AMERICAN UNIVERSITY OF BEIRUT

VACCINES AND ESSENTIAL OIL IN RESPECTIVE PROTECTION AND TREATMENT OF CHICKEN AND RABBIT AILMENTS

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science to the Department of Animal Sciences of the Faculty of Agricultural and Food Sciences at the American University of Beirut

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My recognition and gratitude to all my graduate colleagues in the animal science lab.

To AREC’02 class.

To my family and friends.
AN ABSTRACT OF THE THESIS OF

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Title: Vaccines and Essential Oil in Respective Protection and Treatment of Chicken and Rabbit Ailments.

The first study determined the anti-inflammatory effect induced by *Origanum ehrenbergii* essential oil on *Staphylococcus aureus* skin infection in rabbits. Experimental rabbits were divided into two groups with 5 rabbits per group. Group 1 was challenged with 0.1 ml Staphylococcus aureus intradermally and treated twice topically with 1% *Origanum ehrenbergii* essential oil diluted in 40% DMSO/water. Group 2 received the same challenge as Group 1 but without any treatment. After 24 hours, the mean weight of the ear lesion in the non-treated rabbits was larger than the mean weight of the ear lesion in the treated rabbits (2.72g, 1.24g respectively, p<0.05).

In the second study, serological profiling for disease diagnosis and for vaccine evaluation was conducted for the first time in Lebanon on free-range poultry farms situated in Jezzine area. The humoral immune responses in the chicken sera showed disease exposure to IBV, NDV, EDS, Mg and IBDV. The immune response of chicken towards an inactivated trivalent vaccine was serologically determined by testing paired serum samples from the vaccinated farms. According to the ELISA system, no significant difference in humoral antibody level was observed after 21 days of vaccination. Furthermore, the immune responses to the trivalent vaccine were both qualitatively and quantitatively assessed by reacting the protective polypeptides in the pooled farm sera with the antigens of the trivalent vaccine by Western Immunoblotting. The qualitative immune response was observed in the appearance of new antibodies to the F protein (60KDa) in NDV, and the appearance of new antibodies to the membrane glycoprotein GP36 in IBV. In addition, there were more quantitative levels of antibodies to the large (L) protein (248 KDa) and the matrix (M) protein (40 KDa) in NDV and to the nucleocapsid protein (51 KDa) and the membrane glycoprotein GP28 (28 KDa) in the case of IBV three weeks after vaccination compared to levels at the time of vaccination (P<0.05).

*Key words: Staphylococcus aureus, Origanum ehrenbergii, free-range poultry, serological profiling, ELISA, Western Immunoblotting.*
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ABBREVIATIONS

% Percent
/
µl Micro liter
Ab Antibody
Ag Antigen
AGID Agar Gel Immunodiffusion Test
ALV Avian Leukosis Virus
ANOVA Analysis of Variance
Ark Arkansas
AUB American University of Beirut
BaCl₂ Barium Chloride
C Celsius
CFT Complement Fixation Test
CFU Coliform Forming Unit
Conn Connecticut
Cm Centimeters
CTL Cytotoxic T Lymphocytes
DMSO Dimethyl Sulfoxide
DNA Deoxyribonucleoside Triphosphate
EDS Egg Drop Syndrome
ELISA Enzyme-Linked Immunosorbant Assay
et al. Et allii (and others)
<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>EDTA</td>
<td>Ethylene-Diamine-Tetraacetic Acid</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization of the United Nations</td>
</tr>
<tr>
<td>G</td>
<td>Gram (s)</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutination</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>MSA</td>
<td>Mannitol Salt Agar</td>
</tr>
<tr>
<td>MWs</td>
<td>Molecular weights</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
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<tr>
<td>N</td>
<td>Normality</td>
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<tr>
<td>NDV</td>
<td>Newcastle Disease Virus</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>VN</td>
<td>Virus Neutralization</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
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<tr>
<td>UKROFS</td>
<td>UK Register of Organic Food Standards</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tween-Tris-Buffer Saline</td>
</tr>
<tr>
<td>TPB</td>
<td>Tryptose Phosphate Broth</td>
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<tr>
<td>TEMED</td>
<td>Tetra-Methyl-Ethylene Diamine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-Buffer Saline</td>
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<tr>
<td>S protein</td>
<td>Spike protein</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Means</td>
</tr>
<tr>
<td>SA</td>
<td>Soil Association</td>
</tr>
<tr>
<td>rpm</td>
<td>Rounds per minute</td>
</tr>
<tr>
<td>°</td>
<td>Degree</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>pH</td>
<td>Hydrogen Potential</td>
</tr>
<tr>
<td>O.D.</td>
<td>Optical Density</td>
</tr>
<tr>
<td>NCM</td>
<td>Nitrocellulose Membrane</td>
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<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>HI</td>
<td>Hemagglutination-Inhibition</td>
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<tr>
<td>MOSS</td>
<td>Monitoring and Surveillance System</td>
</tr>
<tr>
<td>M</td>
<td>Molarity</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MG</td>
<td>Mycoplasma gallisepticum</td>
</tr>
<tr>
<td>Mass</td>
<td>Massachusetts</td>
</tr>
<tr>
<td>ma</td>
<td>Milliamperes</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton (s)</td>
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<tr>
<td>IBV</td>
<td>Infectious Bronchitis Virus</td>
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<tr>
<td>IBDV</td>
<td>Infectious Bursal Disease Virus</td>
</tr>
<tr>
<td>HR</td>
<td>Heptad Repeat</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
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<tr>
<td>H-V-R</td>
<td>Hypervariable region</td>
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To
My Beloved Parents
CHAPTER I

INTRODUCTION

Staphylococci are gram-positive spherical bacteria that occur in microscopic clusters resembling grapes. Almost all strains of *Staphylococcus aureus* produce the enzyme coagulase, hence this bacteria is considered a potential pathogen (Todar 2004).

Staphylococcal skin infection restricts the host response to inflammation characterized by elevated temperature at the site, swelling, the accumulation of pus, and necrosis of tissue. In an aim to evaluate the susceptibility of staphylococcal isolates to oregano essential oil, Nostro *et al.* (2004) showed that oregano essential oil is primarily characterized by its phenol constituents, carvacrol and thymol and by there two precursor monoterpenic hydrocarbons, γ-terpinene and p-cymene. However, no antimicrobial activity was reported for γ-terpinene and p-cymene probably due to the absence of the phenolic hydroxyl group in these hydrocarbon monoterpenes (Dorman and Deans 2000). The most beneficial effect that essential oils have on immune modulation is their ability to inhibit the multitude of inflammatory processes that contribute to almost every immune response in illness and disease (Alexander 2001).

In the first study, *Staphylococcus aureus* bacteria were injected intradermally into the skin of the rabbit ears. Then topical treatments of *Origanum ehrenbergii* essential oil were administered on the inflammation areas. The purpose of the first study was to assess the anti-inflammatory effect induced by *Origanum ehrenbergii* essential oil on *Staphylococcus aureus* skin infection in rabbits.

The outgrowing poultry production in the last two decades has forced some small poultry producers to shift their production into free-range/organic chickens, in
addition to the fact that the natural/organic market has largely grown in the last decade (Neufeld 2002). Free-range/organic chickens, also known as “all natural”, contain neither preservatives nor artificial ingredients and are being raised in a friendly environment.

According to Darre (2003), free-range poultry production increased from 1.9 % in the year 1985 to 15.5 % in the year 1999, as compared to cage poultry production that has dropped from 96.1 % in the year 1985 to 80.1 % in the year 1999.

In organic standards, vaccination is the only mean of prevention; where as hormones, growth promoters and prophylactic use of antibiotics are prohibited. The prevention of disease is accomplished by improving the welfare of the animal. Usually, free-range pullets are vaccinated in their rearing stage, specifically against Newcastle Disease Virus, Infectious Bronchitis Virus, and Marek’s Disease and in some occasions Infectious Laryngotracheitis.

Surveillance for animal diseases has been widely used by epidemiologists and other animal health experts to aid in monitoring and controlling health-related measures in animal populations (Salman et al. 2003). Disease surveillance is the device that provides information for scheduling eradication programs and disease control. The data obtained for a monitoring and surveillance system (MOSS) should be used to assess the health status of an animal population and to trigger action. A vital requirement for evaluating a MOSS is by using a transparent, objective, and systemic approach. Integrated quality assurance mechanisms and precise objectives will facilitate in evaluating a well-documented MOSS (Salman et al. 2003).

Many disease surveys require the application of laboratory diagnostic tests to analyze specimens collected from the animal. An example is the use of laboratory tests such as Complement Fixation Tests (CFTs), ELISAs, or Agar Gel Immunodiffusion.
Tests (AGIDs) to evaluate the presence of antigens and antibodies in the blood. Few laboratory tests are ideal, even though most tests give inaccurate results only occasionally. When using laboratory examinations in a disease survey, it is advisable to understand the accuracy of the test, and the errors that might occur (Cameron 1999).

In the second study, a disease surveillance study for the first time in Lebanon was conducted on a group of free-range poultry farms after which serological profiling was conducted to determine the disease outbreaks. Another serological profiling was conducted to the same farms after 3 weeks of vaccination with an inactivated trivalent vaccine in order to assess the immunological responses of the birds to the vaccine. Furthermore, the immune responses to the trivalent vaccine were both quantitatively and qualitatively assessed by Western Immunoblotting, thus reacting the pooled farm sera with the antigens of the trivalent vaccine and antigens of the live vaccine strains including ND clone 30, IB H120 and IB 4-91.
CHAPTER II  
LITERATURE REVIEW  

A. Experiment 1: Anti-Inflammatory Effect of *Origanum ehrenbergii* against *Staphylococcus aureus* Skin Infection in Rabbits  

1. *Staphylococcus aureus*  

a. Introduction  

Staphylococci are gram-positive spherical bacteria that occur in microscopic clusters resembling grapes. Rosenberch (1884) described two pigmented colony types of staphylococci and named them as *Staphylococcus aureus* and *Staphylococcus albus* that later became known as *Staphylococcus epidermitis*. Bergey’s manual (Bergan and Kocur 1986) describes more than 20 species of Staphylococcus, however, *Staphylococcus aureus* and *Staphylococcus epidermitis* are the most likely to interact with animals. *Staphylococcus aureus* is hemolytic on blood agar and forms a yellow colony on rich medium. Most strains of *Staphylococcus aureus* produce the enzyme coagulase, hence this bacteria is considered a potential pathogen (Todar 2004).  

b. Pathogenesis of *Staphylococcus aureus* Infections  

The ability of *Staphylococcus aureus* to avoid the host immune system response and cause pathogenesis is due to an extensive range of virulence factors including two lipases, exotoxins, enterotoxins, four hemolysins, and several proteases (Somerville *et al.* 2002). The production of several virulence factors is regulated by the accessory gene regulatory (*agr*) operon (Morfeldt *et al.* 1988; Peng *et al.* 1988) and several other globular regulatory loci.  

There is a common pathway by which both gram-positive and gram-negative
bacteria induce the production of different inflammatory mediators, such as factors of
the coagulation, complement, and contact systems, which act simultaneously with
cytokines forming a complex inflammatory network (Bone 1993; Glauser et al. 1991).
The contact system through their enzymatic factors release bradykinin (BK) that
induces vasodilatation (Mattsson et al. 2001). The coagulation of plasma is a well-
established phenomenon for the majority of *Staphylococcus aureus* pathogenic strains
(Blair 1939; Bayliss and Hall 1965).

Staphylococcal skin infection restricts the host response to inflammation
characterized by elevated temperature at the site, swelling, the accumulation of pus, and
necrosis of tissue. Around the inflamed area, a fibrin clot may form, walling off the
bacteria and leukocytes as a characteristic pus-filled boil or abscess. More serious
infections of the skin may occur, such as furuncles or impetigo (Todar 2004).

c. Invasion by *Staphylococci*

Staphylococci invasion of host tissues involves the production of a large range
of extracellular proteins. A major membrane-damaging toxin produced by
*Staphylococcus aureus* is α-hemolysin that binds to the membrane of susceptible cells
forming a pore through which cellular contents leak. Another important membrane-
damaging toxin is leukocidin whereby almost 90 % of *Staphylococcus aureus* strains
isolated from dermatonecrotic lesions express this toxin, hence suggesting the
importance of leukocidin in necrotizing skin infection (Todar 2004).

An important extracellular protein that plays a role in Staphylococci invasion is
coagulase that is a traditional marker for identifying *Staphylococcus aureus* in
microbiology laboratories. Coagulase will lead to the conversion of fibrinogen to fibrin;
hence *Staphylococcus aureus* might possibly form localized clotting to protect itself
from phagocytic and immune defenses of the host (Todar 2004).

2. Origanum Species and Their Essential Oils
   a. Introduction

   The genus Origanum (Labiatae family) is characterized by a large morphological and chemical diversity: forty-nine taxa divided into ten sections belong to this genus, most of them having a local distribution around the Mediterranean. In particular, three taxa are restricted to Morocco and south of Spain, two occur in Algeria and Tunisia, three are endemic to Cyrenaica, nine are restricted to Greece, south Balkan and Asia Minor (six are local Greek endemics), 21 are found in Turkey, Cyprus, Syria and Lebanon (21 are local Turkish endemics), and eight are locally distributed in Israel, Jordan and Sinai Peninsula. The essential oils of the members of the Origanum genus vary in respect to the total amount produced by plants (ranging from traces to 8ml/100 g of dry weight) as well as in their qualitative composition. Origanum essential oils are characterized by a number of main components, which are implicated in the various plant odors. A wide chemical diversity is found even within a single Origanum species, like the widely used *O. vulgare*. The pattern of variation of quantitative and qualitative essential oils in the latter species follows its geographical distribution or depends on the time of plant collecting (Padulosi 1997).

