccines has been successful tool to protect birds against predominant v-NDV genotypes, and reduce viral shedding and replication (Afonso and Miller 2013). Globally, there is a rising interest in the use of genotype-matched (Autogenous, Chimeric, or Vector) vaccines for replacing the failing classical NDV vaccines in reducing viral shedding and spread, and in offering better protection against virulent forms of NDV (Hu et al. 2011).

J. Vaccine Evaluation

Cell mediated immunity (CMI) and humoral immunity play a role in the protection

and clearance of NDV infection (Reynolds and Maraqa 2000). The mean death time of v-NDV strains that induce systemic infection in birds is 2-6 days making the contribution of CMI negligible towards providing protection against v-NDV. Protection against v-NDV outbreak requires the presence of preexisting antibodies prior to infection. Studies showed that antibodies against NDV surface proteins, the hemagglutinin (HN), and the Fusion (F) protein, are capable of neutralizing NDV upon infection (Boursnell, Green et al. 1990; Taylor, Edbauer et al. 1990). On the other hand, infection of chickens with lentogenic NDV viruses results in local, humoral and CMI.

In addition to the standard observation of performance, morbidity and mortality, serological tests like HI, ELISA, WB, and viral shedding tests are critical for NDV vaccine evaluation (Alexander 1988; Ahmad and Sharma 1992; Park, Steel et al. 2006). Licensed commercial vaccines are required to offer more than 80% protection in vaccinates upon a v-NDV controlled challenge with Herts 33 in Europe, and Texas GB in US (OIE Manual 2012).

Performance standards checked in vaccine evaluation trials are usually Feed Conversion Ratio (FCR) and Body Weight Gain (BWG) when comparing vaccinated groups to control groups in a controlled challenge trial. Serological vaccine evaluation tests include HI, ELISA and WB tests. HI test is the mostly used serological test since it is relatively cheap to perform compared to other serological tests.

Western Blotting is important for vaccine evaluation, since WB test consists of the separation of the virus into its protein components based on molecular weight. The main

advantage of WB test regarding NDV vaccine evaluation is its ability to measure the specific antibody response against the fusion protein (Reynolds and Maraqa 2000). Studies have shown that rHVT-F vaccines provide better immunity when using F & HN or HN protein alone as a candidate protein in the construction of recombinant NDV vaccines. These results indicate the importance of measuring fusion-specific antibody response of NDV vaccines. WB test for NDV viruses show that the fusion protein F0 has a molecular weight of 66 Kda and is cleaved to F1 (56 Kda) and F2 (10 Kda) by host proteases (Long, Portetelle et al. 1986).

K. *Salmonella* and Public Health

The genus *Salmonella* belongs to the Enterobacteriaceae having more than 2500 *Salmonella* serovars that have been identified and associated with different infectious syndromes. The variation in Salmonellae cell wall polysaccharides and flagellar proteins (O and H) is the foundation for Kauffmann-Le Minor scheme that has been used to identify more than 2610 serovars. According to public health significance, *Salmonella* serovars can be grouped into Typhoidal *Salmonella* and non-typhoidal *Salmonella*. Non-typhoidal *Salmonella* is considered as one of the most bacteria that causes foodborne diseases worldwide (WHO 2013). According to the Centers for Disease Control and Prevention (CDC) non-typhoidal *Salmonella* in US is responsible for approximately 1.2 million illnesses, more than 19,000 hospitalizations and 380 deaths (CDC 2014). In US the economic significance of Salmonellosis including medical costs and productivity loss is

estimated to be more than \$2.8 billion annually (Adhikari, Angulo et al. 2004). Worldwide, non-typhoidal *Salmonella* infections range approximately from 200 million to 1.3 billion cases with 3 million deaths (Coburn, Grassl et al. 2007). *Salmonella* paratyphoid infections cause mild to severe gastroenteritis that can be deadly especially for immunocompromised individuals, elderly and young individuals. The second group of *Salmonella* infecting humans is *Salmonella* Typhi. *S.*Typhi is a host adapted, species-specific, serovar that causes typhoid fever in humans; a major cause of morbidity with an estimated 22 million global outbreaks and 200,000 typhoid related deaths per year (Crump, Luby et al. 2004).

Recently, a global challenge of the emergence of multidrug resistance *Salmonella* serovars is continuously increasing especially for zoonotic *Salmonella* pathogens such as *S.*Typhimurium, *S.*Enteritidis (Hur, Jawale et al. 2012).

L. *Salmonella* Reporting

Most developed countries have a system of annual reporting of the frequency of recovered *Salmonella* serovars from human and other animal hosts (CDC 2015); however, most developing countries rely on sporadic reporting, based on targeted surveillances of *Salmonella* outbreaks (Barbour, Ayyash et al. 2015). The pathogenicity of the different serovars in human and different animal hosts varies significantly, allowing to branch Salmonellosis into different diseases, including Typhoid in human, caused by *S.* Typhi (Crump, Luby et al. 2004), non-typhoids in human caused by serovars other than *S.* Typhi (WHO 2015), Fowl Typhoid and Bacillary White Diarrhea disease in poultry caused by *S.*

Gallinarum and *S. Pullorum*, respectively (WHO 2012), and paratyphoid infections in human and many species of animals, caused by 100(s) of other serovars (WHO 2015).

Only ten of the developed countries present to their public, on their specialized websites, annual reports related to the susceptibility of recovered *Salmonella* serovars to important drugs used in medical and veterinary practices (CDC 2013). Unfortunately, most developing countries, including the ones with huge population size, have a paucity of these reports (Okeke, Laxminarayan et al. 2005). The physicians and veterinarians of the developed countries rely routinely on these web-available reports for a better understanding of the shift in efficiency of the available drugs against *Salmonella* organisms; however, the paucity of these reports in most developing countries deprive the medical and veterinary communities from vital directions to their chemotherapeutic practices (Barbour, Ayyash et al. 2015).

The nowadays resistance of *Salmonella* serovars to drugs is widespread across the globe, including resistance to antimicrobials that were the ‘drugs-of-choice’, since a decade (Hur, Jawale et al. 2012). Accordingly, new members of drugs, under the original generations, are synthesized and marketed, aiming to provide wide leverage of efficiency to prescribed treatments by physicians and veterinarians against Salmonellosis (CDC, 2011).

1. Salmonella Typhi

Salmonella Typhi is a species specific *Salmonella* serovar that is adapted only to humans. *S. Typhi* causes typhoid fever that is considered to be a major source of morbidity

with an estimated 21 million cases and 200,000 typhoid related deaths per year (Crump, Luby et al. 2004). A person infected with typhoid fever will have the bacteria *S. Typhi* in his bloodstream and intestine. Typhoid symptoms in patients include prolonged fever, nausea, headache, loss of appetite, constipation and sometimes diarrhea (WHO, 2013). School aged children, infants, toddlers and travelers are considered among the most susceptible to typhoid infections (Fraser, Paul et al. 2007). When infected with typhoid fever, antibiotics are usually the main source of treatment. However, plasmid mediated resistance to ampicillin, chloramphenicol, and cotrimoxazole have been particularly reported in southern and Southeast Asia strains (Accou-Demartin, Gaborieau et al. 2011). Consequently, fluoroquinolones (such as enrofloxacin and ciprofloxacin) are widely used in Typhoid fever treatment and are recently showing decreased susceptibility to multidrug-resistant *S. Typhi* that is becoming an endemic in India and Southeast Asia (Accou-Demartin, Gaborieau et al. 2011). Two typhoid vaccines are effective and available internationally to control endemic disease and outbreaks (WHO 2013). After recovering from the disease, a person can still be a carrier of *S. Typhi*, and both infected and carrier persons shed *S. Typhi* in their feces (CDC 2013). In US around 5,700 cases of typhoid occurs annually that are mostly acquired during travelling (CDC 2013). Sources of typhoid infection can be by eating food that is prepared by a person that is shedding *S. Typhi*, or by drinking water that is contaminated with sewage water containing the bacteria. *S. Typhi* is less frequent in industrialized countries where there is a high personal hygiene level and is mostly common in developing countries (CDC 2013).

2. *Salmonella* Enteritidis

Salmonella Enteritidis is considered one of the most commonly reported *Salmonella* serovars worldwide (CDC 2010). In the United States, SE is the second most commonly isolated *Salmonella* serovar after *Salmonella* Typhimurium accounting for 17% of Salmonellosis cases (CDC 2010). The economic significance of non-typhoidal *Salmonella* outbreaks in US is estimated to be more than \$2.8 billion annually including medical costs and productivity loss (Adhikari, Angulo et al. 2004). In Europe, *Salmonella* Enteritidis is the most frequently isolated serovar accounting for 60% of the verified Salmonellosis outbreaks. In Australia, *S. Enteritidis* outbreaks are uncommon (WHO 2010) and are linked to travelling (WHO 2009). Most *Salmonella* Enteritidis outbreaks are associated with eggs or egg containing products (Patrick 2004). Studies have shown that *Salmonella* Enteritidis had a significantly higher ability to survive in egg albumin at 42°C for 24 hours compared to other serovars such as *S. Typhimurium*, *S. Heidelberg*, *S. Kentucky* and *S. Gallinarum* (De Vylder, Raspoet et al. 2013). This mechanism of survival explains the prevalence of *Salmonella* Enteritidis in eggs since egg albumin is considered an unfavorable environment for survival having antimicrobial compounds (transferrin and lysozymes), alkaline PH and Iron deficiency (De Vylder, Raspoet et al. 2013). There are two routes for egg contamination with *Salmonella*; the horizontal transmission takes place when the egg shell is contaminated with feces containing *Salmonella* (Howard, O'Bryan et al. 2012). The second route is vertical transmission of *Salmonella* in eggs associated with infection of the reproductive organs. Recent studies showed that *Salmonella* Enteritidis have a special set of genes (81 genes) that are responsible for colonizing the oviduct. The

major group of genes include the *Salmonella* pathogenicity islands 1 and 2 that are responsible for stress responses, cell wall, and lipopolysaccharide structure, and the region-of-difference genomic islands 9, 21, and 40 (Raspoet, Appia-Ayme et al. 2014).

3. *Salmonella* Typhimurium

In US *Salmonella* Typhimurium is the most isolated serovar in humans (CDC 2010). Cattle, Swine and Poultry were found to be potential reservoirs for *Salmonella* Typhimurium (FDA, 2010; Kaldhone, Nayak et al. 2008; Rayamajhi, Kang et al. 2008). *Salmonella* Typhimurium is not only associated with gastroenteritis, but also with an alarming multidrug-resistance (MDR) (Rayamajhi, Kang et al. 2008). *S.* Typhimurium DT104 is the most common MDR profile among veterinary isolates in Europe, North America and Asia that is resistant to ampicillin, Chloramphenicol, Streptomycin, Sulfamethoxazole and Tetracycline (Chiu, Su et al. 2006; Futagawa-Saito, Hiratsuka et al. 2008; Graziani, Busani et al. 2008; Hur, Jawale et al. 2012). *S.* Typhimurium DT104 was the first reported high-level fluoroquinolone resistant *Salmonella* that was reported in Germany (Heisig 1993).

4. *Salmonella* Kentucky

Multidrug resistant *S.* Kentucky ST198 is becoming a global threat. *S.* Kentucky ST198 was found to be resistant to ciprofloxacin and was mostly detected in Egypt before 2005, and is rapidly spreading in Africa and the Middle East (Le Hello, Hendriksen et al.

2011). First it was thought that *S. Kentucky* ST198 only affects poultry and that poultry is the only reservoir. However, recent studies showed that *S. Kentucky* ST198 was found in different animals and foods including contaminated spices in France and US, turkey flocks in Germany and Poland, and wild animals (Le Hello, Hendriksen et al. 2011). *S. Kentucky* ST198 is becoming a great threat due to its growing antibiotic resistance including ciprofloxacin, and its expanding reservoirs in livestock.

5. *Salmonella* Anatum

Salmonella Anatum is known to be a heat resistant serovar (Moats, Dabbah et al. 1971). In a study conducted on 18 different poultry, cattle and swine farms across five states in US, it was found that the most isolated *Salmonella* serovars came from swine farms and the most isolated serovar was *S. Anatum* (Rodriguez, Pangloli et al. 2006).

M. *Salmonella* and Animal Health

In animals, although *Salmonella* can multiply in small intestine, disease is not an inevitable consequence. In poultry and pigs most non-typhoidal *Salmonella* infections are asymptomatic. However, in young poultry non-typhoidal *Salmonella* infection can lead to high mortality. *Salmonella* Gallinarum along with *Salmonella* Pullorum continues to be the major *Salmonella* infections in poultry. *S. Gallinarum* and *S. Pullorum* are highly host adapted *Salmonella* serovars that are responsible for Fowl Typhoid (*S. Gallinarum*) and Pullorum Disease (*S. Pullorum*) that causes a significant morbidity and mortality in poultry.

1. *Salmonella Gallinarum*

Salmonella Gallinarum is host specific *Salmonella* that is adapted to avian species (Eswarappa, Janice et al. 2009). *Salmonella Gallinarum* causes fowl typhoid disease in chickens, turkeys and other avian species that is mostly observed in mature stock and is associated with high morbidity and mortality according to the virulence of the strains. *S. Gallinarum* has been eradicated from commercial poultry in many developed countries including Western Europe, United States of America (USA), Canada, Australia and Japan (OIE Manual 1996). Clinical signs observed are anorexia, diarrhea, dehydration, weakness and death. Red mites are seen to be involved in *S. Gallinarum* transmission and persistence in poultry farms. *Salmonella Gallinarum* is said to be derived from *Salmonella Enteritidis* via a gene deletion event (Thomson, Clayton et al. 2008). However, *S. Gallinarum* is only adapted to avian species and is considered of minimal zoonotic importance (Shivaprasad 2000).

N. Antibiotics

Antibiotics are natural or synthesized molecules that kill or inhibit the growth of bacteria and fungi. Antibiotics that kill bacteria are bactericidal, and usually decrease bacterial counts like penicillin. On the other hand, antibiotics that stop bacterial growth are referred to as bacteriostatic such as chloramphenicol (Walsh 2003) (Figure 4). Some antibiotics can exhibit bacteriostatic or bactericidal effect based on the circumstances

present. Antibiotics originating from natural microorganisms have been mostly used to treat human infections with infectious diseases in the past 60 years. While man-made antimicrobial drugs in clinical use are sulfa drugs, quinolones, and oxazolidinone. Antimicrobial drugs can be antibacterial or antifungal. This review will focus mainly on antibacterial antibiotics.

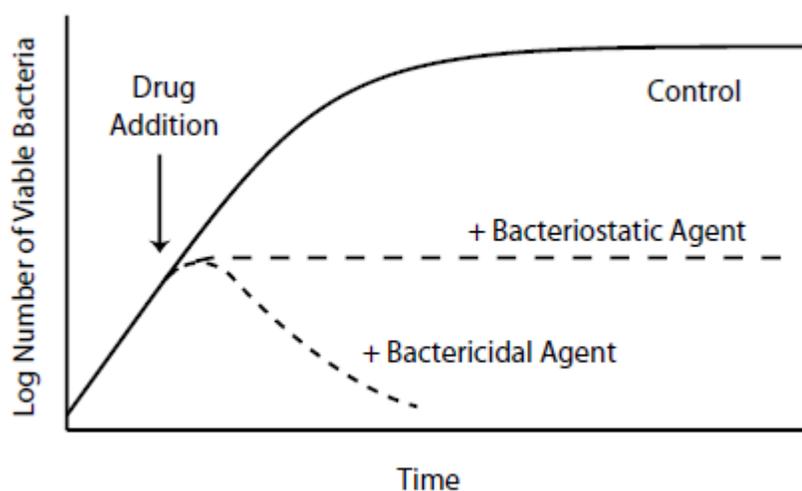


Figure 4. Bactericidal and bacteriostatic effects of antibiotics on logarithmically growing bacteria (Scholar and Pratt 2000).

O. Mechanism of Action

Mechanism of action is the most widely used classification system for antibiotics. Another useful classification of antibiotics is by structure like B-Lactams that have B-Lactam ring (Walsh 2003). Antibiotics are classified based on their mechanism of action

into; cell wall synthesis inhibitors, protein synthesis inhibitors, DNA and RNA synthesis inhibitors, and folate synthesis inhibitors.

Figure 5 summarizes the different mechanisms of action of antibiotics in gram positive and gram negative bacteria and Table 8 lists some antibiotics under each class.

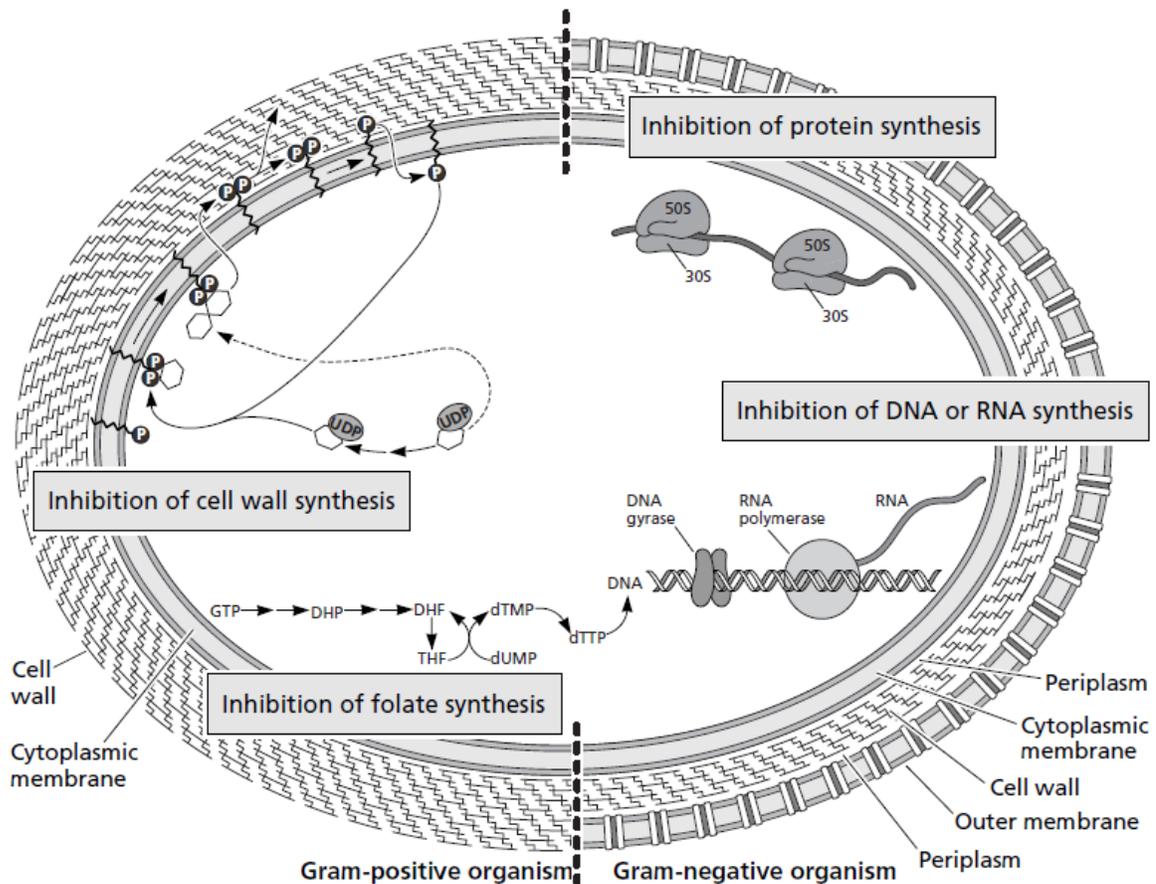


Figure 5. Different mechanisms of action of antibiotics in gram positive and gram negative bacteria (Scholar and Pratt 2000).

1. Cell wall synthesis inhibitors

Gram staining divides bacteria into two groups, gram-positive bacteria, like Staphylococci, Streptococci and enterococci, and gram-negative bacteria, such as

Escherichia coli, *Salmonella*, *Pseudomonas* and *Yersinia*. Both gram-positive and gram-negative bacteria have a peptidoglycan (PG) layer in their cell wall structure. However, the main difference between the two groups is the generally thicker PG layer in gram-positive bacteria that appears as Crystal-violet color upon gram-staining. Cell wall synthesis inhibitors act on constraining inhibiting enzymes or confiscating substances involved in PG assembly (Walsh 2003).

2. Protein synthesis inhibitors

The first step in protein synthesis is the transcription of DNA into RNA by RNA polymerase. The RNA strand is then translated into proteins by the action of ribosomes. Ribosome organelles and the host cytoplasmic accessory factors, are involved in mRNA translation that occurs over three sequential phases (initiation, elongation and termination) (Garrett 2000). Ribosomes are composed of two-subunit nucleoprotein particles, the 30S and 50S, that are involved in organizing the initiation phase that consist of the formation of a complex between mRNA transcript, tRNA, initiation factors, and a free 30S subunit (Nissen, Hansen et al. 2000). Drugs that inhibit protein synthesis are considered to be one of the broadest classes of antibiotics, and are divided into two subclasses: the 50S inhibitors and 30S inhibitors as seen in Table 8.

3. DNA and RNA synthesis inhibitors

Topoisomerase enzymes, such as DNA gyrase, are responsible for DNA

replication and repair. These enzymes catalyze standard breakage and rejoining reactions that are required for DNA synthesis, mRNA transcription, and cell division (Espeli and Mariani 2004; Drlica and Snyder 1978). DNA synthesis inhibitors class of antibiotics work on inhibiting these reactions.

Rifamycins is the only RNA synthesis inhibitor antibiotic in clinical use. This drug inhibits RNA polymerase and results in blocking bacterial transcription. Rifamycins is commonly used in combination regimens aimed at killing the slow-growing pathogen *Mycobacterium tuberculosis* (Kohanski, DePristo et al. 2010).

4. Folate synthesis inhibitors

Sulfa drugs, folic acid inhibitors, are considered to be the longest synthetic chemicals in use as effective antimicrobials. Sulfamethoxazole, the current generation of sulfa drugs, is commonly used with trimethoprim to add synergy to the combination. Sulfamethoxazole- Trimethoprim (SXT) is mainly used to treat human urinary tract infection (Scholar and Pratt 2000). Eukaryotic cells are known to acquire folate from dietary sources and transport it into the cell. However, bacterial prokaryotic cells have to make their own folate acid that is essential for DNA and RNA synthesis. Sulfamethoxazole and trimethoprim drugs acts on blocking different enzymes needed for the biosynthesis of folate coenzyme. Both drugs are known to have bacteriostatic effect alone and bactericidal synergistic effect when combined together (Walsh 2003).

Table 8. Antibiotic grouping by mechanism

| | |
|-----------------------------------|--|
| Cell wall synthesis inhibitors | Penicillins Cephalosporins Vancomycin Beta-lactamase Inhibitors Carbapenems Aztreonam Polymycin Bacitracin |
| Protein synthesis inhibitors | <u>Inhibit 30s Subunit</u> Aminoglycosides (gentamicin) Tetracyclines <u>Inhibit 50s Subunit</u> Macrolides Chloramphenicol Clindamycin Linezolid Streptogramins |
| Nucleic acid synthesis inhibitors | Fluoroquinolones Metronidazole |
| Folate synthesis inhibitors | Sulfonamides Trimethoprim |

P. Antibiotic Resistance: History, Mechanisms and Causes

Natural antibiotics have been produced by bacteria and fungi as a defense mechanism against their neighbors. Since then, evolutionary pressure occurred in the bacteria under attack to develop new survival mechanisms or else perish. Antibiotics have been in use since more than 80 years for the treatment of human infectious diseases. In the

antibiotic era, pathogenic bacteria have developed significant resistance to one class of AB after another as shown in Table 9 (Amyes 2001; Levy 1998).

The second requirement for bacterial resistance, other than evolutionary pressure, is the acquired resistance that mainly arises from the transfer of resistant genes from one resistant bacteria to another by plasmids (conjugation or transformation), transposons (conjugation), integrons and bacteriophages (transduction) (Giedraitienė, Vitkauskienė et al. 2011). After the bacteria acquire resistance genes, it utilizes several resistance mechanisms: antibiotic inactivation, target modification, altered permeability, and "bypass" metabolic pathway (Giedraitienė, Vitkauskienė et al. 2011) as shown in Figure 5. Pathogenic bacteria under pressure can acquire resistance and self-protecting mechanisms from antibiotic-producing bacteria. Thus, the main advantage of synthetic antibacterial is that they do not induce such resistant mechanisms in target bacteria. However, mutation of enzymes is the main resistance mechanism against synthetic AB like SXT (Walsh 2003). The race between AB resistance and AB research and development triggered medicinal chemists to search for new generations in AB classes, like the four generations of cephalosporins, each having a chemical modification aimed at combating resistance, or extending the spectrum (Walsh 2003).

World Health Organization summarized the main causes for increasing AB resistance in an infographic (Figure 6) that shows that AB use in livestock is among the major causes.