   The Origanum species are sub-shrubs or perennial herbs with seven stems, ascending or erect, subsessile or petiolate leaves and flowers in verticillasters aggregated in dense or loose spike which are arranged in a peniculate or corymbiform inflorescence. Origanum plants are widely used all over the world as very popular spice, under the vermicular name “oregano”. They are of great economic importance, which is not only related to their use as a spice. In fact, as recent studies have pointed
out, oregano is used traditionally in many other ways as their essential oils have anti-microbial, cytotoxic, and anti-oxidant activity (Lagouri et al. 1993; Sivropoulou et al. 1996).

The highest yields correspond to plants growing at low altitudes, in Mediterranean ecosystems, as is common for the whole family of Labiatae (Kokkini et al. 1989). It should be noted that these values are the highest essential oil yields reported for any oregano plant.

A number of studies have shown that variation within a single *Origanum* species may occur in its morphological and chemical features. Furthermore, it has been found that the pattern of variation of a single species follows its geographical distribution or it depends on the season of plant collecting. Quantitative and qualitative essential oil analyses have shown that the major constituents are carvacrol and/or thymol, accompanied by p-cymene and γ-terpinene (Vokou et al. 1993).

b. **Morphology of the Origanum Species**

The morphological variation within the genus results in the distinction of 10 sections consisting of 42 species or 49 taxa (species, subspecies and varieties) (Ietswaart 1980; Carlstrm 1984; Danin 1990; Danin and Künne 1996). Following Ietswaart’s classification (1980), Origanum ehrenbergii under section Prolaticorolla was endemic to Lebanon. Section Prolaticorolla comprises 3 species endemic to eastern or western parts of the Mediterranean. These species are characterized by dense spikes and tubular calyces becoming turbinate in fruiting.

c. **Storage Conditions of Origanum Species**

There is little information on the conditions in which the genetic resources of
Origanum are being preserved. Most institutions hold seed collections and only a few maintain field collections. Seed collection of Origanum does not need particular conservation methods: seeds are preserved in the same controlled conditions used for any other orthodox-seeded plant, thus being maintained in short-, medium-, or long term storage rooms. As for most aromatic plants, also for Origanum, long-term storage (-18 C) is a good conservation method, which ensures the safe seed conservation for at least a period of eight years (Montezuma-De-Carvalho et al. 1984).

d. Classification of Essential Oils

The essential oils of Origanum members vary in respect of the total amount produced per plant as well as in their qualitative composition. Based on their essential oil content, the different taxa of the genus can be distinguished as 3 main groups:

- Essential oil “poor” taxa with an essential oil content of less than 0.5% (ml/100g dry weight), e.g. the Greece endemic O. calcaratum (Karousou 1995);
- Taxa with an essential oil content between 0.5 and 2%, e.g. the Cretan endemic taxon O. microphyllum known as Cretan marjoram (Karousou 1995);
- Essential oils “rich” taxa with an essential oil content of more than 2%, as for example the two most well commercially known “oregano” plants, O. vulgare subsp. hirtum (Greek oregano) and O. onites (Turkish oregano) (Kokkini et al. 1991; Vokou et al. 1993).

e. Chemical Composition of Essential Oils

With reference to their essential oil composition, Origanum taxa may be characterized by the dominant occurrence of the following compounds:

- Linalool, terpinen-4-ol, and sabinene hydrate like the essential oil of
O. majorana (Fisher et al. 1987);

- The phenolic compounds, carvacrol and/or thymol, like the essential oils of O. vulgare subsp. hirtum (Kokkini and Vokou 1989; Kokkini et al. 1991; Vokou et al. 1993) and O. onites (Vokou et al. 1988; Ruberto et al. 1993);

- Sesquiterpenes like the essential oil of O. vulgare subsp. vulgare (Lawrence 1984).

A study done by Alma et al. (2003) on the chemical composition of essential oil of Origanum syriacum showed that the phenolic compounds are relatively high. Among those phenolic compounds was carvacrol constituting 26.97 % of the essential oil. Baser et al. (2003) reported that Origanum syriacum grown in Turkey contained carvacrol (64.1 %) and p-cymene (12.3 %) as major components. Fleisher and Fleisher (1991) reported that while essential oil from Origanum syriacum originating from Israel contained thymol (59.87 %) and carvacrol (80.17 %) as a major component, the essential oil from the same plant originating in Egypt contained only carvacrol as the major constituent (76.7 %). These sources of variation are possibly due to growing conditions, harvesting time and geographical origin.

In an aim to evaluate the susceptibility of staphylococcal isolates to oregano essential oil, Nostro et al. (2004) showed that oregano essential oil is primarily characterized by its phenol constituents, carvacrol and thymol and by there two precursor monoterpane hydrocarbons, γ-terpinene and p-cymene. However, no antimicrobial activity was reported for γ-terpinene and p-cymene probably due to the absence of the phenolic hydroxyl group in these hydrocarbon monoterpenes (Dorman and Deans 2000).
f. Characteristics of Oregano

- Utilization as a spice

Records on the use of oregano date back thousands of years: the famous “hyssop”, mentioned in the Bible, is believed to be an *Origanum syriacum* L. plant (Hepper 1987). Today, oregano plant parts are commonly used in the food industry as a spice. In spite of its long European history, oregano was only introduced into America at the beginning of this century, and afterwards to other parts of the world. Today oregano can be considered one of the most important spices both in Mediterranean countries (Carmo et al. 1989; Baser et al. 1992, 1993; Vokou et al. 1988, 1993) and elsewhere (International Organization for Standardization 1985). The overall market of oregano is about 350-500 tons in France, 600 tons in Germany, 500 tons in the UK and 150 tons in the Netherlands (Maftei 1992).

The popularity of oregano is increasingly growing as a result of scientific developments achieved in the area of its cultivation and utilization. More and more new interesting varieties are being produced, thus contributing to broadening the horizon of its actual application.

- Antibacterial and antifungal activity

The antibacterial and fungicidal activity of oregano has been reported in many works; its oil has a bactericidal effect against *Erwinia amylovora* (Scortichini and Rossi 1989 1993) and many other microorganisms such as *Bacillus subtilis*, *Escherichia coli*, *Hafnia alvei*, *Micrococcus luteus*, *Proteus vulgaris*, *Staphylococcus aureus* and *Streptococcus faecalis* (Biondi et al. 1993). Research findings also report inhibiting effects on the growth of some plant and animal pathogens, as well as organisms causing food spoilage (Deans and Svoboda 1990; Deans et al. 1992; Izzo et al. 1995). On honeybee pathogens, the bactericidal and fungicidal effects of oregano extracts are
reported for *Bacillus larvae* (causing American foulbrood), *Ascosphaera apis* (causing chalkbrood) and *Bacillus alvei* (a secondary pathogen involved in European foulbrood). These findings, confirmed by Calderone *et al.* (1994), highlight the important role that oregano can also play in the management of honeybee diseases.

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

The antibacterial/fungicidal activity of oregano oil is used today in a number of practical applications. An interesting example is provided by the following case: an aerosol formulation of its extracts (containing 0.4% mixture of essential oils) was sprayed into three enclosed areas (school hall, analytical laboratory and library) and a drastic reduction of the microorganisms present in the air was registered (a drop of 74-93%) (Panizzi and Pinzuti 1989).

- **Antiviral activity**

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

- **Nematicidal activity**

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

- **Anti-oxidant activity**

  The anti-oxidant activity of natural sources, including Origanum species, has been investigated (Dorofeev et al. 1989; Nguyen et al. 1991; Sawabe and Okamota 1994; Takacsova et al. 1995). Lagouri et al., (1993) have demonstrated the anti-oxidant activity of *O. vulgare subsp. hirtum* (Greek oregano), *O. onites* (Turkish oregano), *Coridothymus capitatus* (Spanish oregano) and *Satureja thymbra*. Results of these findings indicate that the anti-oxidant effect may be related to the presence of carvacrol and thymol in the essential oils. Other chemical compounds such as terpenoids may be responsible for the anti-oxidant activity; this is the case of glucosides (Nakatani and Kikuzaki 1987) or flavonoids (Vekiari et al. 1993), both extracted from the leaves of oregano. Five phenolic acids with anti-oxidant properties were isolated by Kikuzaki and Nakatani (1989), their activity being greater than the one shown by alpha-tocopherol.

### 3. Essential Oils and Immunity

The most beneficial effect that essential oils have on immune modulation is their ability to inhibit the multitude of inflammatory processes that contribute to almost every immune response in illness and disease (Alexander 2001).

The inflammatory process involves a series of events that can be elicited by
numerous stimuli such as infectious agents, ischaemia, antigen-antibody interactions, and thermal and other physical injuries. Inflammatory responses occur in three distinct phases, each apparently mediated by different mechanisms:

- An acute transient phase, characterized by local vasodilatation and increased capillary permeability;
- A delayed, subacute phase, most prominently characterized by infiltration of leucocytes and phagocytic cells; and
- A chronic, proliferative phase, in which tissue degeneration and fibrosis occur (Alexander 2001).

One of the chief functions of the immune system is to respond to pathogenic or inflammatory agents, which activate cytokines, which scavenge for the antigens. The cytokines are a group of diverse proteins produced by lymphocytes and related cells that have a number of discrete roles in regulation of the immune system, as well as hematopoiesis (blood cell formation). Cytokines include interferons, colony stimulating factors like granulocytes and monocytes, and the interleukins (IL), and tumour necrosis factor (TNF) (Alexander 2001).

Responses to inflammation also trigger the expression of eicosanoids (leukotrienes, prostaglandins, thromboxane) and platelet-activating factor (PAF). The eicosanoids play a prominent role in inflammation, are extremely prevalent, and have been detected in almost every tissue and body fluid. Because virtually every cell can form eicosanoids, it is not unreasonable to suspect that each pharmacological effect may reflect a physiological and pathophysiological function (Alexander 2001).

Many essential oils owe their anti-inflammatory effects to blockade of eicosanoids. For example, cardamom essential oil reduces the synthesis of eicosanoid mediators of inflammation, also exhibiting analgesic activity (Al-Zuhair 1996).
Histamine, a hormone/chemical transmitter is involved in local immune responses, regulating stomach acid production and in allergic reactions as a mediator of immediate hypersensitivity. Histamine is often the chief catalyst in an inflammatory process. The histaminergic (binding and liberating histamine) system is thought to function in the regulation of arousal, body temperature, and vascular dynamics. Histamine might be a central neurotransmitter due to the fact that histamines and anti-histamines active in the periphery have been known to produce significant effects on animal behavior. Thus, essential oil acting in a histaminergic capacity has central as well as peripheral activity (Schwartz 1994).

Inflammation caused by hypersensitivity to allergens and upon cellular injury results from a chemical cascade facilitated by histamine activation, by affecting inflammatory mediators of the immune system, which stimulate inflammation. The mast cell is the predominant storage site for histamine; the concentration of histamine outside the brain is particularly high in tissues that contain large numbers of mast cells, such as the mucosa of the bronchial tree, the intestinal mucosa and skin. The actions of histamine on bronchial and smooth muscle and blood vessels account in part for the symptoms of the allergic responses that are caused by mast cell activation. Mast cells (which are leukocytes) contain metachromatic granules that store a variety of inflammatory mediators. These include:

- Histamine and serotonin
- Proteolytic enzymes that can destroy tissue or cleave complement components.
- Heparin or chondroitin sulfate which are anticoagulants.
- Chemotactic factors, such as eosinophil chemotactic factor of anaphylaxis (an important regulator of eosinophil function) and neutrophil chemotactic factor.
Normally, mast cells are not found in circulation.

Allergies are abnormal immune system reactions to things that are typically harmless to most people. Substances that cause allergic reactions, such as certain foods, dust, plant pollen, or medicines are known as allergens. In an attempt to protect the body, the immune system produces IgE antibodies to that allergen. Those antibodies then cause certain cells in the body to release chemicals into the bloodstream, one of which is histamine. The histamine then acts on a person's eyes, nose, throat, lungs, skin, or gastrointestinal tract and causes the symptoms of the allergic reaction. Future exposure to that same allergen will trigger this antibody response again.

Though histamine is a mediator of the inflammatory process, agents that antagonize (block) their releases are useful only for the treatment of vascular events in the early transient phase of inflammation (like burns). There are many essential oils that block the actions of histamine that should respond well to hypersensitivity reactions or other cellular injury, including lavender (LisBalchin & Bart 1999), peppermint (Taylor 1983), rosemary (Aqel 1991), blue chamomile (Miller 1996), clary sage (Moretti 1997), curcuma leaf (Chandra & Gupta 1972), black cumin (El Tahir 1993), croton (Coelho-de-Souza 1997), carum capticum (Boskabady & Shaikhi 2000), origanum compactum (Van Den Broucke & Lemli 1982), Todalia asiatica (Kavimani 1996), Alpinia speciosa and A. japonica (Morita 1996) and eugonol-containing essential oils (Hume 1983). One of the greatest benefits of using essential oils as anti-inflammatory agents is that many essential oils exert multiple anti-inflammatory effects related to inflammatory mediators like histamine, kinin, and prostaglandin by inhibiting their synthesis (Kavimani 1996), as well as inhibit platelet-activating factor (PAF) (Murayama & Kumaroo 1986) and thromboxane production (Saeed and Gilani 1994).

Eugenol, a common and often the most effective constituent of many essential
oils has been shown to be a histamine antagonist, a kinin antagonist (Sharma 1997) and inhibits PAF (Saeed & Gilani 1994; Janssens 1990). The results of Kim and Cho (1997) suggest that eugonol possesses antianaphylactic properties by preventing mast-cell degranulation. The effect of eugenol on vascular resistance was examined by Hume (1983). The results showed it depressed vasoconstrictor responses in response to exogenous histamine.

Histamine antagonists are most useful in dermatology to treat pruritis due to urticaria, atopic dermatitis, contact dermatitis, psoriasis, and many other clinical conditions facilitated by histamine activation. Lavender essential oil applied topically potentially inhibited allergic responses to sensitized skin by inhibiting histamine release from mast cells, which indicated that mast-cell mediated immediate-type allergic reactions were inhibited (Kim and Cho 1997).

The degranulation of mast cells liberates many other inflammatory mediators other than histamine, including prostaglandins, leukotrienes, thromboxane, platelet-activating factor and kinins. Many essential oils inhibit the synthesis of these inflammatory mediators. Prostaglandins are found in almost every tissue and body fluid, and are important factors in the occurrence of inflammation. Prostaglandins are released whenever cells are damaged and they appear in inflammatory exudates (Alexander 2001).

Inflammatory process also involves reactive oxygen species started by leukocyte activation. Grabmann (2000) investigated two common essential oils used to treat irritations and infections of the respiratory tract. They found that eucalyptus and myrtle attenuated leukocyte activation by scavenging hydroxyl radicals, indirectly produced by leukocyte degranulation, thereby interfering with inflammatory processes by acting as antioxidants. It has been shown by many researches that essential oils
possess antioxidant properties in various physiological processes, including rosemary, oregano, geranium, lemongrass, nutmeg and thyme (Deans 1993; Zheng 1993; Aeschbach 1994).

B. Experiment 2: Serological Profiling and Molecular Detection of Protective Polypeptides in Sentinel Free-Range Layers

1. Surveillance and Epidemiology

a. Introduction

Surveillance for animal diseases has been widely used by epidemiologists and other animal health experts to aid in monitoring and controlling health-related measures in animal populations (Salman et al. 2003).

The two terms monitoring and surveillance are often used interchangeably in animal health programs. Animal disease surveillance involves examining an animal population carefully to determine if a particular disease enters that population. Monitoring of animal diseases identifies a disease to establish changes in prevalence and find out the direction and rate of disease spread. The World Organization for Animal Health, OIE defines these terms as follows:

- *Surveillance*: “surveillance means the continuous investigation of a given population to detect the occurrence of disease for control purposes, which may involve testing of a part of the population”.

- *Monitoring*: “monitoring constitutes on-going programs directed at the detection of changes in the prevalence of disease in a given population and in its environment” (Cameron 1999).

According to Cameron (1999), there are 2 types of surveillances, namely the active surveillance and the passive surveillance.
Active surveillance: Unlike passive reporting system, active surveillance differs by using surveys of a relatively small, representative sample of the population. Advantages of active surveillance are better quality of information collected, the information estimated the true situation in the whole population, and it is usually quicker and inexpensive to collect as compared to passive methods.

Passive surveillance: in spite of their disadvantages such as under reporting, cost and non-representative reports, passive reporting systems are an essential source of disease information. Almost all countries are following the passive disease reporting systems; with relatively few exceptional countries following the active surveillance. This is probably because appropriate techniques were previously lacking, and the veterinary staff was unqualified in the necessary skills.

Disease surveillance is the device that provides information for scheduling eradication programs and disease control. The data obtained for a monitoring and surveillance system (MOSS) should be used to assess the health status of an animal population and to trigger action. Documentation is a precondition for evaluating the value of the monitoring and surveillance system. All standards on procedures and methods should be recognized in detail. Documentation includes the following steps:

- Sampling
- Processing the samples
- Recording the data
- Laboratory analysis
- Statistical analysis (Salman et al. 2003).

The monitoring and surveillance system designer must characterize the type of information that the system will gather, and should also describe the proposed uses of the data before the system is in process. An ideal MOSS for an animal disease should
gather data on the agent, the host and the environment (Hueston 1993). The data collected should consist of the following:

- The number of incidences of the disease
- The animal species affected
- The population at risk
- The type of production system in which the incidences were found
- The geographical location of the incidences
- Whether there is laboratory conformation of the disease
- The type of test used for conformation (Hueston 1993).

A vital requirement for evaluating a MOSS is by using a transparent, objective, and systemic approach. Integrated quality assurance mechanisms and precise objectives will facilitate in evaluating a well-documented MOSS (Salman et al. 2003).

Understanding the distribution of the disease is an efficient way to control the disease spread. For this purpose, the following steps should be considered:

- Both intensive and non-intensive animal industries must be located
- The level of disease in different areas, and
- The pattern of animal movements between different areas (Cameron 1999).

b. Disease Surveys

In order to create complete reporting (and precise measures of disease frequency), a passive disease reporting system requires information about every single case of the main diseases present in the country. Hence, each animal must be examined regularly. Such kinds of data collection are recognized as census, where all members of the population are checked (Cameron 1999).

Surveys are capable to collect information rapidly and inexpensively because
as an alternative of requiring a census, where the whole population is examined (by unqualified owners), only a small percentage (a sample) of the population is examined (by qualified veterinary staff) (Cameron 1999).

The sample usually constitutes of a small group of animals that have been chosen from their population. So a survey examines a small group (a sample) selected from the population. Nevertheless, the problem with surveys is that they accurately identify the disease status of the sample; but little is known about the rest of the population that was not examined. Hence, we can only assume the disease status of the population. So inference is the mechanism by which we can estimate the similarity in disease status between the sample and the population (Cameron 1999). In order to exclude the possibility of the inference from being wrong, the sample and the population have to be almost the same with respect to the unit of interest so we can have a representative sample. If not, then the estimate is considered as biased that is caused by systemic error.

Choosing a representative sample is a difficult task in any animal disease survey. To select a representative sample, it is a must to ensure that each animal in the population has an equal probability of being selected in the sample, regardless of its location, size, owner or any other characteristic. Sampling techniques of this kind are recognized as random sampling. Hence random sampling is a reliable technique in selecting a representative sample (Cameron 1999).

c. Measure of Disease

For an effective control of disease, the distribution of the disease must be understood in terms of quantity, location, and the animals affected. Disease surveys are based on considering the number of infected and uninfected animals. Prevalence and
Incidence rate are the two main measures of disease used for active surveillance (Cameron 1999).

Prevalence: “prevalence (sometimes called point prevalence) is a measure of the number of animals with the disease of interest at a specific time, as a proportion of the total number of animals in the population” (Cameron 1999).

Incidence Rate: “incidence rate (specifically true incidence or incidence density rate) is a measure of the average speed at which the disease is spreading. Incidence rate is the total number of new cases of disease divided by the total time that each animal in the population was at risk of getting the disease” (Cameron 1999).

In many occasions, prevalence surveys are sufficient to identify the level of disease present in a population; at times incidence rate surveys might replace the prevalence surveys. In addition, both survey types can be implemented together when laboratory tests fail to differentiate among naturally acquired and vaccine antibodies (Cameron 1999).

d. Seroprevalence versus Clinical Prevalence

Prevalence surveys can determine any aspect of disease, clinical symptoms of disease, indication of subclinical disease, or proof that the animal was infected earlier, but has now recovered. In each situation, the duration of the disease varies. Clinical disease usually lasts for a relatively short duration. Subclinical disease comes before clinical disease in a longer time. Evidence of earlier exposure to a disease, in the form of antibodies, persists for a much longer period of time after the disease (Cameron 1999).

When conducting surveys on rare diseases having a low prevalence, a large number of animals should be evaluated to find a significant number carrying the
disease. Diseases of high prevalence require less animals to be examined, thus the survey will be less expensive and faster. This is why seroprevalence surveys (“surveys to estimate the prevalence of animals with high levels of antibodies indicating previous exposure to the disease or previous vaccination”) are frequently used in animal disease control programs. It is usually more practical to measure the level of disease through a seroprevalence survey rather than a clinical prevalence survey since it requires a smaller sample size. One advantage of clinical prevalence is that only clinical examination of animals is required, while laboratory examinations are not needed. This may be faster and cheaper, but laboratory examinations aid in developing more reliable diagnosis (Cameron 1999).

e. Diagnostic Tests

Many disease surveys require the application of laboratory diagnostic tests to analyze specimens collected from the animal. An example is the use of laboratory tests such as Complement Fixation Tests (CFTs), ELISAs, or Agar Gel Immunodiffusion Tests (AGIDs) to evaluate the presence of antigens and antibodies in the blood. Few laboratory tests are ideal, even though most tests give inaccurate results only occasionally. When using laboratory examinations in a disease survey, it is advisable to understand the accuracy of the test, and the errors that might occur (Cameron 1999).

f. Sensitivity and Specificity

The reliability of a test is assessed by two measures, sensitivity and specificity. “The sensitivity of a test measures the proportion of truly diseased animals that the test correctly identifies as diseased. The specificity measures the proportion of non-diseased animals that the test correctly identifies as non-diseased” (Cameron 1999).
2. Free-Range Poultry Production

a. Introduction

The outgrowing poultry production in the last two decades has forced some small poultry producers to shift their production into free-range/organic chickens, in addition to the fact that the natural/organic market has largely grown in the last decade (Neufeld 2002).

Free-range/organic chickens, also known as “all natural”, contain neither preservatives nor artificial ingredients and are being raised in a friendly environment.

According to Darre (2003), free-range poultry production increased from 1.9 % in the year 1985 to 15.5 % in the year 1999, as compared to cage poultry production that has dropped from 96.1 % in the year 1985 to 80.1 % in the year 1999.

Many important issues must be considered while designing and managing free-range/organic poultry systems, these include:

- Breed origin, suitability and rearing of stock
- Behavior, housing and welfare
- Sources and types of feedstuffs that must provide suitable rations with acceptable costs
- Animal health and treatment, specifically towards coccidiosis, cannibalism, and other infectious diseases
- Stocking densities, manure handling and the access to range
- Market demands
- Financial returns and cost of poultry enterprises (Lampkin 1997).

b. Feeding

Diet is still a point of controversy between free-range and organic standards,
where free-range standards do not concentrate on the feed type and quality. While organic standards suggest that 100% of the diet to be organic with some allowance for non-organic components. The UKROFS (UK Register of Organic Food Standards) (1996) standards state that a minimum of 50% of the diet to be fully organic, while the rest comes from sources that are converting to organic production. The EU draft regulation is proposing additional rules that restrict the use of non-organic materials to 20% while increasing the fully organic materials to 60%. Furthermore, the EU draft regulation suggests that the holdings should produce their own animal feed where a special approval is needed to buy in feeds.

c. Health and Medication

In organic standards, vaccination is the only mean of prevention; where as hormones, growth promoters and prophylactic use of antibiotics are prohibited. The prevention of disease is accomplished by improving the welfare of the animal. However, the UKROFS (1996) gave some exceptions towards the use of anthelmintics, specifically the use of coccidiostats accompanied by managerial practices to minimize the problems.

The EU free-range and barn regulations and the RSPCA (The RSPCA welfare standards for laying hens and chickens) (1995) have no restrictions on vaccination, growth promoters, hormones or the use of medications.

At present, no organic pullet-rearing activity is being held to provide layers for organic egg production, except for small enterprises. According to the Soil Association (SA) standards (1996), layers might be accepted from conventional sources up to the age of 16 weeks where they will be subjected to a six weeks conversion period. This type of management is widely used especially for large-scale producers that would be
minimizing their cost of production.

Usually, free-range pullets are vaccinated in their rearing stage, specifically against Newcastle Disease Virus, Infectious Bronchitis Virus, and Marek’s Disease and in some occasions Infectious Laryngotracheitis. However, it is recommended that the producers do not buy vaccinated birds if they do not have the infection on their unit (Lampkin 1997).

d. Housing

Organic/free-range poultry producers have two approaches for housing systems; static housing systems, where chickens have access to outside areas covered with vegetation, and mobile systems that allow houses to be moved for better utilization of vegetation when resources are limited (Lampkin 1997).

The UKROFS standards (1996) insist on the necessity that birds must have easy access to the outside areas; if such conditions are not fulfilled, then the free-range farms will not be accredited. Organic standards aim to minimize stress by providing an optimum environment for the birds in which they can practice their normal behavior patterns. Minimizing stress on the birds will reflect positively on the production capacity and the health of the flock. Most free-range farms are designed in a way to account for the behavioral needs of the birds especially during night times when the farms are closed to prevent the entry of predators.

e. Behavioral Considerations

Chickens are known for their strong pecking order, where birds have the ability to distinguish each other by their head forms. Hence excessive numbers of birds are threatened by serious pecking problems that will result in a socially unstable group
leading to the formation of subgroups within the flock (Keppler et al. 1996).

Attractive nests are necessary in reducing the number of mislaid and ground eggs, consequently, this will facilitate the time needed for egg collection and will reduce the number of dirty eggs. Nests are usually placed away from the light in a quit area of the barn but not far from the other facilities (Bauer et al. 1994). Nests having an enclosed framework seem to be preferred especially when located undercover in the wild (Appleby 1984). Comfort behavior of free-range hens is associated with sand and sun-bathing to maintain proper hygiene and an effective control of ectoparasites (Lampkin 1997).

f. Lighting

According to the organic standards, natural lighting should be available knowing that direct sunlight causes problems associated with cannibalism and feather pecking. Such problems can be avoided by placing extended roofs facing the windows or by covering the windows with white paint (Lampkin 1997).

Artificial lighting is allowed to reach a maximum of 16 hours of lighting per day, whereby the lighting should be provided in the morning instead of the evening. This will allow the majority of the birds to lay their eggs early in the morning before leaving the barn thus reducing the chances of obtaining dirty eggs (Lampkin 1997).

g. Fencing

Fencing is not a must in free-range farms since birds do not usually wonder far away from their premises and they are shut up at night. However, fencing is recommended to protect both the flock and the vegetation if predators are present. Two kinds of fencing are implemented; the most effective is the electric netting that are
connected to batteries and the permanent non-electric fence that is usually 2 meters high with a portion that is buried to prevent digging by foxes (Lampkin 1997).

h. Animal Health

Most of the organic producers surveyed reported very few or no health problems, emphasizing the need for good management and meticulous care with respect to hygiene and cleanliness, including disinfections between batches. However, there currently appear to be some health problems in organic poultry production for which specific solutions in the spirit of the organic standards have not been found. For other health issues, such as virus diseases, it is assumed that satisfactory solutions can be achieved with prevention through management and/or permitted methods of treatment/vaccination. Day-old chicks and pullets purchased from conventional hatcheries and rearers will be routinely vaccinated against a number of diseases in any case. In practice, few (if any) organic producers used vaccines once the birds had arrived on the holding (Lampkin 1997).