Table 9. Evolution of resistance to antibiotics (Palumbi 2001).

| Antibiotic | Year deployed | Resistance observed |
|-----------------|---------------|---------------------|
| Sulfonamides | 1930s | 1940s |
| Penicillin | 1943 | 1946 |
| Streptomycin | 1943 | 1959 |
| Chloramphenicol | 1947 | 1959 |
| Tetracycline | 1948 | 1953 |
| Erythromycin | 1952 | 1988 |
| Vancomycin | 1956 | 1988 |
| Methicillin | 1960 | 1961 |
| Ampicillin | 1961 | 1973 |
| Cephalosporins | 1960s | late 1960s |

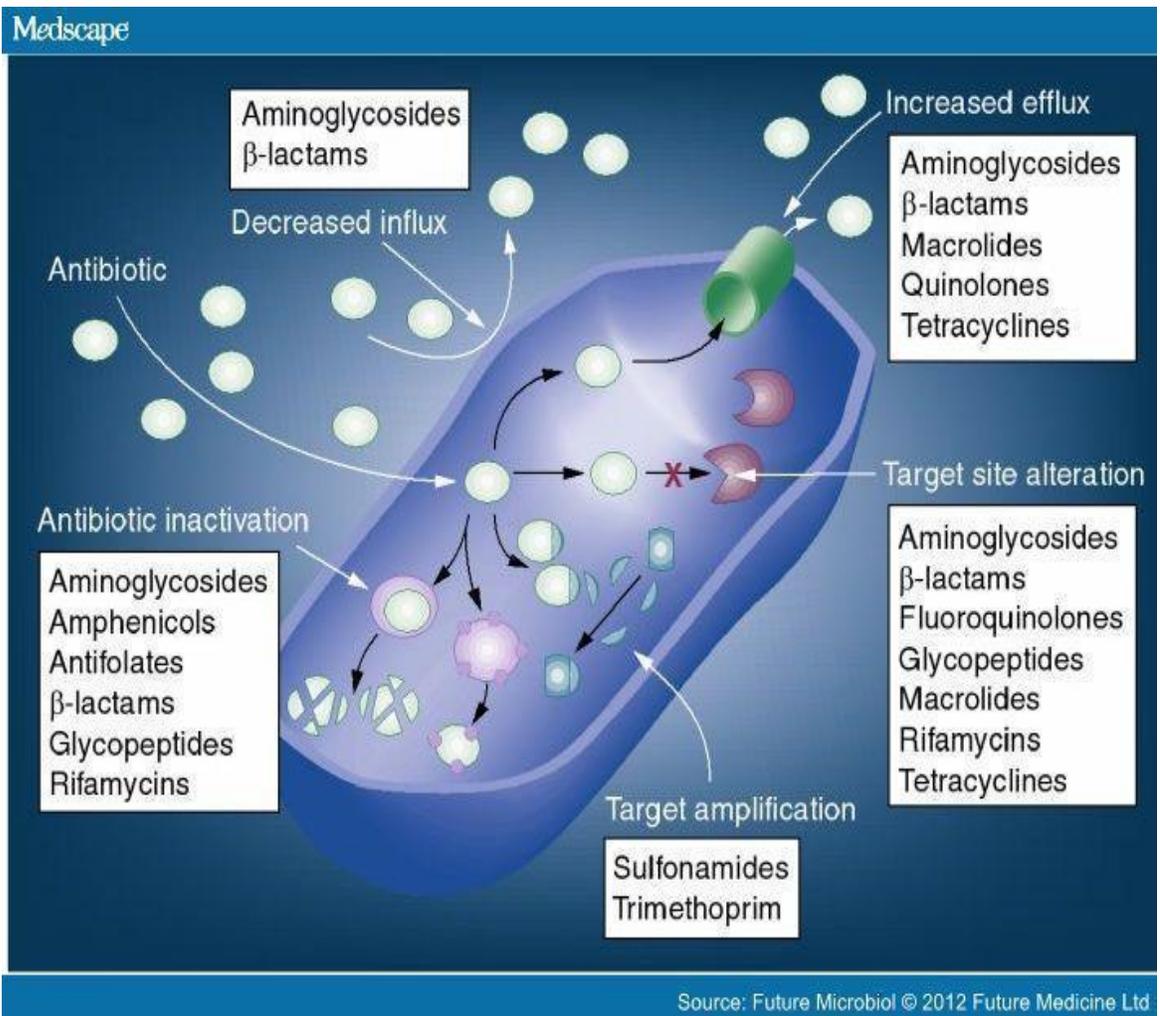


Figure 6. Acquired bacterial resistance mechanisms against antibiotics.

CAUSES OF ANTIBIOTIC RESISTANCE



Antibiotic resistance happens when bacteria change and become resistant to the antibiotics used to treat the infections they cause.



Over-prescribing of antibiotics



Patients not finishing their treatment



Over-use of antibiotics in livestock and fish farming



Poor infection control in hospitals and clinics



Lack of hygiene and poor sanitation



Lack of new antibiotics being developed

www.who.int/drugresistance

#AntibioticResistance



Figure 7. Causes behind the increase in antibiotic-resistant bacteria (WHO).

Q. Antibiotic Use in Livestock

Antibiotics are commonly used in livestock production for treatment, prophylaxis (prevention), and growth promotion. Globally, non-therapeutic use of antibiotics as growth promoters accounts for the largest amount of Antibiotics (AB) used in livestock (Dibner and Richards 2005). Antibiotics are administered to livestock animals in drinking water, feed, and as injectables (Wages 2003). There is a rising public health concern regarding the use of antibiotics in livestock production. One of the concerns is the fear of antibiotic residues in livestock products. In developed countries, certified veterinarians are responsible prescribing antibiotics for on farm use, working on restricting the use of antibiotics for the treatment of secondary infections, and following strict guidelines regarding antibiotic withdrawal periods. Moreover, in these countries poultry products are routinely tested for any antibiotic residues. For example, USDA inspectors are present in every slaughterhouse and frequently analyze representative samples for antibiotic residues and bacterial loads. The second and perhaps the major public health concern regarding the use of antibiotics in livestock, is the fear of antibiotic-resistant foodborne infectious diseases like *Salmonella* and *Campylobacter* in poultry, and MRSA in pigs (Antunes, Réu et al. 2003; Smith, Male et al. 2009). This concern pushed many countries to stop the use of antibiotic in livestock production like Sweden (Pruden 2013), or to strictly monitor the use of antibiotics through promoting the judicious use of AB in livestock. Judicious use principles for poultry includes proper diagnosis, knowledge of antibiotic properties, dosage, spectrum, interactions, and initiation of treatment (Wages 2003). For example, two of the most used *in vitro* tests that assess the susceptibility of isolated pathogens to antibiotics

used in livestock are, the Minimum Inhibitory Concentration test (MIC), and disk diffusion test (Wages 2003). These susceptibility tests are recommended as part of judicious use principles.

Even though antibiotic use in livestock production has been regulated, monitored, and managed, consumers are pushing towards “Zero” use of antibiotics in livestock production. In 2015, one of the largest food chains in the world, McDonald’s, announced that within 2 years, it would only buy chickens raised free of antibiotics that are important for humans. By this announcement, McDonald's will be adopting the more strict WHO regulations that only permit the use of ionophores, antibiotic that are not important to humans, in livestock production. However, FDA rules still permit the use of some antibiotics in poultry like bacitracin that is commonly used in US poultry industry to prevent and control necrotic enteritis.

R. Antibiotic Growth Promoters Mode of Action and Alternatives

According to the Poultry Science Association (PSA), the use of subtherapeutic doses of antibiotics in poultry production aims at reducing subclinical infection, decreasing production of microbial products that have toxic effects and depressing microbial competition in birds allowing for enhanced absorption of nutrients. A study done in UC Davis concluded that feeding antibiotics may permit growth by preventing immunologic

stress and associated metabolic changes (Roura, Homedes et al. 1992). In 2007, a new theory regarding the mode of action of AB in poultry was presented. The theory argues that antibiotics have a non-antibiotic, anti-inflammatory effect that reduces wasted energy and redirect it for production (Niewold 2007). According to the author, Professor Niewold, this theory explains why AGPs results are reproducible, unlike antibiotic alternatives aimed at microflora management.

It is lucid that there is a rising public health concerns regarding antibiotic-resistant “superbugs” which is triggering consumers to lobby for “Zero” use of antibiotics in livestock production. As a result, governments are moving towards monitoring, restricting, and even stopping the use of AB in livestock production. All these facts give importance to the direction of international efforts towards directing research funds for understanding the MOA, exploring, and assessing AGP alternatives. Alternative AGPs includes: probiotics, prebiotics, enzymes, acidifiers, complex minerals, vitamin derivatives, amino acid derivatives, antibodies, short and medium chain fatty acids, plant extracts and others (Sifri, 2016).

S. Essential Oils Safety and Efficacy

Globally, about 300 essential oils are traded at an estimated worth of \$1 billion in 2013 (Tisserand and Young 2013). Food flavoring and pharmaceutical industries are among the largest consumers of EO, representing 20% of EO consumption each. Moreover, the

application of EOs is growing in farm animals due to their remarkable potential of killing resistant bacteria, but also because they can reverse resistance to conventional antibiotics.

Essential oils safety is of major importance to everyone due to the broad use of EO in humans, food, and animals. Ames test, aimed at assessing the mutagenicity of chemicals like EO, is a commonly used EO safety test. Another test in use is the Draize test that is aimed at finding out if specific EO induce skin reactions and irritation (Tisserand and Young 2013).

Consumer pressure regarding the increase of antibiotic-resistant pathogens triggered the development, assessment, and use of non-AGP alternatives such as essential oils. Aromatic plants and their extracts have received great attention as growth and health promoters in poultry. Essential oils use in poultry was proven to enhance the production of digestive secretions, stimulate blood circulation, have antioxidant properties, reduce the level of pathogenic bacteria, and enhance immunity (Brenes and Roura 2010).

Moreover, the use of natural therapeutic antimicrobial plant products, like Essential oils, has become a common practice in poultry production. The antibacterial activity of plant essential oils was first reported in 1881 (Boyle 1955). The mechanism of action of EO cannot be confirmed, since EO are composed of different natural chemical molecules acting in synergism and displaying various antimicrobial activities (Carson, Mee et al. 2002). For this reason, phenolic, volatile and terpene compounds are of great importance when exploring EO. For example, terpenes are known to disrupt and penetrate the lipid structure of bacterial cell wall resulting in changes in cell function, denaturation of

proteins, release of ion molecules, causing starvation. This destruction in cell membrane leads to cytoplasmic leakage, cell lysis, and eventually cell death (Fisher and Phillips 2008).

Eucalyptus is a commonly used antiseptic in folk medicine for relieving cold symptoms like sore throat, cough, and other medical uses. The main volatile components of eucalyptus oil were tested against *Salmonella* by determining the Minimum Inhibitory Concentration (MIC) (% vol/vol). The piperitone component of eucalyptus had 33% MIC, while the terpinen-4-ol had MIC of 0.17%, and was proven to be effective against gram-negative bacteria (Delaquis, Stanich et al. 2002).

Peppermint is another antibacterial commonly used as alternative medicine and well known for its antibacterial, antifungal, anti-inflammatory, and decongestant properties. Essential oils of peppermint were tested against biofilm of *S. Enteritidis* S64 on stainless steel surfaces and showed powerful anti-biofilm effect (Valeriano, De Oliveira et al. 2012). Another study aimed at assessing the antimicrobial activity of peppermint showed that its EO exhibited an inhibitory effect on *Salmonella* and other gram-negative bacteria that were tested (*S. Typhi*, *S. Paratyphi*, *S. Dysenteriae*, *P. Mirabilis*, *P. Vulgaris*, *E. coli*, *K. Pneumoniae*, *P. Aeruginosa*, *Y. Enterocolitica* and *E. Aerogenes*) (Saeed, Naim et al. 2006).

CHAPTER III

MATERIALS AND METHODS

A. Evaluation of an autogenous vaccine and/or a blend of essential oils in the control of v-NDV in broilers

1. Preparation of the v-NDV challenge

The NDV virus was isolated from an outbreak that took place in a broiler farm at Agricultural Research and Education Center, Bekaa, Lebanon. The virus was confirmed as v-NDV using RT-PCR, and the fusion protein was further sequenced with a Genbank accession number KC425723.1. Sequencing revealed the presence of multi-basic amino acids at the cleavage site representing a motif that is highly associated with virulence (Figure 8) (Alexander 2000).

| |
|---|
| DGRPLAAAGIVVTGDKAVNVYTSSQTGSIIVKLLPNMPKDKEACAKAPLEAYNRTLTT LLTPLGDSIRKIQGSVATSGGR ₁₁₂ RQKR *F ₁₁₇ |
|---|

Figure 8. Isolated v-NDV Amino Acid sequence of the fusion protein fragment containing the cleavage site.

2. Phylogenetic analysis

The fusion protein fragment of the isolated v-NDV, which includes the cleavage site nucleotide sequence of virus, was run through BLAST NCBI to detect the genetically similar v-NDV viruses available at Genbank. Twenty-three similar isolates of percent fusion protein fragment similarity ranging from (100-92%), and representing different

temporal, geographical, and host parameters were included in the phylogenetic study. Two additional isolates representing the mostly used NDV commercial vaccine strains namely La Sota and Clone 30 were also included. FASTA format of all NDV isolates was collected and aligned using muscle alignment method on MEGA 7.0 program. The alignment file was exported, and then phylogenetic tree was constructed on MEGA 7.0 program using UPGMA method. Newcastle disease genotypes and sub lineages were recorded from Aldous, Mynn et al. (2003).