Mainstream poultry units have generated the concept of "in house" biosecurity, where disease challenge is met by vaccination for viral infections and environmental controls (air quality etc.) for bacterial problems. Organic animal health standards restrict the use of prophylactic medicines. This, and the consequences of being free-range, means that organic systems are potentially at a greater risk from some diseases. However, low stocking rates and access to outdoor environments reduce the potential for other diseases associated with dense populations, high levels of inputs, enclosed environments and restricted movement. Organic systems can avail themselves of the advantages of vaccinations based on risk assessment and the lessons that have been learned from good basic hygiene and health management practices in conventional
systems (Hovri et al. 2001).

A survey of small-scale organic producers in the UK revealed that producers do not generally perceive a problem with the health of their flocks (Roderick et al. 1996; Roderick and Hovi 1999). Other studies, in particular those described by Permin and Nansen (1996) and Permin et al (1999), suggest that organic poultry are subject to a range of diseases, including internal parasites picked up whilst ranging. Lampkin (1997) identifies coccidiosis, feather pecking and cannibalism, and external parasites as significant potential problems in organic and other free-range systems. Swarbrick (1986) identified a range of diseases and welfare problems in free-range systems in southern England. In Canada, Herenda and Jakel (1994) recorded significantly higher levels of disease related condemnation rates in "vegetarian" (also referred to as organic) as compared to other free-range and "standard" systems.

Contamination from exotic birds is also a possibility. Infections of virulent forms of Newcastle disease have been associated with birds purchased as pets. Free-range chickens are at a greater risk than intensively managed birds, as they are likely to be in close contact with wild and migratory birds that carry the disease (Alexander and Gough 1986).

i. Feather Pecking and Cannibalism

Feather pecking and cannibalism currently appear to be a big problem for larger-scale organic egg producers. Cannibalism is a problem in many conventional free-range systems, sometimes, but not always associated with feather pecking which is only one of several pre-disposing factors (along with group size and light intensity) (Lampkin 1997).

Feather pecking alone might not necessarily represent a serious problem, even
though it leads to a partial loss of feathers and can represent a visual problem. However, feather pecking often is followed by cannibalism, where hens inflict injuries mainly to the cloacal region of other hens, which can lead to further health problems because of infection and also has negative impacts on production. Producers therefore try to reduce feather pecking in the first instance in order to avoid the problem of cannibalism (Lampkin 1997).

A variety of factors have been suggested as causes for feather pecking including exposure to sunlight, insufficient protein supply, excessive egg sizes, excessive group size, stress and boredom. In addition, attention should also be focused on breeds. A tendency to engage in feather pecking is partly inherited and it appears that most modern lines of hybrids selected under intensive conditions have similar problems when housed in free-range systems (Bauer and Keppler 1996).

Cannibalism can also be related to parasite problems and infections in the ovaries and cloacal region of the layers. Birds already infected might draw the attention of other hens, and the wounds resulting from cannibalism can be the cause of further infection. It is clear that a wide range of factors are potentially involved, and that producers need to pay attention to all of these if they are to avoid cannibalism problems in free-range systems without the use of beak clipping. In summary they should pay attention to the rearing of pullets, enriched housing systems, offering a variety of feed sources (including scratch feed) in a balanced ration, equal distribution of light (including daylight) and regular checks of climate and general health of the flock (Bauer and Keppler 1996).

Permin and Nansen (1996) view the incidence of cannibalism, with the risk of secondary Escherichia coli infection, as a growing problem on organic farms. Keeling et al. 1988 observed an outbreak of cannibalism in a free-range flock that occurred
primarily in the last 8 weeks of lay. Cannibalism and pecking have also been observed in other free-range flocks in England. An extensive postal survey of "alternative" poultry systems in the UK and showed that over 55% of respondents reported feather pecking in laying flocks (Swarbrick 1986).

Flock size and density factors were also observed in research on laying hens in percheries, which showed that mild feather pecking increases with age, flock size and stocking density. However, although rare, aggressive pecking was most common in small flocks of low stocking density. This was related to birds attempting to form social hierarchies, whereas large flocks at high density appear to adopt non-social, non-aggressive behavioral strategies (Nicol et al. 1999).

j. Egg Drop Syndrome (EDS)

Egg drop syndrome causes decreased egg production (Van Eck el. 1976). It is caused by a duck adenovirus belonging to the genus Atadenovirus. The EDS virus is nonenveloped, hemagglutinating, DNA virus, 74-80 nm in diameter, which replicates in the nucleus of host cells (Jordan 1990).

The infected birds lay soft-shelled or shell-less, discoloured and miss-shapened eggs. EDS virus may cause 40-50% decrease in egg production. If the disease is due to reactivation of latent virus, the fall usually occurs when production is between 50% and peak level. In acute cases there may be mild depression, however general appearance, feed and water intake of the affected bird remain normal (Yamaguchi et al. 1980). EDS virus spreads both vertically through the embryonated eggs (Adair et al. 1979) and horizontally. The horizontal transmission of the disease in the flock is usually faster. The virus can survive in the litter of an infected poultry house for many weeks. Both domestic and wild ducks may act as a carrier and play a vital role in the transmission of
disease (McFerran 1999).

The EDS virus agglutinated avian erythrocytes but not mammalian erythrocytes, due to the presence of hemagglutinin molecule on its surface (Baxendale et al. 1980; Jordan 1990). It was also observed that EDS virus mediated HA did not show elution on 24 hours post incubation. This property might be due to the lack of neuraminidase molecules on the surface of EDS virus (Spalatin et al. 1970).

The incidence of the disease has declined as the classical form has largely been eliminated from primary breeder flocks. Organic and free-range flocks may be at a greater risk than conventional ones, as there is likely to be more contact with wild-fowl and geese or duck carriers. EDS has been encountered in organic flocks in Denmark, and is considered a risk (Permin and Nansen 1996). Swarbrick (1986) recorded EDS in three out of 16 free-range farms, and of these at least one was infected from wild ducks. Christensen and Stanislawek (1994) reported high levels of antibodies to EDS 76 in a free-range flock that had contact with ducks on a farm dam and was supplied with untreated drinking water from the dam.

Spread of EDS may occur through three possible routes. Classical EDS may be introduced by means of a vaccine that has been grown in duck eggs and spread by vertical transmission via the egg. An endemic form may also spread laterally, from bird to bird, through a flock. A sporadic form can spread to chickens via direct contact with ducks, geese or infected wild birds, or indirectly through contaminated water. The virus can also be transmitted via humans, needles and egg trays (Lampkin 1997).

The lateral spread of the disease can be slow. The virus has been shown to take 11 weeks to spread through a battery laying house, while affecting 4% of eggs produced (Cook and Darbyshire 1980). Birds with antibodies slow the spread of the virus. Chicks hatched from eggs of infected hens possess maternal antibodies that confer a passive
immunity to challenge for approximately the first 4 weeks of life (Darbyshire and Peters 1980).

k. Infectious Bronchitis Virus

- **Introduction**

IBV is the cause of highly contagious respiratory disease that produces high morbidity in all ages of chickens and high mortality in chicks less than 6 weeks old (Case et al. 1983). IBV can cause losses to the poultry producer due to poor feed conversion, condemnations, and mortality, especially when the birds are co-infected with other pathogens (Andrade et al. 1983; Perrotta et al. 1988).

- **Morphology of IBV**

IBV particles tend to be circular, although some pleomorphism of the stained virus is observed. The virus particle has characteristic club-shaped projections uniformly distributed on its surface. According to McIntosh et al. (1967), these are approximately 20 nm long and 10 nm wide at their outer edge, with a narrow base. They are spaced loosely around the particle in contrast to the closely spaced, rod-shaped projections on paramyxoviruses. In a study of 12 strains of IBV, Harkness and Bracewell (1974) found differences in the morphology of the projections.

Berry et al. (1964) reported the size of IBV to be 80-120 nm in diameter including projections. Cunningham (1966) found the sedimentation constant of IBV to be 344S, from which he estimated the diameter of the particle to be 80-100 nm. However, McIntosh et al. (1967) reported the size variation from 120 to 200 nm. Cunningham (1966) reported the specific gravity of IBV to be 1.24 in cesium chloride and 1.19 in sucrose.
Chemical composition of IBV

IBV is a member of the Coronaviridae family. The nucleic acid of IBV is a single-stranded RNA (Cunningham 1966), this positive RNA genome codes for three structural proteins: nucleocapsid (N) protein, membrane (M) protein, and spike (S) protein. The molecular weights (MWs) of the three proteins are approximately 50, 23-35, and 180 KDa, respectively (Cavanagh 1981). The surface of the particle contains an essential lipid. Purified virions contain approximately 4% by weight of RNA.

Sixteen polypeptides with molecular weights ranging from 15 to 135 KDa were observed by polyacrylamide gel electrophoresis of disrupted purified IBV on 7.5% acrylamide gels (Alexander and Collins 1977). Stern and Kennedy (1980) analyzed extracted nucleic acid from IBV-infected chicken embryo infected kidney cells. They found six RNA species designated A-F, the latter having the same electrophoretic mobility as purified viral genome.

The S protein of IBV is posttranslationally cleaved into S1 and S2 subunits with molecular weights of 90 and 84 KDa, respectively (Stern & Sefton 1982). The S2 subunit anchors the spike in the membrane, whereas the S1 subunit forms the globular head of the spike protein. The S1 subunit encodes amino acids involved in the induction of neutralizing, serotype specific, and hemagglutination-inhibition antibodies (Collison et al. 2001).

It has been shown that the epitopes that induce virus-neutralizing (VN) antibody and determine the IBV serotype are associated with the S1 fraction (Cavanagh & Davis 1986). Chickens inoculated with purified S1 fraction, and mice inoculated with a recombinant vaccinia virus containing S1 gene, produced IBV VN Abs (Tomley et al. 1987). Also, a majority of VN monoclonal antibodies produced against IBV have been shown to react with S1 protein and not with M or N proteins (Karaca et al. 1992).
Two clusters of amino acid substitutions (between positions 56 to 69 and 117 to 133) were observed in the S1 protein sequences of several Mass serotype isolates. These were designated hypervariable regions 1 and 2 (HVR-1 and HVR-2), respectively (Cavanagh et al. 1988). This led to the proposal that most VN epitopes were located in the first and the third quarters of the S1 protein (Jia et al. 1996).

- **Replication of IBV**

  Becker et al. (1967) studied the morphogenesis of IBV in infected embryonated eggs. The virus appears to develop in the cytoplasm by a process of budding into cisternae or vesicles, incorporating intracytoplasmic cellular membrane material into its outer coat. The club-shaped projections observed on the virus particle in negatively stained preparations were not observed on the surface of the virus in tissue sections of infected chorioallantoic membrane (CAM). The complete virus particle has a double outer shell surrounding a core of amorphous material.

- **Biological properties of IBV**

  Untreated IBV in allantoic fluid does not agglutinate avian erythrocytes. Bingham et al. (1975) found the hemagglutinin of the Connecticut strain to be detectable after sucrose gradient purification. The Massachusetts strain required an additional incubation with the enzyme phospholipase C after the purification step to reveal hemagglutination activity. Alexander and Chettle (1977) produced a satisfactory hemagglutination antigen by concentrating the virus, followed by treatment with phospholipase C type I at a final concentration of 1 unit/ml for 2 hr at 37 C.

- **Strain classification of IBV**

  Prior to 1956 avian IB was considered to be caused by a single antigenic type of virus as exemplified by the pathogenic Massachusetts strain and the Beaudette, nonpathogenic, embryo lethal strain. However, since that time a number of isolates have
been identified from outbreaks of IB in chickens that are different antigenically from the original Massachusetts type (Cunningham 1966).

More than 20 serologically distinguishable IBV strains have been identified worldwide (Gelb et al. 1981). The majority of IBV field isolates in the United States belong to serotypes Massachusetts (Mass), Arkansas (Ark), and Connecticut (Conn) (Hopkins 1974). Vaccination with one serotype most often results partial or no immunity to heterologous types (Andrade et al. 1983; Gelb et al. 1981; Gelb et al. 1983; Hofstad 1981; Johnson & Marquardt 1975; Marquardt et al. 1982). This is important, since the Ark-99 strain has been isolated numerous times on the Delmarva Peninsula, where common vaccination regimens often don’t include this serotype (Gelb et al. 1981; Gelb et al. 1983). Furthermore, although eyedrops, course spray, and intratracheal instillation are all effective methods of vaccination (Andrade et al. 1983), both Mass and JMK serotypes of IBV have been isolated from vaccinated flocks (Gelb et al. 1981; Perrotta et al. 1988).

Since IBV immunity is serotype specific, vaccine failures are often associated with the emergence of antigenic variants that differ from the vaccine viruses. Therefore, a thorough understanding of the serotype-specific epitopes on the S protein is required for developing strategies to improve currently used vaccines (Jia et al. 1996).

- **Natural and experimental hosts of IBV**

  The chicken is the only natural host for IBV. All ages are susceptible, but the disease is most severe in baby chicks were it causes some mortality. Following aerosol exposure of chicken to IBV there is rapid multiplication of the virus in the respiratory tract. Virus can be isolated from the trachea and lungs at 24 hr through the 8th day. Virus also multiplies in nonrespiratory tissues such as the kidney and bursa where it may persist longer than in the lung and trachea (Hofstad 1984).
• **Transmission of IBV**

IBV spreads rapidly among chickens in a flock. Susceptible birds placed in a room with infected chickens usually develop symptoms within 48 hr. It is assumed that the virus spreads among flocks where farms are close and in the direction of the prevailing wind (Hofstad 1984).