3. Standardization of the viral propagation technique in chicken

embryonated eggs

Serial 1:2 dilutions of v-NDV viral stock with 500 and 250 v-NDV particles/inoculum were performed. A volume of 100 µl of each dilution was inoculated in triplicate in 11-days old embryonated eggs. Samples were pooled and HA test was performed. The pool was preserved in transport medium at -80°C.

- The v-NDV stored in transport medium inoculated in 9 day old embryo eggs at a rate of 100µl of 250 particles of v-NDV /egg.
- Eggs were incubated in an incubator for 3 days at a temperature of 37.5°C And 70 % Relative humidity.
- Embryos were sacrificed by putting them in 4°C for a minimum of 3 hours and the allantoic fluid was harvested.
- HA test was performed on pooled allantoic fluid

4. Autogenous vaccine preparation

a- Inactivation and preparation of aqueous solution

- The viral load was taken from the viral challenge as prepared by the previous paragraph.
- Viral inactivated in its aqueous phase was done by adding 0.3 % formalin. The aqueous solution was stirred overnight at room temperature.
- The aqueous solution containing the inactivated virus was inoculated in embryonated eggs at an amount of 100µl/egg as a quality control measure of viral inactivation.
- HA Test was performed to make sure that the HA titer is zero indicating that viral inactivation was successful.
- A 250 ml of inactivated virus were mixed with 250 µl of Tween 80 for 3 minutes.

b- Oil phase preparation

250 ml of mineral oil were added to 11 ml of Arlacel C (emulsifier). The solution was mixed using the Pro250 homogenizer (Pro Scientific Inc., Monroe, CT, USA) and sterilized by autoclaving at 121°C for 15 minutes then it was left to cool.

c- Mixing aqueous viral suspension into an Oil Phase

- The Aqueous solution containing the inactivated v-NDV was added slowly onto the Adjuvant solution accompanied by slow and continuous mixing via a

mixer with a shaft and sharp edges propeller. This was followed by pouring the emulsion in a colloidal mill (Greerco, Ohio 45401 USA) at a stator gap setting of 0.002”

- The stability of the emulsion was watched overnight and the emulsion solution was stored in fridge until use.
- Before use, the emulsion solution was put at room temperature to lower its viscosity.
-

5. Animals

Eighty day-old broiler chicks belonging to the Ross 308 commercial strain were used in this study. Birds were given feed and water ad libitum and as per the National Research Council (NRC) 1993 nutritional requirements.

6. Experimental design

Day-old chicks were subdivided into 8 groups of 10 birds each as detailed in the below Table 10.

Table 10. The v-NDV controlled challenge experimental design.

| Group | Essential Oil Treatment ¹ | v-NDV challenge ² | Cocci Challenge ³ | NDV Vaccination ⁴ |
|-------|---|---------------------------------|---------------------------------|---------------------------------|
| 1 | + | + | + | + |
| 2 | M+ ⁵ | + | + | + |
| 3 | - | + | + | + |
| 4 | AC | + | + | - |
| 5 | - | + | + | - |
| 6 | AC | - | - | + |
| 7 | + | - | - | + |
| 8 | M+ | - | - | + |

¹Essential oils made from a blend of eucalyptus and peppermint

²v-NDV challenge was administered intramuscularly at 28 d of age, by v-NDV of genotype VI containing 1.2×10^8 TCID₅₀/0.5 ml/bird. The challenge was administered to two birds that were marked in every challenged group.

³Cocci challenge was administered to mimic field conditions. The challenge was at 21 d of age, by equivalent number of Sporulated Oocyst of 8 *Eimeria* spp (1×10^6 sporulated oocysts/bird). The 8 *Eimeria* spp, were *E. necatrix*, *E. acervulina*, *E. mivati*, *E. tenella*, *E. brunetti*, *E. maxima*, *E. hagani* and *E. praecox*.

⁴The Autogenous vaccine is a water-in-oil emulsion, in which the aqueous phase contained 128 HA units of formalized v-NDV of genotype VI. Vaccinated birds received 2 subcutaneous injections of 0.5 ml each (priming at 1d) and (boosting at 14d).

⁵Modified blend of essential oils made up of a blend of eucalyptus, peppermint and other unspecified substances.

⁶AC = Anticoccidial drugs commercial combination of narasin and nicarbazin

7. Observed parameters

Performance and pathologic observations were recorded namely, FCR and BWG

(d 1-35), HI titers (d 1, 14, 28 & 34), and fusion protein specific antibodies by WB.

8. Hemagglutination Inhibition (HI) Test

a- Preparation of Saline

- 8.5 g NaCl were dissolved in 1000 ml distilled water

b- Preparation of 25 % RBC suspension

- Withdraw blood from commercial chicken free of diseases using anticoagulant-coated tubes
- Centrifuge blood and spin for about ten minutes at 2000 rpm
- Discard the supernatant (Plasma + buffy coat) and wash the RBC three times with saline
- Saline was added, finally, to the RBC resulting in a 25 % RBC stock suspension
- Prepare 1 % RBC working suspension from the 25 % stock

c- Antigens preparation

- Prepare 4 Hemagglutinating (HA) units of the virus by performing HA test (*Fenner et al. 1987*) using serial dilutions of the antigen stock with a dilution factor of $\frac{1}{2}$, and with RBC suspension of 1 %.
- N.B To ensure the specificity of the HI test, the antigen stock should be prepared from the isolated local field strains

d- HI Test Procedure

- Deliver 50 μ l of saline per well of a microtiter plate (96 wells, U bottoms) using 12 tip multichannel pipette

- Add 50 μ l of tested chicken serum per well in column 1
- Dilute serum from column 1 to column 12 with a carried volume of 50 μ l from one well to the other using the same multichannel pipette
- Discard last 50 μ l (the dilution factor is 1:2)
- Add 50 μ l of the 4 HA antigen for Virus in each well with gentle rotation of plates
- Incubate at 37°C for about 45 minutes.
- Add 50 μ l of 1 % RBC suspension to each well and rotate plates.
- Leave plates at room temperature for 30 minutes
- Antibody titer of each serum sample was read
- **HI titer** is defined as the highest dilution of the serum that had enough antibodies to inhibit the viral-RBC agglutination

9. Viral purification

- Centrifuge 300 ml of allantoic fluid at 5,000 xg for 1 hr.
- Discard the pellet and collect the supernatant.
- Subject the supernatant to ultracentrifugation at 35,000 xg for 1 hr at 4°C.
- Discard the supernatant and suspend the pellet containing the virus in a small volume (3 ml) of NTE and mix well for 24 hrs in ice bath for homogeneity.
- Overlay the 3 ml homogenous virus over 18 ml of a higher concentration of sucrose (33% w/v of tube).
- Ultracentrifuge at 95,000 xg for 80 minutes (27,500 rpm).

- Discard the supernatant and collect the pellet of pure virus (to solubilize and charge for SDS and Western Immunoblotting).
- Virus is suspended in 300 μ l of NTE buffer mixed for 24 hrs in ice bath
- Determine HA of the virus to dilute on laurel sulfate amount to solubilize and charge before SDS PAGE and Western.
- NTE Buffer: 0.15 M NaCl, 0.001M EDTA, 0.05M Tris with pH 7.4.

10. Protein assay

- Six dilutions of Bovine Serum Albumin in saline were made for the preparation of a standard curve. The dilutions were 0.5, 1, 1.5, 2, 2.5 and 3 mg/ml.
- Volume of 5 μ l of standards and samples were pipetted into a clean, dry microtiter plate containing 25 μ l of reagent A and 20 μ l of reagent B in every well (Bio-Rad Lab. 2000 Alfred Nobel Dr., Hercules, CA, USA).
- After 15 minutes, absorbance was read at wavelength of 750 nm using a spectrophotometer
- The protein assay revealed that the purified v-NDV virus has a concentration of 3.4 μ g/ μ l.

11. Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

All SDS-PAGE and WB reagents and equipment mentioned below were purchased from Bio-Rad (Bio-Rad Lab. 200 Alfred Nobel Dr., Hercules, CA, USA) unless otherwise indicated.

a- Preparation of 12% separating gel (for 27 x 8.5cm gels)

The separating gel was prepared by adding 4.8 ml of Acrylamide, 3 ml of 1.5 M Tris-HCl, 4.2 ml of DDW(double distilled water), 210 μ l APS(ammonium persulfate), and 30 μ l of TEMED.

b- Preparation of 4% stacking gel (for 27 x 8.5cm gels)

The stacking gel was prepared by adding 750 μ l Acrylamide, 1.25 ml of 0.5 M Tris-HCl, 3.5 ml of DDW, 140 μ l APS(ammonium persulfate), and 20 μ l of TEMED.

c- Gel Electrophoresis

A Mini-PROTEAN Tetra transfer system was assembled according to the procedure described by the Bio-Rad manual. This electrophoresis cell is designed to run 4 gels simultaneously, however the below methods will only include running 2 gels simultaneously. The 12% separating gel was added using a pipette in between the assembled glass plate sandwiches. Distilled water was added on top of the separating gel in order to level the gel that was left for 20 minutes to polymerize. After drying the area above the separating gel by a filter paper, the stacking gel was added while placing the 10 wells comb in the gel sandwich. The stacking gel was allowed to polymerize for 20 minutes.

After removal of the comb, the electrophoretic cell was filled with running buffer. After following the NDV viral purification procedure, and conducting a protein assay that yielded a 3.4 µg/µl of purified v-NDV concentration, 25 µl of reduced sample buffer were added to each well insuring that each well contains at least 7 µg/µl of the purified v-NDV virus. The sample buffer for two gels was prepared by adding 48 µl of the purified NDV virus to 144 µl of sterile saline, 383 µl of Laemmli buffer, and 23 µl of β-mercaptoethanol at a dilution rate on 1:2 antigen/reducing buffer. All the reduced sample buffer mixed reagent were heated at 95°C for 5 minutes aiming at denaturing the virus into its protein components. An amount of 8 µl of molecular ladder (Precision Plus Protein Kaleidoscope, Bio-Rad, cat # 161-0375) was added in the first lane of each of the 2 prepared stacking gels. The gels were run at a constant electric current of 60 mA for 45 minutes. The gels was stained with Coomassie blue for 30 minutes with continuous shaking and then detained with a destainer solution for 1 to 3 hours to check the protein bands of NDV and document it by taking a photograph. After using 1 gel to standardized the SDS-PAGE protocol, all other gels were directly stored, after SDS-PAGE, in transfer buffer at 4°C overnight in order to proceed with the immunoblotting procedure the day after.

12. Immunoblotting procedure

A new Trans-Blot Turbo machine manufactured by Bio-Rad was used in the blotting procedure. This new system accelerates the semi-dry blotting process that is known to be cumbersome in conventional semi-dry techniques, without sacrificing performance.

The other advantage of this new system is that it accommodates traditional semi-dry consumables such as filter paper and transfer buffer.

The SDS-PAGE gel and Nitrocellulose membrane, and blotting filters were all soaked in transfer buffer for 10 minutes. The transfer buffer was prepared by adding 3 g of tris base, 14.4 g of glycine and 1 g of SDS into 800 ml of water. A volume of 200 ml of methanol was added reaching a 1 L volume of transfer buffer. The NCM was placed on the filter paper that is placed towards the bottom (+) cassette electrode (anode). The gel was placed on top of the NCM towards the top (-) cassette electrode (cathode). Blot roller was used to remove any bubbles formed between the gel and NCM and a layer of filter paper was added. The Cassettes holding the gel in contact with NCM placed between two ion reservoir stacks were locked. Loaded cassettes were inserted into the instrument to access the power supply for protein transfer. A preprogrammed transfer protocol (Standard semi dry protocol: 25 V, 1.0 A, 30 min) was easily selected from the firmware menus using the navigation and selection bottoms. During the run transfer conditions and run progress were displayed on the LCD screen. After finishing the protein transfer from SDS-PAGE gel to NCM, the NCM was immersed in Ponceau S stain for 5 minutes with continuous shaking. The NCM was rinsed with distilled water for 2-3 minutes in order to resolve the transferred polypeptides. Lanes were numbered using a sharp pencil and were then carefully cut. The NCM lanes were immersed in TBS for 10 minutes shake. Blocking of the active site was done by immersing the NCM in 5% gelatin-TBS for 1 hour at 37°C with continuous shaking. The NCM lanes were washed twice with TTBS for 5 minutes shake per each wash.

Serum samples were diluted with 1% gelatin-TTBS to 1:250 (40 µl of every Sera added to 10 ml of 1% gelatin-TTBS). The NCM lanes were incubated in each diluted serum sample for 1 hour at 37°C allowing NDV-specific antibodies to bind. The Unbound antibodies were removed by washing the NCM lanes twice with TTBS for 5 minutes shake per wash. The NCM lanes were immersed in a sheep anti-chicken IgG (H+L) Peroxidase Conjugate (Sigma, St. Louis, MO, USA) 1:1000 diluted solution in in 1% gelatin-TTBS (32 µl of secondary antibodies added to 40 ml of 1% gelatin-TTBS) and were incubated at 37°C for half an hour shake.

The NCM lanes were washed thoroughly twice with TTBS for a 5 minutes shake per wash. The NCM lanes were further washed twice with TBS similarly. The lanes were dried over a filter paper. The NCM lanes were then immersed in DAB peroxidase Substrate solution (Sigma, St. Louis, MO, USA) using 2 tablets of DAB in 10 ml of distilled water, and were shaken at 37°C to obtain brown-colored bands. The NCM lanes containing the bands were rinsed with distilled water and dried over a filter paper. The lanes were pasted on the laboratory copybook using a UHO in order to proceed for scanning.

13. Fusion protein band intensity

Antibodies specific to v-NDV fusion protein and that formed a band on NCM lanes were quantitatively measured by reading the optical densities of the Bands formed in vaccinated birds, non-vaccinated birds, and negative control NDV ELISA Serum. The intensities were measured using Quantity One 1D Analysis Software (Bio-Rad Lab. 200

Alfred Nobel Dr., Hercules, CA, USA).

Three 5 by 10 squares of every fusion protein band were compared to NDV negative control ELISA serum. The program works on measuring the optical density with respect to the chosen area (OD/mm²) and calculates the mean optical density (OD). The fusion band intensities of vaccinated, non-vaccinated birds, and negative control ELISA serum were recorded. Areas used for measuring intensities in each band were picked randomly.

14. Statistical analysis

One way ANOVA and Tukey's test) were used to compare means of egg quality parameters using SPSS version 22 (SPSS Inc., USA). The confidence interval was 95%.

Chi-square test was used to compare the % of compliance of the farms with EU standard practices.

B. Susceptibility of *Salmonella* serovars recovered from hospitalized Saudis to commercial drugs and to a chemically and safety-characterized essential oil

1. Salmonella isolates

Six *Salmonella* Isolates were used in this experiment. *S. Enteritidis*, *S.*

Typhimurium, *S. Kentucky*, *S. Anatum*, and *S. Typhi* were isolated from 16 separately admitted Saudi patients in addition to *S. Gallinarum* serovar that was isolated from poultry flocks in Nigeria and Brazil.

2. Kirby-Bauer (Disk Diffusion) Test

The disk diffusion tests aimed at evaluating the susceptibility of *Salmonella* isolates to 23 antimicrobials and a blend of eucalyptus and peppermint essential oils.

a- Antibiotics used

The 23 different antimicrobial disks were purchased from Oxoid Ltd, Basingstoke, Hampshire, England. The disks and their potencies are listed below, according to their mode of antibacterial inhibition:

- Cell wall inhibitors

Amoxicillin/Clavulanic acid (30 mcg), Ampicillin (10 mcg), Aztreonam (30 mcg), Cefamandole (30 mcg), Cefixime (5 mcg), Cefotaxime (30 mcg), Cefoxitin (30 mcg), Ceftazidime (30 mcg), Cefuroxime Sodium (30 mcg), Cephalothin (30 mcg), Imipenem (10 mcg), Cefepime (30 mcg), and Piperacillin/Tazobactam (85 mcg).

- Protein Synthesis Inhibitors

Amikacin (30 mcg), Chloramphenicol (30 mcg), Gentamicin (10 mcg), Kanamycin (30 mcg), Tetracycline (30 mcg), and Tobramycin (10 mcg).

- Nucleic Acid Synthesis Inhibitors

Ciprofloxacin (5 mcg), Nitrofurantoin (300 mcg), and Norfloxacin (10 mcg).

- Folate Pathway-Inhibitors

Sulfamethoxazole/Trimethoprim (25 mcg).

b- Disk Diffusion Test Procedure

- A volume of 100 µl of bacterial suspension at log phase was spread onto Mueller Hinton Agar plates
- Four different antibiotics were used for each plate, and each having a duplicate plate
- Plates were incubated overnight at 37°C and the inhibition zones were measured and recorded according to Oxoid Ltd, Basingstoke, Hampshire, England charts
- As for the essential oil, a volume of 20 µl of EO was added on a blank disk to assess the efficacy of the blend against the same *Salmonella* isolates.

3. Minimum Inhibitory Concentration (MIC) Test of the essential oil blend

The chemically-characterized essential oil blend was provided by EWABO Chemikalien GmbH & Co, Wietmarschen, Germany. The essential oil was a blend of eucalyptus and peppermint (1/1, v/v). The percentages of the main active ingredients, provided by EWABO Co., were: 1,8-cineol (8 %), Menthol (4 %), Menthone (2.0-4.6 %), Pinene (0.1-1.0 %), Phellandrene (0.1-1.0 %), Limonene (0.1-1.0 %), gamma-Terpinene

(0.1-2.0 %), Methyl acetate (0.3-1.0 %), and Menthofuran (0.3-1.0 %). This blend is chemically-engineered to form an oil-in-water emulsion, enhancing its homogenous dispersion in aqueous diluent. The MIC protocol followed a previously documented (Salmon and Watts 2000) procedure ,with dilutions of the blend ranging between 0.02 to 8.0 %. Briefly, each dilution of the blend was incorporated in triplicates of Tryptose Phosphate broth medium, followed by inoculation of the *Salmonella* serovar culture and incubation overnight at 37°C. The MIC values were recorded according to the minimum dilution of the blend incorporated in the medium that enables the growth-inhibition of the *Salmonella* culture.

4. Safety assessment of the blend

The safety assessment of the essential oil blend was determined by Rabbit-Draize Eye (Wilhelmus 2001) and Ames tests (Zeiger and Mortelmans 1999).

a- Rabbit-Draize Eye test

The safety of the essential oil blend, in its inability to induce conjunctival inflammatory reaction, was assessed by the Rabbit-Draize Eye test (Wilhelmus 2001) Briefly, nine male rabbits of one year old were divided evenly into three treatments. Rabbits of Treatment 1 were the controls, deprived of any application of the blend on their eyes. Rabbits of Treatment 2 received a 2 % dilution of the blend, in a volume of 50 µl/each of their left eye, and repeating the application for three consecutive days. Rabbits of Treatment 3 received a 6 % dilution of the blend in a similar manner to that used in

Treatment 2. The right and left eyes of the 9 rabbits were examined daily and for a period of 7 days, effective the first day of application. The eye examination included the observation of inflammation, manifested in redness, swelling, discharge, ulceration, hemorrhaging, cloudiness, and blindness.

b- Ames test for mutagenicity

The procedure of the Ames test, applied on the essential oil blend, was adopted from previously documented protocols. Briefly, the test organism was *Salmonella* Typhimurium (ATCC ® 29629-Strain Designations: TA 1535), an auxotrophic mutant, provided by ATCC, Manassas, USA. The provided mutant requires histidine for growth. The test of mutagenesis involves the addition of the blend at different dilutions (0.02, 2.0, 3.0, 4.0, 5.0, and 6.0 %) to a histidine-free medium, and observing the ability of the blend to revert back the mutated *S. Typhimurium* to a prototrophic state, enabling it to synthesize histidine and to grow. The negative control medium was not supplemented with the oil blend, while the two control-positive media were supplemented with either 20 or 200 nmoles of the mutagen Ethidium Bromide. The *S. Typhimurium* mutant was plated in triplicates on each of the previously described media, incubated at 37°C for 24 hrs, and the number of colonies was recorded to calculate the % increase in colony count in the medium supplemented with the blend in relation to that growing on non-supplemented medium, using the Ethidium Bromide as a positive control mutagen.

The below detailed procedure of the test is as follows based on Current Protocols in Toxicology:

Preparation of the Trace elements solution

The trace element solution (containing citric acid as a solubilizing agent) is made up as follows: In 95 ml. distilled water, dissolve successively with stirring at room temperature:

| | |
|--|------------|
| Citric acid, 1 H ₂ O | 5.00 grams |
| ZnSO ₄ , 7 H ₂ O | 5.00 grams |
| Fe(NH ₄) ₂ (SO ₄) ₂ , 6 H ₂ O | 1.00 gram |
| CuSO ₄ , 5 H ₂ O | 0.25 gram |
| MnSO ₄ , 1 H ₂ O | 0.05 gram |
| H ₃ BO ₃ , anhydrous | 0.05 gram |
| Na ₂ MoO ₄ , 2 H ₂ O | 0.05 gram |

Preparation of Biotin Solution

Dissolve 5 mg of Biotin in 50 ml of distilled water and filter sterilize the solution.

Histidine solution preparation

Dissolve 1.94 mg of Histidine in 250 mL of Distilled water and filter sterilize the solution.

D.2.4 Preparation of 50X Vogel Bonner salts:

Vogel's 50X salts as given in the Microbial Genetics Bulletin, 1956. In 750 ml. distilled water, dissolve successively with stirring at room temperature:

| | |
|---|-----------|
| Na ₃ citrate, 2H ₂ O | 125 grams |
| KH ₂ PO ₄ , anhydrous | 250 grams |
| NH ₄ NO ₃ , anhydrous | 100 grams |
| MgSO ₄ , 7 H ₂ O | 10 grams |

| | |
|---------------------------------------|---------|
| CaCl ₂ , 2H ₂ O | 5 grams |
| Trace Element Solution | 5 ml. |
| Biotin Solution | 2.5 ml. |

Preparation of the Minimal Agar

Dissolve 1.5 g of Agar agar in one liter of distilled water, and autoclave at 121°C and 21 PSI for 15 minutes. Following sterilization, place flask in 45.5o C water bath to cool for 5 Minutes, then carefully add 25 ml of sterile 40% glucose and 10 ml of sterile 50X Vogel Bonner salts solution. Swirl to mix well and pour approximately 20-25 mls per plate (stop pouring when plate surface is covered).

Preparation of the soft overlay agar

- a. Tubes containing an amount of 3 mL of the minimal agar are cooled to 45.5°C in a water bath. Pipette 150 µl of *Salmonella* typhimurium strain (ATCC- 29629) and 300 µl of histidine/biotin solution into each tube. Add test substances, including varying amounts of Mentofin, namely 0.02, 2, 3, 4, 5, and 6% over the soft agar
- b. To mix, roll tubes between hands. Pour immediately on top of glucose-minimal media agar plates. Tilting and rotating plates, distribute the melted top agar evenly over the surface. Allow the inoculated soft agar to set for several minutes.
- c. incubate at 37C overnight.
- d. count the colonies and record the results.
- e. select few colonies from the soft agar, and subculture them onto BGA plates to confirm the presence of *Salmonella* only.

Table 11. Ames test experimental design.

| Treatment | Number of replicates | Test substance |
|-----------|----------------------|-----------------------------------|
| 1 | 2 | - |
| 2 | 2 | Mentofin (0.02%) |
| 3 | 2 | Mentofin (2%) |
| 4 | 2 | Mentofin (3 %) |
| 5 | 2 | Mentofin (4%) |
| 6 | 2 | Mentofin (5%) |
| 7 | 2 | Mentofin (6%) |
| 8 | 2 | Ethidium Bromide (200 nmol/plate) |

CHAPTER IV

RESULTS AND DISCUSSION

A. Evaluation of an Autogenous Vaccine and/or a Blend of Essential Oils in the Control of v-NDV in Broilers

1. Molecular characterization of the Lebanese v-NDV isolate

Phylogenetic analysis of the isolated v-NDV fusion protein revealed that it belongs to NDV genotype VIc (Figure 9). It is closely related to the Pakistani strains; 2004/PK/22 (100%), 2005/PK/26 (98%) and 1995/PK/16 (98%). The phylogenetic similarity decreased with viral strains isolated from United Arab Emirates and Saudi Arabia, and United Kingdom, although they all belong to genotype VIc that included isolates originating from the Middle East (Aldous, Mynn et al. 2003). The old circulating Lebanese strain 1970 was more related to NDV strains that were circulating in neighboring countries such as Kuwait, Iraq and Israel being all of genotype VIa (Aldous, Mynn et al. 2003). This might indicate that the current v-NDV virus was different from the old 1970 strain and could have originated from the 1995-2005 outbreaks in Pakistan. It is worth noting that La Sota and Clone 30 vaccine strains, known to be of genotype II, were genetically distinct from the Lebanese and other Middle Eastern NDV field isolates. This indicates clearly the genotypic difference between the mesogenic/lentogenic vaccine strains and circulating field strains. Therefore, the need to incorporate genotype-matched vaccines for an enhanced protection and reduced viral shedding which is of great importance to v-

NDV endemic developing countries.