• **Immunity to IBV**

Chickens recovered from the natural disease are resistant to intratracheal inoculations with the homologous strains as soon as symptoms have subsided. It requires about 3 wk for chickens to reach a high level of antibodies following exposure to IBV. Flocks that have experienced the outbreak have some degree of immunity, and antibodies can be demonstrated for at least one year (Hofstad 1984). However, observations by Van Rockel *et al.* (1950) have suggested that immunity may decline sufficiently for reinfection to occur in some flocks following exposure to the virus. Plurality of strains has complicated immunity studies with IBV.

Eggs laid by recovered hens will carry antibodies later absorbed by the hatched chick. The passive antibody levels are highest soon after hatching and decline steadily to negligible levels by the forth week. Passive antibodies serve to reduce the severity of the disease but do not prevent respiratory infection following exposure to the virus (Hofstad 1984).

Immunity to IBV has most often been assessed using traditional serological assays such as HI and VN tests (Alexander *et al.* 1976; Garcia & Bankowski 1981; Hatcher *et al.* 1983; King & Hopkins 1983; Lukert 1966). However, the ELISA is now being used on a more frequent basis to measure IBV Abs (Case *et al.* 1983; Garcia & Bankowski 1981; Marquart *et al.* 1981). The use of an ELISA offers a number of advantages compared with traditional serological assays, including increased sensitivity.
(Garcia & Bankowski 1981; Marquart et al. 1981), ease of automation (Snyder et al. 1983; Snyder et al. 1984), and the fact that the antibody levels to a number of different viruses and/or strains can be measured simultaneously (Snyder et al. 1984). These factors all support the idea that ELISA is a promising tool for serological studies, especially for use in evaluating the efficacy of vaccination regimens and in monitoring the immune status of birds in a flock (Perrotta et al. 1988).

A number of authors have reported success in using the IBV-ELISA to measure the antibody levels following vaccination with IBV (Case et al. 1983; Garcia & Bankowski 1981; Snyder et al. 1983). However, although ELISA is extremely sensitive, it is not serotype-specific with regard to IBV antibody detection (Perrotta et al. 1988).

Although HI and VN can detect type- as well as group- specific Abs to IBV strains, they, like ELISA, are of little epidemiological value because of the confusion associated with cross reacting antibodies (King & Hopkins 1983). If a reliable type-specific ELISA could be developed, it would greatly assist diagnosticians and epidemiologists in the establishment of serotype causation of IBV field outbreaks (Perrotta et al. 1988).

In addition, lack of serotype specificity combined with great sensitivity make the ELISA ideally suited for monitoring the pressure of IBV in specific pathogen free (SPF) flocks. Furthermore, the IBV-ELISA could be used as a screening test to monitor for IBV outbreaks among previously vaccinated or infected flocks. In this case, once an increase in anti-IBV antibody titer is detected, a more specific assay could be utilized to identify the serotype responsible for the increase (Perrotta et al. 1988).

- Isolation of IBV

Isolation of IBV is most frequently accomplished by inoculating 10- or 11-day-old embryonating chicken eggs via the allantoic sac with a broth suspension of exudates
collected by swabbing the trachea of several birds in the acute stage of the disease. Penicillin and streptomycin (10,000 units and 10 mg respectively per ml) are usually used to control bacterial contamination. However, filtration through the 0.65, 0.45, and 0.3 μm Millipore filters can also be used to eliminate bacteria from the broth suspension (Hofstad 1984).

- **Serology for IBV**

  Diagnosis of avian IB by serologic methods is based on demonstration of an ascending serum antibody level. The first sample of blood is collected during initial stages of the disease and the second is taken in 2 or 3 weeks. Both samples of serum are tested for neutralizing antibodies against IBV. Demonstration of a negative or low antibody titer in the serum collected early and a higher titer after recovery constitutes a diagnosis of IBV. The plurality of strains may necessitate using strains of IBV other than the Massachusetts type, the most frequently encountered (Hofstad 1984).

  The primary aim of IBV diagnostic techniques is to determine the serotype of the virus. Serotype is defined as a grouping of viruses that are serologically related. Classically, strains of IBV have been placed into serologically related groups on the basis of their reactivity with known serotype-specific antiserum in virus neutralization tests (Collison *et al.* 2001).

1. **Newcastle Disease Virus**

   - **Introduction**

     Five clinical forms or pathotypes of Newcastle disease (ND)-Doyle’s, Beach’s, Beaudette’s, Hitchner’s, and avirulent enteric- are caused by ND viruses that are morphologically and antigenically similar.

     Doyle’s form, first recognized in 1926 (Doyle 1927), is an acute lethal
infection of all ages of chickens. Hemorrhagic lesions of the digestive tract, which lead to description of this form of the disease as VVND (viscerotropic velogenic Newcastle disease), are a prominent pathologic feature.

Beach’s form, described in 1942 (Beach 1942) is an acute and frequently lethal infection of chickens of all ages characterized by lesions in the respiratory tract and nervous system. Hemorrhages are conspicuously absent from the digestive tract. It was initially called nervous respiratory disease or pneumoencephalitis and is also caused by certain velogenic strains. This pathotype is frequently termed “neurotropic Newcastle”.

Beaudette’s form, recognized a few years later (Beaudette and Black 1946), is an acute respiratory and sometimes lethal nervous infection of young chickens. In older birds mortality is rare. Mesogenic viruses cause this pathotype of ND. Some of these strains are used as viable vaccines.

Hitchner’s form (Hitchner and Johnson 1948) is a mild or unapparent respiratory infection of chickens caused by lentogenic strains. Mortality is rare in birds of any age. Several lentogenic strains are widely used as vaccines.

The asymptomatic enteric form of infection (Lancaster 1981) is caused by lentogenic strains that result in no clinical signs or pathology and is detectable only by virus isolation from the gut or feces and by presence of specific antibodies.

High mortality of 50-100% among adult birds has characterized Doyle’s form of the disease in many countries where flocks have been devastated and market operations upset. Severe direct and indirect losses occurred from VVND viruses in southern California in 1971-72 (Walker at al. 1973). Less lethal forms of Beach and Beaudette have also been the source of major economic losses. Crippling, impaired growth and poor feed use often occur among surviving birds. Greatest loss among layers frequently results from reduced production and impairment of eggshell and
albumin quality. Reduction of fertility and hatchability of eggs has also been reported, and additional losses have come from restrictions on export of eggs (both hatching and table), chicks, breeding and other stock, and dressed poultry (Beard and Hanson 1984).

- **Classification and molecular nature of the ND virus**

  NDV is a paramyxovirus. This genus, according to the classification presented by Mathews (1979), includes the parainfluenza viruses of mammals 1-5, mumps virus, and the avian paramyxoviruses (PMVs), of which there are at least 7 serotypes, NDV being serotype PMV-1. PMVs contain a single strand of RNA within protein arranged in a helical symmetry. This nucleocapsid is contained within a lipoprotein envelope (Beard and Hanson 1984).

  The virus is enveloped and contains a negative –sense, single stranded RNA genome that codes for six proteins including an RNA-dependent RNA polymerase (L), fusion (F) protein, hemagglutinin-neuraminidase (HN) protein, matrix (M) protein, phosphoprotein (P), and nucleoprotein (NP) (Kapczynski and Tumpey 2003).

  The HN and F proteins are glycosylated and form two projections on the lipoprotein envelope of the virion. Studies with monoclonal antibodies to these proteins have demonstrated neutralization to NDV in vitro and in vivo by preventing virus attachment and cell fusion activity. The M protein is located in the inner surface of the envelope and provides structural integrity for the virion. The NP, P, and L proteins are associated with genomic RNA to form the nucleocapsid. The role of antibodies to NP/P and M proteins in protection against NDV infection is unclear (Reynolds and Maraqa 2000).

- **Morphology of the ND virus**

  The virion or mature NDV unit, which varies in size from 120 to 300 nm but is usually about 180 nm, consists of an envelope and an internal component. The ether-
sensitive and osmotically deformable envelope has a pattern of projection or spikes (80 Å long) and contains the antigenic components that stimulate the host to produce hemagglutinin inhibiting and virus neutralizing antibodies (Rott 1964).

The internal component (nucleocapsid), also known as the G antigen or nucleoprotein (NP) soluble antigen, consists of a long and much coiled tube of a diameter of 180 Å. The protein structural units of the tube are arranged in a helix around the central hollow axis; within them and determining the entire configuration is the RNA. The oligonucleotide fingerprinting of NDV strains (McMillan and Hanson 1982) has allowed precise determination of sameness or differences among isolates.

Two glycoproteins of the envelope and seven other polypeptides have been identified by investigators and compared among other avian paramyxoviruses (Alexander and Collins 1981). The two glycoproteins are the HN (containing hemagglutinin and neuraminidase) and the F (responsible for cell-fusing activity and polykarocytes). The HN and F polypeptides are derived from precursors, HN0 and F0, by proteolytic cleavage. Susceptibility of these precursors to cleavage by enzymes determines ability of the virus to spread in the host and organ tropism. Therefore, it now appears that cleavage of these surface glycoproteins is a requirement for pathogenicity (Nagai et al. 1979).

The HN protein has three functions: it recognizes sialic acid–containing receptors on cell surfaces, it promotes the fusion activity of the F protein, allowing the virus to penetrate the cell surface, and it acts as a neuraminidase (sialidase), removing sialic acid from progeny virus particles to prevent virus self-agglutination. Many studies show that only hemotypic HN and F can induce fusion suggesting that there is a specific interaction between HN and F, involving both the stalk and globular head regions of HN. The multifunctional HN molecule makes it an attractive target for structure-based
Unlike many viral F proteins, paramyxovirus F proteins do not require the acid pH of endosome for the activation of fusion activity; thus, other mechanisms for F protein activation must be invoked. Because of this acid pH independence, infected cells expressing both HN and the F proteins can fuse with adjacent cells to form multinuclear cells or syncytia, a process that is assumed to be similar to virus cell fusion (Gravel and Morison 2003).

For paramyxoviruses, fusion of the viral and host cell membranes occurs at the cell surface, in contrast to the influenza virus, which undergoes endocytosis where the lower endosomal pH triggers a dramatic structural change in the influenza virus hemagglutinin. In the case of paramyxoviruses, binding of HN to sialic acid receptors must trigger the fusion protein into its fusogenic state through an association between HN and F (Zaitsev 2004).

The NDV F protein is synthesized as a precursor, F0 that must be proteolytically cleaved to activate F protein fusion activity. Cleavage at amino acid 117 produces disulfides-linked F2 and F1 polypeptides derived from the amino-terminal and carboxyl-terminal domains of F0, respectively. The F1 polypeptide has one acid perhaps two-fusion peptides. Upon initiation of fusion, fusion peptides are thought to insert into target membranes, docking the protein to these membranes. Paramyxovirus F1 polypeptides have 2 heptad repeat (HR) regions, one (HR1) located just carboxyl-terminal to the more amino-terminal fusion peptide and the other adjacent to the transmembrane domain (HR2). Studies of peptides with sequences of these HR domains, characterization of mutations within the domains, and results of similar studies of human immunodeficiency virus and influenza virus have led to hypothesis that F proteins are synthesized and transported to cell surfaces in a metastable conformation in drug design for diseases caused by paramyxoviruses (Zaitsev et al. 2004).
which the HR domains are not associated and the fusion peptides are masked. Upon
fusion activation, F proteins are thought to undergo a series of conformational changes
that result in the insertion of fusion peptides into target membranes and the interaction
of the HR1 and HR2 domains to form a very stable complex. The formation of this
complex is thought to pull target and attack membranes in close proximity, allowing
subsequent fusion events (Gravel and Morison 2003).

The HN protein binds to sialic acid-containing molecules found on cell
surfaces and has demonstrated neuraminidase activity. The F and HN transmembrane
glycoproteins are the principle antigens that elicit a protective immune response.
Monoclonal antibodies directed against these glycoproteins neutralize NDV by
inhibiting cell fusion and attachment (Kapczynski and Tumpey 2003).

Because of evidence which suggested an interaction between HN and F
protein, we have explored their association during NDV infection since there have been
no reports of such an interaction in naturally infected cells. We were able to
demonstrate an interaction between the HN and F protein in NDV-infected cells by
using coimmunoprecipitation and chemical cross-linking. To stabilize the potential
association of HN and F proteins on infected cell plasma membranes, use of the
chemical cross-linker DTSSP was investigated. DTSSP is a membrane-impermeable
cross-linker; therefore, incubation of intact cells with this cross-linker will link only
proteins expressed on the cell surface. We report that a complex between HN and F
protein could be immunoprecipitated with antisera against either protein, and this
complex could be stabilized by chemical cross-linking. Precipitation of this complex
was not dependent on cleavage of Fo (uncleaved fusion protein) into F1 and F2 (cleaved
form of fusion protein), the complex did not dissociate immediately after HN protein
attachment to uninfected cells, and it was not dependent on attachment of the HN
protein to its receptors (Stone-Hulslander and Morison 1997).

In most paramyxovirus systems, F protein cleavage is not sufficient to activate these conformational changes in the F protein; rather, coexpression of the attachment protein is also required. The HN protein provides more than an attachment or membrane-docking function, since mutants with mutations in the HN protein can retain attachment activity but are defective in fusion promotion. It has been proposed that the attachment of the HN protein to its receptor serves to activate the F protein, but the nature of this activation is poorly understood. The HN protein and the F protein must be from the same virus, with a few exceptions, indicating that virus-specific interactions between HN and F proteins are required for fusion directed by the F protein. Indeed, an interaction has been shown in several systems. It seems likely that this interaction is somehow linked to F protein activation (Gravel and Morison 2003).