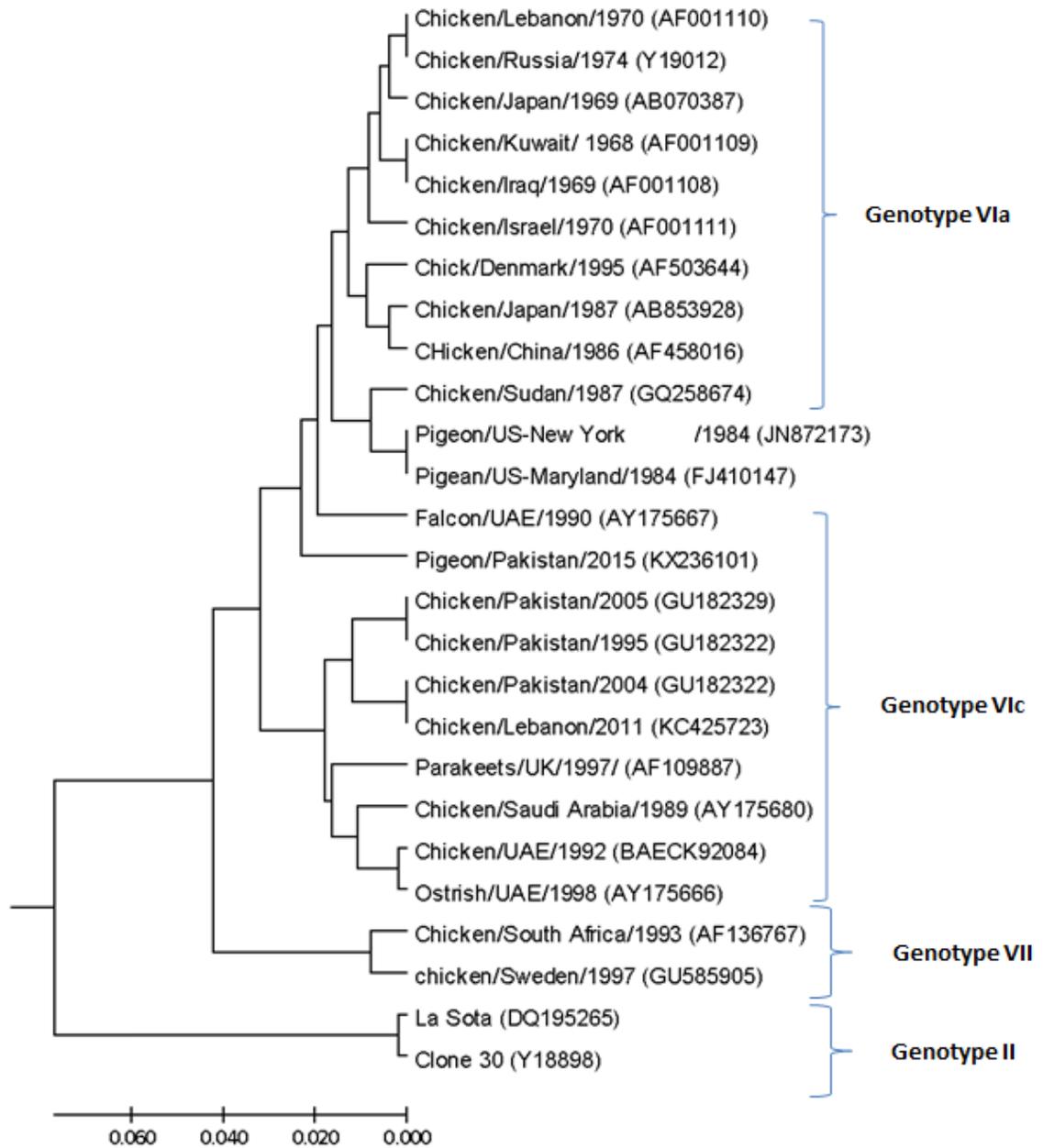


Figure 9. Phylogenetic analysis of the v-NDV isolate on MEGA 7.0 program using the UPGMA method, and based on the fusion protein fragment containing the cleavage site (Aldous, Mynn et al. 2003).

2. In vivo evaluation of an autogenous vaccine and/or a blend of essential oils in the control of v-NDV in broilers

The broiler mortality rates in the 8 different treatments are shown in Table 12. The broiler mortality rates in treatments 1, 2 and 3, receiving autogenous vaccine at d1 and d14 and challenged with v-NDV at d28 and with eight *Eimeria* spp. at d21, was 0%. On the contrary, the mortality percentages in the positive control birds of treatments 4 and 5, that were deprived of the autogenous vaccine and received the same challenge as that of the other treatments, were 100% and 70% respectively. This significant survival of vaccinated and challenged birds of treatments 1, 2 and 3 is in agreement with other researchers that documented a full protection against devastating diseases in poultry by autogenous killed vaccines incorporating in it the homologous strain that is used in the challenge (Ronco, Stegger et al. 2016; Gharaibeh and Amareen 2015). Moreover, other researchers working on the optimization of NDV vaccination program concluded that homologous genotype-matched vaccines offer Immunogenic protection and reduced viral shedding (Miller et al., 2007; Hu et al., 2009), recommending their use in countries that are having increased reporting of the failure of NDV commercial vaccines (Kapczynski, Afonso et al. 2013; Roohani, Tan et al. 2015). The high mortality rates of 70% and 100% in non-vaccinated v-NDV challenged birds reflect the devastating effect of v-NDV. It has been demonstrated that v-NDV is considered to be the second poultry disease in terms of economic significance and has a negative impact on the human livelihoods in developing countries due to its impact on both commercial and backyard chicken (Alexander and Senne 2008).

Table 12. Mean frequency of mortality, FCR, and percent weight gain in eight different treatments of broilers.

| Group | EO Trt ¹ | v-NDV & Cocci challenges ^{2,3} | NDV Vaccination ⁴ | % Cumulative Mortality (d1-d35) | FCR (d1-35) | % WG (d1-d35) |
|-------|---------------------|---|------------------------------|---------------------------------|-------------|---------------|
| 1 | + | + | + | 0 ^a | 2.05 | 35.67 |
| 2 | M+ ⁵ | + | + | 0 ^a | 2.03 | 39.46 |
| 3 | - | + | + | 0 ^a | 1.74 | 43.98 |
| 4 | AC ⁶ | + | - | 100 ^b | 2.66 | 26.65 |
| 5 | - | + | - | 70 ^b | 2.70 | 25.29 |
| 6 | AC | - | + | 0 ^a | 1.84 | 40.20 |
| 7 | + | - | + | 0 ^a | 2.26 | 42.73 |
| 8 | M+ | - | + | 0 ^a | 1.84 | 40.17 |

^{a-c}Means in a row with different Arabic numerical are significantly different (P <0.05).

¹⁻³Means in a column with different alphabet superscripts are significantly different (P<0.05).

¹Essential oils made from a blend of eucalyptus and peppermint.

²v-NDV challenge was intramuscular at 28 d of age, by v-NDV of genotype VI containing 1.2×10^8 TCID₅₀/0.5 ml/bird. The challenge was administered to two birds that were marked in every challenged group.

³Cocci challenge was administered to mimic field conditions. The challenge was at 21 d of age, by equivalent number of Sporulated Oocyst of 8 *Eimeria* spp (1×10^6 sporulated oocysts/bird). The 8 *Eimeria* spp, were *E. necatrix*, *E. acervulina*, *E. mivati*, *E. tenella*, *E. brunetti*, *E. maxima*, *E. hagani* and *E. praecox*.

⁴The Autogenous vaccine is a water-in-oil emulsion, in which the aqueous phase contained 128 HA units of formalized v-NDV of genotype VI. Vaccinated birds received 2 subcutaneous injections of 0.5 ml each (priming 1d) and (boosting 14 d).

⁵Modified blend of essential oils made up of a blend of eucalyptus, peppermint and other unspecified substances.

⁶AC = Anticoccidial drugs commercial combination of narasin and nicarbazin

Despite the fact that *Eimeria* spp. in chicken causes suppression of acquired immunity to vaccines, and more specifically to NDV vaccines (Akhtar, Awais et al. 2015), still the autogenous vaccine was highly protective against a homologous challenge with v-NDV of genotype VI. The difference in mortality percentages between positive control group 4, receiving an anticoccidial commercial drug combination of Narasin and Nicarbazin in addition to the challenge, and group 5 that was v-NDV challenged and deprived of any treatment, proves for the first time in literature that a failure in protection against v-NDV will interact negatively with the presence of anticoccidial drug supplementation that is in disagreement with other researchers (Munir et al. 2007).

The survival of all birds in Treatments 6, 7 and 8 that received the vaccine, and were deprived of both challenges, indicates the safety of the administered vaccine. Actually, the use of killed vaccines in poultry husbandry is favored, since it is devoid of living organisms that might cause a vaccine reaction or even significant loss in production under certain stressful situations. Regarding the cumulative production parameters between 1-35d of age, non-challenged groups showed the similar compatibility between the Modified EO Treatment 8 and the anticoccidial Treatment 6 in obtaining the lowest FCR (1.82) (Table 12). While the highest mean percent weight gain (42.73%) among the non-challenged was obtained by the EO treatment (Table 12). These results indicate that essential oils could have a promising growth promotion effect in broiler chicks, and can improve FCR and weight gain in v-NDV and *Eimeria* challenged broilers. However, among the challenged groups, and for the whole period of 1-35 days, the challenged Treatment 3 receiving the anticoccidial drug and the autogenous NDV vaccine had the lowest feed

conversion ratio of 1.74 and the highest mean % weight gain of 43.98%. This result is in agreement with reported literature on the augmentation of anticoccidial drugs on the anti-NDV immune response in broiler chicks (Munir et al. 2007). Moreover, anticoccidial drugs still have an important impact on FCR and are known to work on, maintaining gut health, controlling *Eimeria* oocysts, and directing nutrients towards growth (Williams 2005). This fact indicates why ionophores use in poultry is of great importance, and is still adopted in many countries around the world (De Gussem, 2007).

Table 13 shows the HI titers of birds from 8 different treatments at d1, d14, d28 and d34. The HI titers showed the same pattern of humoral antibody decay at 14d of age, and the successful acquired vaccine-immunity at d28 (14 days post the booster for NDV vaccine), and the successful seroconversion to challenge by v-NDV in treatments 1, 2 and 3 at d34. The EO treatment 1 birds gave the maximum titers of 20969.6 specific to hemagglutinin protein of v-NDV at 34 days of age, an observation that is shown in previous studies related to immunopotential of immune response by EO (Barbour, Shaib et al. 2013).

The absence of challenge at d28 in treatments 6, 7, and 8 led to significantly low decaying titers at d34 compared to NDV vaccinated and challenged groups of treatments 1, 2 and 3. These results also indicate that HI titers as low as 1:48, prior to v-NDV challenge, can still offer acceptable protection against v-NDV challenge in broilers. Kapczynski and King (2005) showed that broilers with HI titers greater than 16 after multiple vaccinations survived v-NDV challenge. The authors concluded that commercial NDV vaccines used in USA offered full protection for broiler breeders, but failed in protecting broilers (66%

mortality) upon CA02 v-NDV challenge.

Table 13. Mean Hemagglutination-Inhibition titers specific to Hemagglutinin protein of the v-NDV.

| Group | EO Trt ¹ | v-NDV & Cocci challenges ^{2,3} | NDV Vaccination ⁴ | HI d1 | HI D14 | HI D28 | HI D34 |
|-------|---------------------|---|------------------------------|---------------------|-----------------------|-------------------------|-------------------------|
| 1 | + | + | + | 23.0 ¹ | 7.2 ^{ab,1} | 112.0 ^{ab,1} | 2,969.6 ^{c,2} |
| 2 | M+ ⁵ | + | + | 23.0 ¹ | 5.4 ^{a,1} | 172.8 ^{b,1} | 883.2 ^{ab,2} |
| 3 | - | + | + | 23.0 ¹ | 4.4 ^{a,1} | 185.6 ^{b,1} | 1,996.8 ^{bc,2} |
| 4 | AC | + | - | 23.0 ¹ | 5.8 ^{a,1,2} | 0 ^{a,2} | 0 ^{a,2} |
| 5 | - | + | - | 23.0 ¹ | 11.3 ^{b,1,2} | 0 ^{a,2} | 0 ^{a,2} |
| 6 | AC | - | + | 23.0 ^{1,2} | 4.2 ^{a,1} | 138.4 ^{ab,2,3} | 173.6 ^{a,3} |
| 7 | + | - | + | 23.0 ^{1,2} | 4.2 ^{a,1} | 48.0 ^{ab,2} | 82.3 ^{a,3} |
| 8 | M+ | - | + | 23.0 ¹ | 9.2 ^{ab,1} | 106 ^{ab,1} | 24.4 ^{a,1} |

^{a-c}Means in a row with different Arabic numerical are significantly different (P <0.05).

¹⁻³Means in a column with different alphabet superscripts are significantly different (P<0.05).

¹Essential oils made from a blend of eucalyptus and peppermint

²v-NDV challenge was intramuscular at 28 d of age, by v-NDV of genotype VI containing 1.2x10⁸TCID₅₀/0.5 ml/bird. The challenge was administered to two birds that were marked in every challenged group.

³Cocci challenge was administered to mimic field conditions. The challenge was at 21 d of age, by equivalent number of Sporulated Oocyst of 8 *Eimeria* spp (1x10⁶ sporulated oocysts/bird). The 8 *Eimeria* spp, were *E. necatrix*, *E. acervulina*, *E. mivati*, *E. tenella*, *E. brunetti*, *E. maxima*, *E. hagani* and *E. praecox*.

⁴The Autogenous vaccine is a water-in-oil emulsion, in which the aqueous phase contained 128 HA units of formalized v-NDV of genotype VI. Vaccinated birds received 2 subcutaneous injections of 0.5 ml each (priming 1d) and (boosting 14 d).

⁵Modified blend of essential oils made up of a blend of eucalyptus, peppermint and other unspecified substances that remain the proprietary information of the producer.

⁶AC = Anticoccidial drugs commercial combination of narasin and nicarbazin

Table 14 shows the intensity of specific antibody response to fusion protein. Among the NDV challenged groups, groups 2 and 3 with respective modified EO and anticoccidial drug treatments, showed significantly higher intensities in comparison with EO treated group at d 28 of age. Apparently at 28 days of age the HI titers and the mean intensity to the fusion protein band had the same trend among the experimental groups.

As for the non-challenged groups 6, 7, and 8 receiving anticoccidial drug, EO and modified EO respectively, there were no significant differences in the fusion band intensities at d 28 of age.

Table 14. Mean fusion protein intensities in eight different treatments of broilers.

| Group | EO Trt ¹ | v-NDV & Cocci challenges ² | NDV Vaccination ⁴ | Mean Intensity of F band Day 14 | Mean Intensity of the F band- Day 28 | Mean Intensity of the F band- Day 34 |
|-------|------------------------|---|---------------------------------|---------------------------------------|--|--|
| 1 | + | + | + | 9,708.0 ^{a,1} | 23,734.2 ^{a,1} | 113,620.4 ^{ab,2} |
| 2 | M+ ⁵ | + | + | 98,800.9 ^{c,1} | 114,321.5 ^{bc,1} | 182,476.0 ^{bc,2} |
| 3 | - | + | + | 73,853.7 ^{b,1} | 107,478.3 ^{b,1} | 184,140.4 ^{bc,2} |
| 4 | AC | + | - | 60,729.3 ^{b,1} | 84,703.8 ^{b,2} | 84,558.2 ^{a,2} |
| 5 | - | + | - | 22,138.9 ^{a,1} | 22,140.3 ^{a,1} | 44,705.7 ^{a,2} |
| 6 | AC | - | + | 137,462.2 ^{d,1} | 157,240.1 ^{d,1} | 229,904.7 ^{c,2} |
| 7 | + | - | + | 144,466.7 ^{d,1} | 144,320.1 ^{cd,1} | 169,696.6 ^{bc,2} |
| 8 | M+ | - | + | 108,027.5 ^{c,1} | 146,835.3 ^{cd,1} | 204,576.6 ^{c,2} |

^{a-c}Means in a row with different Arabic numerical are significantly different (P < 0.05).

¹⁻³Means in a column with different alphabet superscripts are significantly different (P < 0.05).

¹Essential oils made from a blend of eucalyptus and peppermint

²v-NDV challenge was intramuscular at 28 d of age, by v-NDV of genotype VI containing 1.2×10^8 TCID₅₀/0.5 ml/bird. The challenge was administered to two birds that were marked in every challenged group.

³Cocci challenge was administered to mimic field conditions. The challenge was at 21 d of age, by equivalent number of Sporulated Oocyst of 8 *Eimeria* spp (1×10^6 sporulated oocysts/bird). The 8 *Eimeria* spp, were *E. necatrix*, *E. acervulina*, *E. mivati*, *E. tenella*, *E. brunetti*, *E. maxima*, *E. hagani* and *E. praecox*.

⁴The Autogenous vaccine is a water-in-oil emulsion, in which the aqueous phase contained 128 HA units of formalized v-NDV of genotype VI. Vaccinated birds received 2 subcutaneous injections of 0.5 ml each (priming 1d) and (boosting 14 d).

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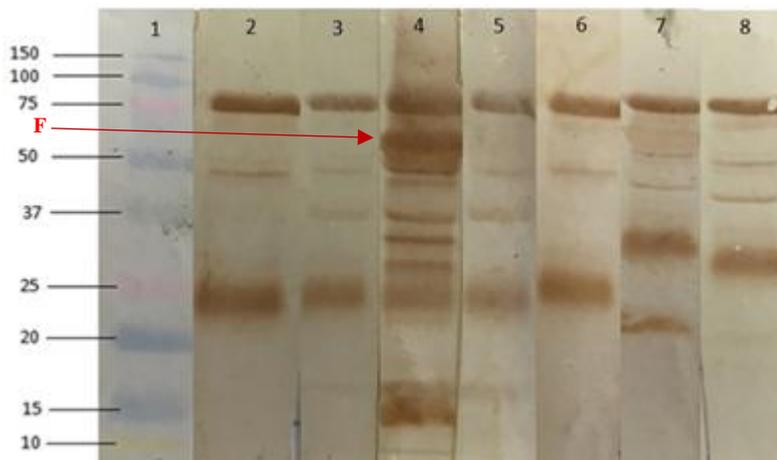


Figure 10. Western Immunoblotting showing the different intensities of the reaction between humoral antibodies, collected from the broilers at different times before and after the vaccination, and the banded fusion (F) protein of genotype VIc v-NDV.

- Lane 1: Molecular Ladder (Precision Plus Protein Kaleidoscope Standard, Biorad).
- Lane 2: treatment 1 (v-NDV vaccinated at d 1& 14) d 14.
- Lane 3: Trt 4 (non-vaccinated) d14.
- Lane 4: Trt 1 d 34 showing Antibodies specific to Fusion Protein (62 Kda).
- Lane 5: Trt 4 d 34.
- Lane 6: negative control provided by ELISA Kit (IDEXX Laboratories, Inc. Westbrook, Maine, US).
- Lane 7: Trt 1 d 28. Lane 8: Trt 2 d 28.

It is worth noting that all vaccinated groups showed significantly higher fusion protein mean intensities in comparison to the non-vaccinated at d34 of age (Table 14 and Figure 10). These results indicate the importance of the NDV fusion protein and the fact that Fusion-specific antibodies have a significant viral neutralization effect (Taylor, Edbauer et al. 1990; de Leeuw and Peeters 1999).

B. Susceptibility of *Salmonella* Serovars recovered from Hospitalized Saudis to Commercial Drugs and to a Chemically and Safety-characterized Essential Oil

The frequency of effective antimicrobials, according to their mode of action, and the MIC values of the essential oil blend against different isolates of each *Salmonella* serovar recovered from Saudi patients are presented in Table 15. The range of frequencies of drugs against the *S. Enteritidis* isolates are bracketed as follows: cell wall inhibitor-drugs (1/13-8/13), protein synthesis inhibitors (0/6-2/6), nucleic acid synthesis inhibitors (0/3-1/3) and folate pathway inhibitor (0/1-1/1). This apparent variability in susceptibility of *S. Enteritidis* to drugs was associated with a wide range of the blend MIC values against isolates of this serovar, equivalent to dilutions from 0.64 to > 8.0 %. The wide variation in the frequency of effective antimicrobials against the *S. Enteritidis* isolates recovered from different individual cases (Table 15) is most likely due to the different history of exposure to the drugs by the humans or animals that hosted this serovar (Velge, Cloeckert et al. 2005).

Table 15 also showed the ranges of frequencies of drugs that are effective against the *S. Typhimurium* isolates. The ranges of frequencies were: cell wall inhibitors (4/13-7/13), protein synthesis inhibitors (0/6-2/6), nucleic acid synthesis inhibitors (0/3-2/3), and absence of sensitivity to the folate inhibitor. The blend MIC values against the *S. Typhimurium* isolates ranged between 2.0- > 8.0%. The variation in the frequency of effective antimicrobials against *S. Typhimurium* isolates was narrower, which could be due

to the lower rate of infection and exposure to drugs of this serovar in poultry and other animal hosts compared to that of *S. Enteritidis*. Actually, the documented prevalence of *S. Enteritidis* in relation to *S. Typhimurium* in sampled poultry of Saudi Arabia was 10 to 1 (Al-Nakhli, Al-Ogaily et al. 1999).

In animal hosts, the frequency of infection by *S. Enteritidis* is higher than by *S. Typhimurium* an indication of the higher adaptability of *S. Enteritidis* to a wide range of hosts, rendering it more exposed to antimicrobial agents and hence more resistant to drugs (CDC, 2010).

The effective ranges of frequencies of drugs against the *S. Kentucky* isolates were: cell wall inhibitor-drugs (0/13-3/13), protein synthesis inhibitors (0/6-1/6), nucleic acid synthesis inhibitors (0/3-1/3), and absence of sensitivity to the folate inhibitor. The MIC of the essential oil blend against the *S. Kentucky* was always > 8.0 %.

The ranges of effective frequencies of drugs against the *S. Anatum* were: cell wall inhibitors (0/13-6/13), protein synthesis inhibitors (0/6-2/6), nucleic acid synthesis inhibitors (0/3-2/3), and folate acid inhibitor (0/1-1/1). The MIC of the essential oil blend against the *S. Anatum* was always > 8.0 %.

Unfortunately, the frequency of effective antimicrobials against the other two non-typhoids (*S. Kentucky* and *S. Anatum*) diminished significantly, resulting in much narrower variation of such frequencies among their isolates. These two non-typhoid serovars are reported previously to infect humans in the United States, 1968-2011 (Control and Prevention 2015), with an alarming resistance of *S. Kentucky* to ciprofloxacin. Future investigation in Saudi Arabia should involve an epidemiology study

of these two highly drug-resistant serovars in both animals and human (Le Hello, Hendriksen et al. 2011) and investigation of their pathogenesis in serious human outbreaks.

The ranges of effective frequencies of drugs against the *S. Typhi* isolates were: cell wall inhibitor-drugs (5/13-7/13), protein synthesis inhibitors (1/6), nucleic acid synthesis inhibitors (0/3-2/3), and folate acid inhibitor (0/1-1/1). The MIC of the essential oil blend against the *S. Typhi* isolates was of wider range namely, 0.32 to more than 8.0 %.

The higher frequency of effective drugs against the *S. Typhi* recovered from Saudi patients compared to that of the non-typhoids, is most likely due to the inadaptability of this serovar to animal hosts, thus avoiding the pressure of antimicrobial use in different animal species that selects for drug-resistant organisms (Crump, Luby et al. 2004).

It is worth noting that the two control-poultry *Salmonella* isolates, recovered from poultry of Brazil and Nigeria, with long history of antimicrobial applications in feed and drinking water, were confirmed with high multiple drug-resistance (Table 16). Only one out of 23 drugs was effective in vitro against the Brazilian *S. Gallinarum* isolate involved in economic fowl typhoid outbreaks, while no drug out of the 23 was effective against the Nigerian isolate of *S. Gallinarum*. Fortunately, the MIC of the blend against both control isolates was low (0.32 %).

A clear example of the impact of overuse of antimicrobials in poultry husbandry on

emergence of drug-resistant *Salmonella* is seen in the Brazilian and Nigerian isolates of *S. Gallinarum* (Table 16). The fact that this serovar causes Fowl Typhoid, resulting in flock mortality between 90-100 %, obliges the farmers to overuse different antimicrobials, trying to save the affected flocks, leading to emergence of resistant strains to a wide range of antimicrobials. The drugs-of-choice, uncovered in this research, are located under their mode of action in Table 17. The Cefepime, a cell wall inhibitor of the 4th generation cephalosporins, is effective against most serovars targeted in this study. Actually, the high Cefepime efficacy against *Salmonella* is reported from many parts of the world, in which physicians refer to it in treating both the non-typhoid and typhoid human cases (WHO 2015).

Table 17 shows the most effective drugs against different serovars of *Salmonella* involved in human outbreaks in Saudi Arabia. Among the 13 cell wall synthesis inhibitors, only the Cefepime was effective against all human non-typhoid and typhoid serovars. The chloramphenicol was persistent as the drug-of-choice among protein synthesis inhibitors against the human non-typhoid serovars, but not against the typhoid isolates, in which Amikacin and Tobramycin were the effective protein synthesis inhibitors of all typhoid cultures. The Norfloxacin was the most effective nucleic acid inhibitor of non-typhoid and typhoid isolates, while the frequency of the folate-inhibitor effectiveness was scarce against both the non-typhoid and typhoid isolates.