Passive administration of anti-serum against intact NDV, or the surface glycoproteins HN/F, provided protection to susceptible chicken against NDV challenge. However, no report has been published on the protective role of the other NDV polypeptides (Reynolds and Maraqqa 2000). In studies with simian virus 5 (SV5), Randall et al. (1988) found that in vitro neutralization was provided by antibodies directed against the surface HN glycoprotein, but neutralization was not achieved when antisera against the internal protein NP, P, or M were used. Furthermore, similar findings have been reported for IBV. Immunizing birds with purified nucleocapsid and membrane proteins of the IBV did not protect them against virulent challenge despite high antibody titers, whereas birds immunized with the surface S1 glycoprotein were protected (Reynolds and Maraqqa 2000).

- **Replication of NDV**

Replication of NDV has been studied in cell culture systems. After the virion
adsorbs to the cell, the virion envelope fuses with the cell membrane, enabling extrusion of the nucleocapsid into the cell. The RNA-dependent RNA polymerase synthesizes RNA complementary to that of the viral nucleocapsid (Huang et al. 1971), which then acts as messenger RNA. Virus production occurring in the cytoplasm reaches its maximum in 5-8 hours. During the 3-4 hours noninfectious period, NDV-specific antigen can be demonstrated by compliment fixation and by use of fluorescent antibody. First to be detected is the NP in the cytoplasm near the nucleus (Rott 1964). Then the hemagglutinating antigen and neuraminidase can be found throughout the cytoplasm. Both the HN and F polypeptides are synthesized in rough endoplasmic reticulum (Schwalbe and Hightower 1982).

NDV interferes with multiplication and pathologic expression of certain other viruses and in turn is interfered with by certain viruses such as infectious bronchitis virus (Raggi et al. 1963). Mixed infections in chickens can result in aborted disease and immunologically reduced response (Hanson et al. 1956). In cell cultures, interference induced by other viruses on multiplication of NDV has been used in development of procedures to detect noncytopathic viruses (Beard 1967). Conditions under which NDV induces formation of interferon in many host systems have been extensively studied (Baron 1964). NDV is frequently used as an elicitor of interferon, being quite resistant to its effects (Lomniczi 1973).

- **NDV- strain classification**

A strain of NDV is a culture that has been recovered from a chicken or other animal by inoculation of a suitable laboratory host system such as embryonated chicken eggs or cell cultures (Beard and Hanson 1984).

Among the strains that are almost avirulent (lentogenic) are B1, F, and LaSota, which have been extensively used as vaccines. Ulster and V4, more recently isolated,
are heat-stable lentogens that replicate in the intestinal mucosa. Among the strains of highest virulence (velogenic) are Milano, Herts, and GB, which have been used as challenge strains. Ca-1083 and Largo are VVND isolates that are frequently used in laboratories with adequate biosafety provisions to test efficacy of vaccines in challenge trials. From some virulent and milder strains, sublines can be isolated that are differentiable from the parent culture on the basis of plaque morphology (Granoff 1964).

- **Immunity to NDV**

  All NDV strains are capable of provoking antibody response in chickens, rabbits, and other species into which they are introduced. Antigens that induce the virus-neutralization (VN) antibody, which is apparently also the hemagglutination-inhibition (HI) antibody, are associated with virus envelope (Beard and Hanson 1984).
CHAPTER III
MATERIALS AND METHODS

A. Experiment 1: Anti-Inflammatory Effect of *Origanum ehrenbergii* Essential Oil against *Staphylococcus aureus* Skin Infection in Rabbits

1. Preparation of *Staphylococcus aureus* Inoculum

The *Staphylococcus aureus* strain was provided by the bacteriology lab of the American University Hospital (AUH), Beirut, Lebanon and grown in a nutrient broth at 37°C for 3-4 hours until reaching to the log phase. A volume of 0.1 ml of *Staphylococcus aureus* was struck for isolation on Mannitol Salt Agar (HiMedia Laboratories, Limited, Mumbai, India) and incubated at 37°C for 24 hours. The incubated MSA was scraped aseptically using a sterile loop and the inoculum was suspended in 5 ml sterile Triptose Phosphate Broth (TPB) tube, and then inoculated for 5 hours at 37°C. After 5 hours, the percent transmittance of the original bacterial culture was 21.75. From the *Staphylococcus aureus* logarithmic standard curve equation \( y = -46.507x + 410.889 \) the inoculum count was deduced as \( 2.33 \times 10^8 \) CFU/ml (according to Microsoft Excel 2000 program).

2. Standard Curve Preparation

For the *Staphylococcus aureus* bacterium, and with the use of a metal loop, sterilized over a flame, a colony was taken from the MSA and placed in TPB. The inocula were incubated for four to five hours, time that is needed for the bacteria to reach the log phase in bacterial growth curves. The turbidity of the inocula was compared to Mc Ferland Turbidity Standard, which consists of 0.5 ml of 1 % \( \text{BaCl}_2 \) and 99.5 ml of 1 % Sulfuric Acid (0.36 N). This \( \text{BaCl}_2 \) suspension matches with a
Staphylococcus aureus culture that contains approximately 1 to 4 x 10⁶ CFU/ml.

Serial dilutions from 10¹ to 10⁸ were made by adding 0.5 ml of each inoculum to 4.5 ml sterile saline. In parallel, percent transmittance for each dilution was read using Spectronic 20 D+ spectrophotometer at a wavelength of 540 nm. The aim was to end up with a standard curve of bacterial count (CFU/ml) versus percent transmittance. However, transmittance reached to a plateau for almost all the dilutions, thus, failed to obtain a linear slope.

Serial dilutions were made from 1:2 to 1:64 in which percent transmittance of the bacterial dilutions could be read but the colonies were too many to count. So serial dilutions from 1:2 to 1:1,048,576 were performed and percent transmittance was recorded for each bacterial dilution. Levels of the bacteria were calculated as follows: bacterial count (CFU/ml) = number of colonies formed x the dilution factor x 10 (since 0.1 ml of the corresponding dilution was plated). The number of colonies considered corresponds to the plate with a count ranging from 30 to 300 colonies. For Staphylococcus aureus, a standard curve of the logarithm of colonies’ count versus percent transmittance was plotted (Figure 1).

3. Optimization of In Vitro Analysis of Antimicrobial Activity of Essential Oil

a. Antimicrobial Activity of Essential Oil from Origanum Ehrenbergii Harvest of Three Different Locations

Three sources of Origanum ehrenbergii essential oil were from three different locations in Lebanon. The essential oils from the three different locations were tested for their inhibitory effect against Staphylococcus aureus by using the Kirby Bauer Test (Bauer et al. 1966).
Fig. 1. Logarithmic standard curve of *Staphylococcus aureus*
X-axis = log$_2$ of the bacterial count and Y-axis = percent transmittance.

Two different essential oil dilutions were tested for each location,

- \(1/100 = 10 \, \mu l \text{ oil} + 2 \, \mu l \text{ DMSO} + 980 \, \mu l \text{ BHI}\)
- \(1/200 = 10 \, \mu l \text{ oil} + 4 \, \mu l \text{ DMSO} + 1960 \, \mu l \text{ BHI}\)

From the *Staphylococcus aureus* logarithmic standard curve equation \((y = -46.507x + 410.889)\) the inoculum count in the major seed stock of *Staphylococcus aureus* was deduced as \(8.013 \times 10^8\) CFU/ml (according to Microsoft Excel 2000 program). For preparation of 100,000 CFU/ml, 1.3 \(\mu l\) of the stock containing *S. aureus* were reconstituted in 10 ml of sterile saline.

A volume of 0.1 ml of the culture was seeded on 6 Muller Hinton (MH) Agar
(Mast group Ltd, Merseyside, U.K.) plates, using sterile L-glass rod. A volume of 20 µl of either 1/100 or 1/200 essential oil dilution was loaded on each blank sterile disk of 6.5 mm diameter (Mast group Ltd, Merseyside, U.K.). The diameter of the area of inhibition around the disk was measured in cm, after the Muller-Hinton Agar cultures were incubated for 24 hours at 37°C.

b. Impact of DMSO Level on Antimicrobial Activity of Dispersed Essential Oil in Aqueous Medium

From the *Staphylococcus aureus* logarithmic standard curve equation ($y = -46.507x + 410.889$) the inoculum count in the major seed stock of *Staphylococcus aureus* culture was deduced as $2.3 \times 10^8$ CFU/ml. For preparation of 1,000,000 CFU/ml, 43.48 µl of the stock containing *S. aureus* were suspended in 10 ml sterile saline.

The essential oil dilution used at variable levels of DMSO in water was 1/100 dilution. The different prepared dilutions of DMSO ranging from 1 to 5% with the constant essential oil level were each loaded on blank sterile disks at a volume of 20 µl/disk and spread on Muller Hinton Agar plates that were already subjected to 0.1 ml-spread of *Staphylococcus aureus* culture standardized at a suspension of $10^6$ CFU/ml.

The same procedure was repeated again but with different levels of DMSO ranging from 5 to 40%. From the *Staphylococcus aureus* logarithmic standard curve equation ($y = -46.507x + 410.889$) the inoculum count in the major seed stock of *Staphylococcus aureus* culture was deduced as $2.33 \times 10^8$ CFU/ml. For preparation of 1,000,000 CFU/ml, 42.9 µl of the stock containing *S. aureus* were suspended in 10 ml sterile saline.
c. Optimized Antimicrobial Activity of Essential Oil and DMSO Dilution against Different Staphylococcus aureus Densities

After determining the best location that resulted in Origanum ehrenbergii with essential oil containing the highest antimicrobial activity and the proper DMSO level (40%), different dilutions of Staphylococcus aureus were included in this following test.

From the Staphylococcus aureus logarithmic standard curve equation \( y = -46.507x + 410.889 \) the inoculum count in the major seed stock of Staphylococcus aureus culture was deduced as \( 5.47 \times 10^7 \) CFU/ml. For preparation of 1,000 CFU/ml, 0.6 \( \mu l \) of the stock containing S. aureus were suspended in 33.3 ml sterile saline. For preparation of 10,000 CFU/ml, 1.828 \( \mu l \) of the stock containing S. aureus were suspended in 10 ml sterile saline. For preparation of 100,000 CFU/ml, 18.28 \( \mu l \) of the stock containing S. aureus were suspended in 10 ml sterile saline. For preparation of 1,000,000 CFU/ml, 182.8 \( \mu l \) of the stock containing S. aureus were suspended in 10 ml sterile saline.

The different levels of Staphylococcus aureus \( (10^3, 10^4, 10^5, \text{ and } 10^6 \text{ CFU/ml}) \) were spread individually, using 0.1 ml inocula, on Muller Hinton Agar plates. Blank disks loaded each with 20 \( \mu l \) of 1/100 essential oil in 40% DMSO in water were overlaid the surface of the Muller Hinton Agar plates that were previously seeded with different levels of Staphylococcus aureus.

4. Disinfection and Preparation of the Animal Rooms

Walls and floors of four animal rooms were cleaned, flushed with water and disinfected by soaking overnight with 10% sodium hypochlorite. Samples for bacterial count were collected from the walls and the floor of each room by rubbing a 25-cm\(^2\) area with sterile swabs previously soaked in Tryptose Phosphate Broth (Mast Group
Each swab was struck for seeding onto Nutrient Agar and McConkey Agar plates (Mast Group Ltd, Merseyside, U.K) and the plates were then incubated for 24 hours at 37 °C. Disinfection was repeated until no colony forming units were observed on both agar plates. Wood shavings were then spread over the floor of each room to a depth of around 5 cm. Each animal room was supplemented with two feeders of 60 cm length, one waterer of 35 cm diameter and one height-adjustable 250-Watts infrared lamp.

5. Antimicrobial Effect of Origanum ehrenbergii Essential Oil on Rabbits Challenged Intradermally with Staphylococcus aureus

a. Experiment 1

On April 29 2004, twenty-one experimental rabbits were distributed equally in 4 groups. The objective of this experiment was to challenge intradermally the right ears of the rabbits with *Staphylococcus aureus* followed by topical treatment with essential oil for a period of three days. Each group was placed in a separate room; the experimental design is presented in Table 1.

On April 30, May 1 and May 2 2004 (three consecutive days after the challenge), the diameter of the inflammation area was measured on the left control ear and the right ear (challenged ear) of each rabbit, twice a day.

b. Experiment 2

On May 10 2004, another approach was conducted with the same experimental design; however, this time the aim was to assess the difference in bacterial counts between the essential oil treated and the non-treated rabbit ears that have been challenged with *Staphylococcus aureus*. 
Table 1. Experimental design of rabbits challenged with *Staphylococcus aureus* and treated with essential oil

<table>
<thead>
<tr>
<th></th>
<th>Group I Room 1</th>
<th>Group II Room 2</th>
<th>Group III Room 3</th>
<th>Group IV Room 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Non-challenged</td>
<td>Challenged</td>
<td>Challenged</td>
<td>Challenged</td>
</tr>
<tr>
<td></td>
<td>Non-treated</td>
<td>Non-treated</td>
<td>Treated with</td>
<td>Treated with</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>essential oil in</td>
<td>40% DMSO/water</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40% DMSO/water</td>
<td></td>
</tr>
<tr>
<td>Left ears</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(control)</td>
<td>No treatment</td>
<td>0.1 ml sterile</td>
<td>0.1 ml sterile</td>
<td>0.1 ml sterile</td>
</tr>
<tr>
<td></td>
<td></td>
<td>saline/rabbit</td>
<td>saline/rabbit</td>
<td>saline/rabbit</td>
</tr>
<tr>
<td>Right ears</td>
<td></td>
<td>0.1 ml of stock</td>
<td>0.1 ml of stock</td>
<td>0.1 ml of stock</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10^5 CFU/ml)*</td>
<td>(10^5 CFU/ml)</td>
<td>(10^5 CFU/ml)</td>
</tr>
<tr>
<td>Oil Treatment on</td>
<td>No</td>
<td>No</td>
<td>100 µl of 1:100</td>
<td>100 µl of 40%</td>
</tr>
<tr>
<td>both ears</td>
<td></td>
<td></td>
<td>essential oil in</td>
<td>DMSO/water***</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40% DMSO/water</td>
<td></td>
</tr>
<tr>
<td>Average Weight/rabbit</td>
<td>1221.43 g</td>
<td>1131.43 g</td>
<td>1137.14 g</td>
<td>1194.29 g</td>
</tr>
</tbody>
</table>

* From the *Staphylococcus aureus* logarithmic standard curve equation (y = -46.507x + 410.889) the inoculum count in major *Staphylococcus aureus* seed stock was deduced as 1.71 x 10^8 CFU/ml. For preparation of 100,000 CFU/ml, 5.9 µl of the stock containing *S. aureus* were suspended in 10 ml sterile saline.

** This solution contains 10 µl essential oil + 396 µl DMSO + 594 µl distilled water.

*** This solution contains 800 µl DMSO + 1200 µl distilled water.

Eight rabbits were distributed into two equal groups (4 rabbits/group); both groups were challenged with *Staphylococcus aureus* and one group was treated with essential oil while the other group was left untreated. The experimental design is presented in Table 2.

In the afternoon of the same day (May 10), only one topical treatment with essential oil was given to Group I. The next day (May 11), Group I was treated in the morning, at noon and in the afternoon with essential oil and the diameter of the inflammation area was measured twice in both groups.

On May 12, the rabbits were anesthetized in desiccators full with ether and the diameter of the inflammation area was measured once. The ears were cleaned aseptically, and then cuts were taken from both ears and put in 2 ml PBS. A
homogenate of the cut ear was ground with a tissue grinder and then serial dilutions were made to tubes each containing 4.5 ml PBS to end up with dilutions of 1:1, 1:10, and 1:100. Finally, 0.1 ml of each dilution was streaked on MSA plates with a sterile L-glass rod and incubated for 48 hours at 37°C to get the bacterial count.

Table 2. Experimental design of rabbits challenged intradermally with *Staphylococcus aureus* and treated with essential oil

<table>
<thead>
<tr>
<th></th>
<th>Group I, Room 2</th>
<th>Group II, Room 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Challenged&lt;br&gt;Treated with essential oil</td>
<td>Challenged&lt;br&gt;Non-treated</td>
</tr>
<tr>
<td>Left ears (control)</td>
<td>0.1 ml sterile saline/rabbit</td>
<td>0.1 ml sterile saline/rabbit</td>
</tr>
<tr>
<td>Right ears</td>
<td>0.1 ml of stock&lt;br&gt;(10^5 CFU/ml)*</td>
<td>0.1 ml of stock&lt;br&gt;(10^5 CFU/ml)</td>
</tr>
<tr>
<td>Oil Treatment</td>
<td>100 µl of 1:100 essential oil in 40% DMSO/water**</td>
<td></td>
</tr>
<tr>
<td>Average Weight/ rabbit</td>
<td>1625 g</td>
<td>1730 g</td>
</tr>
</tbody>
</table>

* From the *Staphylococcus aureus* logarithmic standard curve equation (y = -46.507x + 410.889) the inoculum count in the major seed stock of *Staphylococcus aureus* was deduced as 1.02 x 10^8 CFU/ml. For preparation of 100,000 CFU/ml, 9.8 µl of the stock containing *S. aureus* were suspended in 10 ml sterile saline.

** This solution contains 10 µl essential oil + 396 µl DMSO + 594 µl distilled water.

c. **Experiment 3**

In the previous experiments, the inflammation resulting from *Staphylococcus aureus* challenge was weak and it was not lasting more than three days. Hence, an in vivo passage helping in increasing the virulence of *S. aureus* was followed as detailed below (Peters and Paterson 2003). Ten rabbits were divided into two equal groups and challenged intradermally in the ear with the passaged *S. aureus*, and then one group was treated with essential oil (Table 3). The objective of this experiment was to assess the
difference in the weight of the ear lesion and the bacterial count between the treated and non-treated rabbits that were challenged with *Staphylococcus aureus* (Stock E).

Table 3. Experimental design of rabbits challenged with the *Staphylococcus aureus* (Stock E) and treated with essential oil

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group I, Room 2</th>
<th>Group II, Room 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left ears (control)</td>
<td>Challenged Treated with essential oil</td>
<td>Challenged Non-treated</td>
</tr>
<tr>
<td>Right ears</td>
<td>0.1 ml sterile saline/rabbit</td>
<td>0.1 ml sterile saline/rabbit</td>
</tr>
<tr>
<td>Oil Treatment</td>
<td>0.1 ml of stock E equivalent to 3.45 x 10^7 CFU/ml</td>
<td>0.1 ml of stock E equivalent to 3.45 x 10^7 CFU/ml</td>
</tr>
<tr>
<td>Average Weight/ rabbit</td>
<td>1755 g</td>
<td>1690 g</td>
</tr>
</tbody>
</table>

* This solution contains 10 µl essential oil + 396 µl DMSO + 594 µl distilled water.

Preparation of *Staphylococcus aureus* Challenge (Stock E)

- The *Staphylococcus aureus* strain was provided by the bacteriology lab of the American University Hospital (AUH), Beirut, Lebanon and grown in a nutrient broth (3-4 hours until reaching to the log phase).

- A 0.1 ml volume of *Staphylococcus aureus* was streaked for seeding on Mannitol Salt Agar (HiMedia Laboratories, Limited, Mumbai, India) and incubated at 37°C for 24 hours (day 1).

- The incubated MSA was scraped aseptically using a sterile loop and suspended in 1 ml sterile saline (Stock A), and then injected in one rabbit in its right ear with 2 sites (0.1 ml/site) of Stock A (day 2). After 24 hours of injection, the rabbit was anesthetized in desiccators full with ether; the ears were cut, homogenized in 2 ml sterile saline to obtain Stock B (day 3).
• The passage was repeated for another two times to end up with Stock D.

• Seed the homogenate (Stock D) on 5 plates of MSA; incubate for 24 hours at 37°C (day 5).

• Scrape all cells with a sterile scalpel and reconstitute in 2 ml saline aseptically to obtain Stock E (day 6).

• From the *Staphylococcus aureus* logarithmic standard curve equation \(y = -46.507x + 410.889\) the inoculum count of stock E was deduced as \(3.45 \times 10^8\) CFU/ml.

In the noon and the afternoon of the same day, two topical treatments with essential oil were given to Group I. The next day, the rabbits were anesthetized in desiccators full with ether, the ears were cleaned aseptically then an equal area of the inflamed tissue of the ears were cut and put in empty sterile plates of known weight. The plates were weighed after the cuts were put in them. After that, each cut was put in 2 ml PBS and weighed again. The PBS solution was ground with a tissue grinder, and then counting was done by dilution of the PBS up to \(10^{-6}\) on MSA, using a 1:10 dilution factor (Cork and Halliwel 2002). Finally, 0.1 ml of each dilution was streaked on MSA plates with a sterile L-glass rod and incubated for 48 hours at 37°C to get the bacterial count per gram of the ear weight.

6. Statistical Analysis

Analysis of variance of the rabbits’ ear weights and bacterial counts were carried out by the One Way Analysis of Variance (ANOVA-1) using the MSTAT software (MSTATC 1991). Means were then separated by Duncan’s Multiple Range Test (\(\alpha = 0.05\)).
B. Experiment 2: Serological Profiling and Molecular Detection of Viral Protective Polypeptides by Sera of Sentinel Free-Range Layers

1. Serological Profiling On Sentinel Free-Range Chicken Layers Uncovering Prevalent Pathogen Exposure

a. Farm Surveillance and History

Serological profiling is a technology that is followed in many countries of the world to detect exposure of unvaccinated sentinel birds to field strains of devastating diseases. A representative sample consisting of 10 out of 35 free-range chicken layer farms in Jezzine area were included in this study.

The 10 farms were in the following districts of Jezzine area:

- 2 farms from the north.
- 2 farms from the south.
- 2 farms from the west.
- 2 farms from the east.
- 2 farms from the mid-region.

On August 5, 8, and 12, screening of the ten representative farms was done. From each of the selected farms, ten blood samples were drawn randomly from ten individual chickens (5% of the total number of birds per farm). Serum was extracted from the blood samples and serological analyses were conducted using the ELISA method and Hemagglutination-Inhibition (HI) for an indirect assessment of exposure to pathogens of economical reproductive diseases (IBV, NDV, EDS, and MG) and to other immunosuppressive and neoplastic pathogens namely, IBDV and ALV. Data from each of the chosen farms was recorded (Table 4).
Table 4. Data Collection from sentinel farms that were surveyed before vaccination for exposure to IBV, NDV, EDS, MG, IBDV, and ALV

<table>
<thead>
<tr>
<th>Case #</th>
<th>Farm Name</th>
<th>Bleeding Date</th>
<th>Region</th>
<th>Age (Month)</th>
<th>Location</th>
<th>% Egg Production</th>
<th>Mortality</th>
<th>Previous Outbreaks</th>
<th>Present signs</th>
<th>Feed Consumption grams/bird</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Elias Awwad</td>
<td>August 5 2003</td>
<td>Remat</td>
<td>15</td>
<td>West</td>
<td>40</td>
<td>High</td>
<td>Cannibalism</td>
<td>Weak feather, egg discoloration, torticollis</td>
<td>120</td>
</tr>
<tr>
<td>2</td>
<td>Charbel Hnaini</td>
<td>August 5 2003</td>
<td>Aray</td>
<td>15</td>
<td>East</td>
<td>9</td>
<td>Moderate</td>
<td>Cannibalism</td>
<td>Egg discoloration, small eggs</td>
<td>117</td>
</tr>
<tr>
<td>3</td>
<td>Abed Ramadan</td>
<td>August 8 2003</td>
<td>Al-Rehan</td>
<td>16</td>
<td>South</td>
<td>53</td>
<td>Low</td>
<td>Cannibalism</td>
<td>Poor feathering, egg discoloration</td>
<td>117</td>
</tr>
<tr>
<td>4</td>
<td>Sami El-Hagg</td>
<td>August 8 2003</td>
<td>Aishiyye</td>
<td>11</td>
<td>South</td>
<td>60</td>
<td>Low</td>
<td>None</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>Josef Harb</td>
<td>August 8 2003</td>
<td>Jezzine</td>
<td>16</td>
<td>East</td>
<td>55</td>
<td>Low</td>
<td>Cannibalism</td>
<td>Small eggs, egg discoloration, poor feathering</td>
<td>91</td>
</tr>
<tr>
<td>6</td>
<td>Jobran Maroun</td>
<td>August 8 2003</td>
<td>MazraetAzour</td>
<td>15</td>
<td>North</td>
<td>2</td>
<td>Low</td>
<td>Cannibalism, mites</td>
<td>Poor feathering, egg discoloration</td>
<td>114</td>
</tr>
<tr>
<td>7</td>
<td>Hadi Nammour</td>
<td>August 12 2003</td>
<td>Bkassin</td>
<td>18</td>
<td>North</td>
<td>3</td>
<td>Low</td>
<td>None</td>
<td>Egg discoloration</td>
<td>115</td>
</tr>
<tr>
<td>8</td>
<td>Nicolas Nicolas</td>
<td>August 12 2003</td>
<td>Baisor</td>
<td>7</td>
<td>Middle</td>
<td>86</td>
<td>Low</td>
<td>None</td>
<td>None</td>
<td>95</td>
</tr>
<tr>
<td>9</td>
<td>Nabil Bwaridi</td>
<td>August 12 2003</td>
<td>Lebaa</td>
<td>7</td>
<td>West</td>
<td>66</td>
<td>Low</td>
<td>None</td>
<td>None</td>
<td>97.5</td>
</tr>
<tr>
<td>10</td>
<td>Josef Shalhoub</td>
<td>August 12 2003</td>
<td>Kfarjarra</td>
<td>7</td>
<td>Middle</td>
<td>79</td>
<td>Low</td>
<td>None</td>
<td>None</td>
<td>95</td>
</tr>
</tbody>
</table>
On September 1, 2003, all the chicken in the free-range farms, which belonged to groups three and four (less than 24 weeks old), were given an oil emulsion inactivated vaccine subcutaneously. This vaccine is against three viruses namely, Newcastle Disease Virus, Infectious Bronchitis Virus, and Egg Drop Syndrome Virus. The vaccine name is Bio New EDS+IB, Merial, CHIGNOLO PO (PV), Italy.

Three weeks later (on September 23, 2003), a visit was taken to four of the farms that were given the dead vaccine and 10 blood samples were collected from each farm. The serum was extracted from the blood samples and the analyses for the three viruses were conducted using the ELISA method (for IBV and NDV) and Hemagglutination-Inhibition (HI) test (for EDS). These analyses were performed to assess the change in the total antibody titer of the chicken’s blood, specific to each of IBV, NDV, and EDS, after the vaccine was injected by three weeks. Also data was recorded from these farms as shown in the following Table.

<table>
<thead>
<tr>
<th>Case #</th>
<th>Farm Name</th>
<th>Bleeding Date</th>
<th>Region</th>
<th>Age (weeks)</th>
<th>Location</th>
<th>% Egg Production</th>
<th>Mortality</th>
<th>Previous Outbreaks</th>
<th>Present Signs</th>
<th>Feed Consumption (grams/bird/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>Bshara Jdeid</td>
<td>September 23, 2003</td>
<td>Isnayya</td>
<td>8</td>
<td>East</td>
<td>97</td>
<td>Low(^1)</td>
<td>None</td>
<td>None</td>
<td>110</td>
</tr>
<tr>
<td>12</td>
<td>Marie Sleiman</td>
<td>September 23, 2003</td>
<td>Jnsnaya</td>
<td>8</td>
<td>South</td>
<td>90</td>
<td>Low</td>
<td>None</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>Nabil Bwaridi</td>
<td>September 23, 2003</td>
<td>Lebaa</td>
<td>8</td>
<td>West</td>
<td>66</td>
<td>Low</td>
<td>None</td>
<td>None</td>
<td>115</td>
</tr>
<tr>
<td>14</td>
<td>Josef Shalhoub</td>
<td>September 23, 2003</td>
<td>Kfarjarra</td>
<td>8</td>
<td>Middle</td>
<td>89</td>
<td>Low</td>
<td>None</td>
<td>None</td>
<td>111</td>
</tr>
</tbody>
</table>

\(^1\)Low mortality means ≤ 1
b. **Serum Collection**

Ten individual blood samples were collected from the brachial vein of individual birds on each farm, containing a flock size of 200 free-range layers. The samples were centrifuged at 720 xg (International Equipment Company, Needham Heights, Mass., USA) for 10 minutes and the sera were collected and kept frozen at -20ºC for later serological analysis.

c. **Serology for Disease Diagnosis**

- **Hemagglutination-Inhibition (HI) for screening of chicken exposure to EDS**

  The Hemagglutination-inhibition (HI) test was used to detect the antibody titers in sera specific to Egg Drop Syndrome (EDS) virus (Thayer and Beard 1998). The EDS virus used in this test had a 4 HA strength; the antigen was prepared by Charles River SPAFAS Laboratory products, Connecticut, USA; the chicken RBC suspension was set at 0.5%.