Among the studied protein synthesis inhibitors (Table 15), the chloramphenicol is effective against all isolates of non-typhoid serovars, a fact that is in agreement with the reported high efficacy of this drug against Salmonellosis by WHO. In addition, the

Amikacin and Tobramycin-protein synthesis inhibitors are found effective against the isolates of the *S. Typhi*, a data that is in agreement with previous reports related to these two drugs (WHO 2012).

The studied nucleic acid inhibitors showed that Norfloxacin is the drug-of-choice for these investigated human non-typhoid and typhoid *Salmonellae*. This quinolone is actually rated at the top of the list of antimicrobials used against human Salmonellosis (WHO 2012).

The presence since more than four decades of Sulfamethoxazole/Trimethoprim combination in the global markets of veterinary and human medicine led to its lower effectiveness against *Salmonella* organisms compared to its high reported efficiency in the eighties (Su, Chiu et al. 2004). The recommendation by the WHO to limit the use of some efficient drugs to humans (WHO 2012) is nowadays respected by many veterinary communities, in attempts to avoid the emergence of highly drug-resistant *Salmonella* that threatens the public health.

The correlation, using multiple regression analysis, between the MIC values of the essential oil blend and the frequency of drugs that are effective against the human *Salmonella* isolates is shown in the R² value of +0.46, with a significant correlation at $p < 0.05$.

The repeated application of the 2 and 6 % dilutions of the essential oil blend on the rabbit's eyes did not result in any gross inflammation lesion. In addition, the Ames test proved the absence of mutagenesis on the test organism of *S. Typhimurium* at

concentrations between 0.02-4.0% (Table 18). However, induced mutations by the blend started weakly at 5 % dilution, and raised sharply at 6 %. The positive control, Ethidium Bromide supplemented medium; showed a clear mutagenesis at 200 nmol but not at a lower concentration of 20 nmol.

The obtained MIC and safety data (Table 15 & Table 18) related to antimicrobial activity of the essential oil blend against non-typhoid and typhoid salmonellosis is within the second goal of WHO Traditional Medicine Strategy 2014-2023, quoting ‘strengthening safety, quality, and effectiveness (Qi and Kelley 2014). The wide range of dilutions of the essential oil blend, needed to inhibit different isolates, within most of the same serovars (Table 15), is indicative that the susceptibility of *Salmonella* serovars to essential oils varies in a similar trend as that of their susceptibility to modern synthetic drugs. Actually, the multiple regression analysis did prove the positive correlation between the frequencies of the drugs that the *Salmonella* isolates are resistant to and the MIC values of the essential oil blend ($p < 0.05$). Future investigations should relate the *in vitro* efficacy of the essential oil blend to their *in vivo* efficacy in mice or rat models.

The studied essential oil blend safety was manifested in the absence of conjunctival inflammation in the rabbit’s eyes following multiple application of the essential oil at 2 and 6 % dilution. Previous researches confirmed the presence of anti-inflammatory substances in eucalyptus (Silva, Abebe et al. 2003) and peppermint oils (Horváth and Ács 2015).

The protocol of the Ames test used in this study allowed confining its safety, based on its inability to induce mutagenesis, at the range of dilutions between 0.02 to 4.0 %

(Table 18). Actually, the extracts of these two plants are nowadays incorporated in lozenges that are bought over the counter, without prescription by physicians, and are documented as FDA-approved additives in foods.

The targeted research related to Salmonellosis in Saudi Arabian patients revealed the presence of both, the typhoid and non-typhoid isolates. The non-typhoid cases were dominated by *S. Enteritidis* and *S. Typhimurium* serovars; all non-typhoid isolates were sensitive to Cefepime, Chloramphenicol, and Norfloxacin. Patients with typhoid organisms had more leverage of treatments, since their isolates were sensitive to 10 out of 23 tested antimicrobials. The *in vitro* efficiency of the essential oil blend was dependent on its dilution. Moreover, the absence of induction of conjunctival inflammation and mutagenesis by the essential oil blend were determined at 4.0 and 6.0 %, respectively.

Table 15. The ranges of effective antimicrobial-frequencies and MIC values of essential blend against isolates¹ of *Salmonella* serovars recovered from Saudi patients.

| AB ² | Range of effective antimicrobial frequencies or MIC ³ values against isolates of | | | | |
|-----------------------------------|---|-----------------------|--------------------|------------------|-----------------|
| | <i>S. Enteritidis</i> | <i>S. Typhimurium</i> | <i>S. Kentucky</i> | <i>S. Anatum</i> | <i>S. Typhi</i> |
| Cell Wall inhibitors | 1/13 - 7/13 | 4/13 - 7/13 | 0/13 - 3/13 | 0/13 - 6/13 | 5/13 - 7/13 |
| Protein synthesis inhibitors | 0/6 - 2/6 | 0/6 - 2/6 | 0/6 - 1/6 | 0/6 - 2/6 | 1/6 |
| Nucleic acid synthase inhibitors | 0/3 - 1/3 | 0/3 - 2/3 | 0/3 - 1/3 | 0/3 - 2/3 | 0/3 - 2/3 |
| Folate pathway inhibitor | 0/1 - 1/1 | 0/1 | 0/1 | 0/1 - 1/1 | 0/1 - 1/1 |
| MIC ranges of Essential oil blend | 0.6 - >8.0 | 2.0 - 8.0 | >8.0 | >8.0 | 0.32 - >8.0 |

¹The bracketed numbers of isolates, tested for their antimicrobial susceptibility, and affecting individual patients with specific serovars are: *S. Enteritidis* (4), *S. Typhimurium* (4), *S. Kentucky* (3), *S. Anatum* (3), and *S. Typhi* (2)

²The bracketed numbers of tested antimicrobials, under each mechanism of activity are: cell wall inhibitors (13), protein synthesis inhibitors (6), nucleic acid inhibitors (3), and folate pathway inhibitor (1)

³The MIC value is the minimum dilution of the essential oil blend that inhibits the growth of a *Salmonella* isolate

Table 16. The frequency of effective antimicrobials and MIC values of the essential oil blend against the control *S. Gallinarum* isolates recovered from Brazilian and Nigerian poultry farms with long history of antimicrobial administration.

| Antimicrobials ¹ | Frequency of effective antimicrobials or MIC ² values against <i>S. Gallinarum</i> recovered from poultry of | |
|-----------------------------------|---|---------|
| | Brazil | Nigeria |
| Cell Wall inhibitors ³ | 1/13 | 0/13 |
| Protein synthesis inhibitors | 0/6 | 0/6 |
| Nucleic acid synthesis inhibitors | 0/3 | 0/3 |
| Folate pathway inhibitor | 0/1 | 0/1 |
| MIC of Essential oil blend | 0.32 | 0.32 |

¹The bracketed numbers of tested antimicrobials, under each mechanism of activity, are: cell wall inhibitors (13), protein synthesis inhibitors (6), nucleic acid inhibitors (3), and folate pathway inhibitor (1)

²The MIC value is the minimum dilution of the essential oil blend that inhibits the growth of a *Salmonella* isolate

³The only cell wall inhibitor that is effective against the Brazilian *S. Gallinarum* isolate was the Imipenem

Table 17. The most effective drugs against different serovars of *Salmonella* involved in human outbreaks of Saudi Arabia.

| | Most effective drugs with inhibitory mechanism to synthesis of | | | |
|------------------------|--|-------------------------------|------------------------------|------------------|
| | Cell wall | Protein | Nucleic acid | Folate |
| Human- Non Typhoids | | | | |
| <i>S. Enteritidis</i> | Cefepime Imipenem | Chloramphenicol | Norfloxacin | SXT ¹ |
| <i>S. Typhimurium</i> | Cefepime | Gentamicin Chloramphenicol | Ciprofloxacin Norfloxacin | None |
| <i>S. Kentucky</i> | Cefepime | Chloramphenicol | Norfloxacin | None |
| <i>S. Anatum</i> | Cefepime | Chloramphenicol | Norfloxacin Ciprofloxacin | SXT |
| Human- Typhoid | | | | |
| <i>S. Typhi</i> | Cefepime Cefoxitin Ceftazidime Aztreonam Imipenem | Amikacin Tobramycin | Norfloxacin Ciprofloxacin | SXT |

¹Sulfamethoxazole-Trimethoprim

Table 18. Mutagenicity¹ in histidine-deficient *S. Typhimurium*² by supplementation of its growth medium with different dilutions of the essential oil blend.

| Supplementation of growth medium ³ | Mean ⁴ % increase in <i>S. Typhimurium</i> colony count compared to its count on minimal histidine-supplemented medium ³ |
|--|--|
| EO ⁵ - 0.02% | -16.7 |
| EO - 2.0% | -07.7 |
| EO - 3.0% | 00.0 |
| EO - 4.0% | -16.7 |
| EO - 5.0% | +16.7 |
| EO - 6.0% | +90.9 |
| EB ⁶ - 20 nmole | 00.0 |
| EB - 200 nmole | +12.5 |

¹Mutagenicity is detected by the ability of the *S. Typhimurium* mutant to revert back to an organism that can synthesize its own histidine (Ames test)

²*Salmonella enterica subsp. Enterica serovar Typhimurium* (ATCC ® 29629-Strain Designations: TA 1535), an auxotrophic mutant, provided by ATCC, Manassas, USA

³The growth medium was prepared in reference to Current Protocols in Toxicology (1999)

⁴Mean colony count growing on triplicate plates

⁵EO is the essential oil blend

⁶EB is a control positive substance that induces mutagenesis namely, Ethidium Bromide

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

The conclusions that are drawn from this thesis are:

- 1- The phylogenetic analysis of the isolated and sequenced v-NDV revealed that the viral strain involved in economic outbreaks of Lebanese poultry belongs to genotype VIc, which is considered to be originating from neighboring Middle Eastern countries. There is a high possibility that the v-NDV Lebanese 2011 isolate could be originating from Pakistan with 100%, 98% and 98% fusion protein fragment antigenic match to isolates from Pakistan in 1995, 2004 and 2005.
- 2- The developed genotype VIc autogenous vaccine offered 100% protection upon challenge with the homologous strain. Non-vaccinated groups challenged with v-NDV had 70% and 100% mortality rates, reflecting the devastating effect of Lebanese v-NDV etiologic agent.
- 3- Essential oils namely, that of Eucalyptus and Peppermint blend, offered an interesting immunopotentiating effect that is mostly evident in v-NDV and *Eimeria* challenged birds.
- 4- The developed autogenous NDV vaccine, inducing HI titers as low as 1:48, was able to offer high protection in v-NDV challenged birds.
- 5- Birds vaccinated with autogenous v-NDV vaccine had significantly higher fusion band intensities when compared to non-vaccinated birds at d34 (6 days post challenge), indicating the importance of incorporating Fusion Protein of v-NDV in

- the vaccine, that results in boosting the specific protective antibodies post challenge.
- 6- Regarding the non-typhoid Saudi *Salmonella* isolates, out of the 23 used antibiotics, only Cefepime, Chloramphenicol, and Norfloxacin were effective *in vitro*. This decreased “drug-of choice” susceptibility in an alarming public health threat. While concerning the Typhoid *Salmonella* isolates 10 drugs out of 23 were effective, giving wider range of drugs to choose from.
 - 7- The multidrug resistant *Salmonella* Gallinarum serovars isolated from poultry in Brazil and Nigeria had 1 out of 23, and 0 out of 23 effective drugs, respectively. These results support the WHO and OIE recommendation regarding decreasing and restricting the use of Antibiotics in Livestock production fearing the rise of “superbugs” originating from animal reservoirs.
 - 8- The blend of Eucalyptus and Peppermint essential oils used in this study is proven efficacious and safe.

The following are the recommendations from the two parts of this thesis:

Regarding the NDV study, it is recommended to have routine epidemiological survey, reporting to OIE, and international control measures that sustain the Poultry Industry within National Poultry Improvement Plans (NPIP) of developing and developed countries. The genotype-matched vaccines, that could be chimeric, recombinant, or autogenous, are highly recommended for developing countries reporting the failure of NDV commercial vaccines like that observed in Lebanon. Essential Oils use in high challenge areas (high disease challenge, hot climates, open system housing, poor biosecurity, and bad management practices), offer great immunopotentiating effect that helps in ameliorating

protection against certain pathogenic diseases, and improving performance.

Regarding the *Salmonella* study, continuous surveillance, monitoring and reporting is recommended in developing countries as a prerequisite for any Infectious Diseases control programs. WHO, FAO and OIE judicious principles of antibiotics use in livestock should be strictly adopted by governments all around the world. Drugs that are “critically important” for humans should be prohibited in livestock production. The One health approach, an interdisciplinary approach aimed at maintaining the health of humans, animals and the environment, represents a promising strategy in the fighting against infectious diseases. This approach forms a roadmap towards reaching the millennial goal of combating infectious diseases that are of great concern to the people and World organizations such as the WHO, OIE, and FAO.

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