  The Hemagglutination-Inhibition (HI) test is a quantitative test; it measures the level of antibodies in chicken serum that are specific to the hemagglutinin protein present in the virus. In the HI test, the adsorption or agglutination between hemagglutinin protein of the antigen and chicken red blood cells (RBC) is inhibited by the binding between hemagglutinin of the antigen and its specific antibody present in serum, thus allowing the RBCs used as an indicator in the test to settle down. The HI test consists of many steps:

  **Reagent preparation**

  - Preparation of Saline

    8.5 g NaCl were dissolved in 1000 ml distilled water (0.85% saline solution).
- Preparation of 25 % RBC suspension

A syringe of 10 ml capacity and a needle of 20-gauge size were used to draw whole blood from a commercial chicken layer. Four ml of Alsever’s solution were put in the syringe before drawing about 2-3 ml of the blood from the brachial vein. This solution is an anticoagulant (2 % dextrose, 0.8 % sodium citrate, 0.44 % sodium chloride and 0.05 % citric acid; pH = 6.1). The blood was then put in centrifuge tubes and spun for about ten minutes at 405 xg using centrifugation (Eppendorf AG 22331, Hamburg, Germany). After the first spinning, the supernatant (plasma + buffy coat) was discarded. The saline was added to the RBC at equal volume, mixed gently, centrifuged, and the supernatant was discarded again. This washing was repeated for another two times. At the end of the third washing, saline was added to the RBC resulting in a 25 % RBC stock suspension, which was kept in the refrigerator for not more than 4 days (the volume of the saline in the 25 % RBC suspension was three times that of the packed erythrocytes).

- The 0.5 % RBC suspension was prepared from the 25 % stock just before each test.

- Antigens preparation:

A four Hemagglutinating (HA) units of the Egg Drop Syndrome virus was prepared by performing HA test (Fenner et al. 1987) using serial dilutions of the antigen stock with a dilution factor of ½, and with RBC suspension of 0.5 %.

HI Test Procedure

- Twenty five µl of saline were delivered per well of a microtitration plate (96 wells, U bottoms) using 12 tip multichannel pipette (Digital
Multichannel, Finnpipette, Helsinki, Finland).

- Twenty five µl of the chicken serum per well were put in column number one.

- Dilution of the serum was performed from column 1 to column 12, using the same multichannel pipette, with a carried volume of 25 µl from one well to the other. The last 25 µl were then discarded (the dilution factor is 1:2).

- Twenty five µl of the 4 HA antigen for Egg Drop Syndrome was added in each well and the plates were rotated gently in a circular manner for about one minute, then incubated at 37ºC for about 30 minutes.

- Fifty µl of 0.5 % RBC suspension were then added to each well. The plates were also shaken in a circular way for about one minute.

- The plates were left at room temperature for 45 minutes.

- The antibody titer of each serum sample was read, using a special microplate reading mirror (Titertek, Microtitration equipment, Flow Laboratories); the titer is defined as the highest dilution of the serum that had enough antibodies to inhibit the agglutination between the agglutinin antigen of the virus and the receptors of agglutinin on the RBC.

- ELISA for screening of chicken exposure to IBV, NDV, MG, ALV, and IBDV

Commercial ELISA systems designed by ProFLOCK® Synbiotic Corporation, San Diego, USA, were made to quantitate the relative levels of antibodies in chicken serum that are specific to IBV, NDV, MG, ALV, and IBDV.

ELISA for IBV

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

The assay is designed to measure IBV antibody bound to IBV antigen coated plates. The principle of the test is as follows: Serum obtained from chickens exposed to IBV antigens contains specific anti-IBV antibodies. Serum, diluted in Dilution Buffer, is added to an IBV antigen coated plate. Specific IBV antibody in the serum forms an antibody-antigen complex with the IBV antigen bound to the plate. After washing the plate, an affinity purified goat anti-chicken IgG (H+L) peroxidase conjugate is added to each well. The antibody-antigen complex remaining from the previous step binds with the conjugate. After a brief incubation period, the unbound conjugate is removed by a second wash step. Substrate, which contains a chromagen (ABTS), is added to each well. Chromagen color change (from clear to green-blue) occurs in the presence of peroxidase enzyme. The relative intensity of color developed in 15 minutes (compared to controls) is directly proportional to the level of IBV antibody in the serum. After the substrate was incubated, Stop Solution is added to each well to terminate the reaction and the plate is read using an ELISA plate reader at 405-410 nm.

Sample Collection

Blood samples were collected from poultry farms in Jezzine; serum was extracted from the blood samples and stored (4°C for up to four days or at -20°C for longer periods). This is needed to provide reliable test results.

Sample Dilution Procedure

Dilute serum samples using Dilution Buffer in a clean, uncoated 96 well microtiter plate. This plate has 12 columns labeled with Arabic numbers, and 8 rows labeled alphabetically from A to H.
**Preparation of the Serum Dilution Plate**

- Add 300µl Dilution Buffer to each well of an uncoated 96 well microtiter plate. This plate is referred to as the serum dilution plate.

- Add 6µl unknown serum per well (producing a 1:50 dilution). Start with well A4 and end with well H9 (moving left to right, row by row of wells). It is worth noting that wells A1, A3, and H11 are reserved for positive control serum, while wells A2, H10, and H12 are reserved for the negative control serum.

**Preparation of IBV positive control**

An IBV Positive Control Serum has been provided with this kit. Dilute the appropriate volume of IBV Positive Control Serum with Dilution Buffer (1:50) in a tube, mix well. 150µl of diluted IBV Positive Control are needed per ELISA plate.

**Preparation of Conjugate Solution**

The horseradish peroxidase conjugated anti-chicken IgG (H+L) is supplied in HRP stabilizer. Dilute 100µl stock conjugate in 10ml Dilution Buffer, mix well. This 10ml preparation will supply sufficient conjugate for one 96 well ELISA plate.

**Preparation of Wash Solution**

Dilute 20ml concentrated Wash Solution in 380ml distilled water (1:20), mix well. This 400ml preparation is needed for one 96 well ELISA plate.

**Preparation of Substrate Solution**

The Substrate Solution is ready to use.

**Preparation of Stop Solution**

Dilute 2.5ml concentrated Stop Solution in 10ml distilled water (1:5), mix well. This 12.5ml preparation is needed for one 96 well ELISA plate.

**ELISA Test Procedure**
Preparing the Test Plate

- Remove an IBV antigen coated test plate from the protective bag and label according to dilution plate identification.
- Add 50μl Dilution Buffer to all wells on the test plate.
- Add 50μl diluted IBV Positive Control Serum to wells A1, A3 and H11.
- Add 50μl diluted IBV Negative Control Serum to wells A2, H10 and H12.
- Using a 12 channel pipette transfer 50μl/well of each of the diluted serum samples from the dilution plate to the corresponding wells of the IBV coated test plate.
- Incubate plate for 30 minutes at room temperature.

Wash Procedure

- Tap out liquid from each well into an appropriate vessel containing bleach or other decontamination agent.
- Using a 12 channel pipette, fill each well with 300μl wash solution. Allow soaking in wells for three minutes, and then discarding contents. Repeat wash procedure two more times.

Addition of Anti-Chicken IgG Peroxidase Conjugate, Substrate and Stop Solution

- Using a 12-channel pipette, dispense 100μl diluted conjugate into each assay well.
- Incubate for 30 minutes at room temperature.
- WASH as in steps a and b.
- Add 100μl Substrate Solution into each test well.
- Incubate 15 minutes at room temperature.
- Add 100µl diluted Stop Solution to each test well.
- Allow bubbles to dissipate before reading the plate.
- Read the plate using an ELISA plate reader set at 405 nm.

The ELISA plate reader determined the optical density (O.D.) of the serum samples, and then those values were introduced to a software program (SYNBIOTICS corporation). This program converts the optical densities into titers based on installed regression equation correlating the O.D. values to titers.

ELISA for NDV, MG, and IBDV has the same procedure as that to IBV. However, each kit is coated with the antigen specific to the virus, and each has a different installed regression equation in the used software.

ELISA for ALV differs from the previous ones. The difference is that there is no need for the dilution plate; hence the serum samples are put directly in the wells of the ALV ELISA kit. It is worth noting that the ALV is a direct ELISA and not indirect, like the previous ones, thus detecting the presence of the Retrovirus in the serum. More specifically, the wells are coated with antibodies specific to capture the protein (p27) present on the ALV.

d. Serology for Vaccine Evaluation

- 
  *HI for convalescent evaluation of EDS titers*

  Serum samples collected three weeks after administering the trivalent vaccine and those collected at vaccination were again assessed for their antibody response to the EDS antigens by the HI test. The HI test procedure was conducted as mentioned earlier under Materials and Methods.

- 
  *ELISA for convalescent evaluation of IBV and NDV titers*
Serum samples collected three weeks after administering the trivalent vaccine and those collected at vaccination were assessed also for their antibody level specific to IBV and NDV antigens by the indirect ELISA method. The ELISA test procedures used are the same as the ones used earlier for IBV and NDV, respectively.

e. **Statistical Analysis**

Analysis of variance of outbreak titers (among regions) was carried out using SPSS version 10. The experimental layout was Complete Randomized Design. Means were then separated by Duncan’s Multiple Range test ($\alpha = 0.05$).

Analysis of variance of vaccine titers (at vaccination and 3 weeks after vaccination) was carried out by the One Way Analysis of Variance (ANOVA-1) using the SPSS version 10.

2. **Separation and Identification of the Protective Polypeptides of IBV and NDV**

a. **Separation of the Virus-Aqueous Portion from Oil Emulsion Vaccine**

- A volume of 20 ml of the trivalent oil emulsion vaccine (NDV+IBV+EDS) is added over 20 ml of acetone.
  - Vortex (Vortex Mixer, Industries Inc. Bohem, N.Y., USA) for 1 minute.
  - Centrifuge at a speed of 15557 xg for 15 minutes.
  - Remove all the above liquid layers and keep the pellets in the tube bottom.
  - Add 1 ml saline over the pellets to reconstitute.
  - Vortex for 1 minute.
  - Centrifuge at a speed of 15557 xg for 15 minutes.
  - The reconstituted volume was distributed over 3 Eppendorf tubes.
  - Centrifuge at a speed of 3645 xg for 15 minutes.
• Remove the upper turbid layer and discard it.
• Collect the lower clear layer that contains the antigens (virus stock).
• The samples (virus stock) were pooled and collected in one tube.

b. Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) for Detecting Protein Bands Present In the Viral Vaccine Antigens

• Preparation of 12% separating gel
  - An amount of 3.35 ml of distilled water is mixed with 2.5 ml of 1.5M Tris-HCl, 100µl of 10% sodium dodecyl sulfate (SDS), 4 ml of acrylamide/Bis, 50µl of 10% ammonium persulfate and 5µl of TEMED (N,N,N,N’-Tetra-Methyl Ethylene Diamine) (BioRad Lab. 2000 Alfred NobelDr., Hercules, CA, USA).
  - The 12% separating gel was added using a pipette in between the assembled glass plate sandwiches of the Mini-PROTEAN II Electrophoresis cell (BioRad Lab. 2000 Alfred Nobel Dr., Hercules, CA, USA) that can run two gels at the same time. Distilled water is added at the end to straighten the gel surface. The gel is allowed to polymerize for one hour then the water is removed from above by using filter paper.
• Preparation of 4% stacking gel
  - A volume of 6.1 ml of distilled water is mixed with 2.5 ml of 0.5M Tris-HCl, 100µl of 10% SDS, 1.33 ml of acrylamide/Bis, 50µl of ammonium persulfate and 10µl of TEMED. Stacking gel is added in the area above the separating gel after placing a comb in the gel sandwich. The stacking gel is allowed to polymerize for one hour. The gel is ready, after removing the comb, for sample loading and electrophoresis.
• **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. An individual volume of 5µl of standards or samples were pipetted in a clean, dry microtiter plate into a well containing 25µl of reagent A and 20µl of reagent B (BioRad Lab. 2000 Alfred Nobel Dr., Hercules, CA, USA). The plate is then placed in a microplate reader and mixed for 5 seconds and absorbance is read after 15 minutes using a wavelength of 640 nm. A standard curve equation was established allowing the determination of the protein concentration in the viral stock namely, a concentration of 1.8 mg/ml was obtained using the Excel program (Microsoft 1997).

• **Gel Electrophoresis**

  - All the samples were diluted 1:2 in the SDS reducing sample buffer (25µl of β-mercaptoethanol and 475µl of stock sample buffer that was prepared by mixing 4.8 ml of distilled water, 1.2 ml of 0.5M Tris-HCl, 1 ml glycerol, 2 ml of 10% SDS and 0.5 ml of 0.1% Bromophenol blue). They were heated at 95°C for 5 minutes and 20µl of each of the reduced samples and 5µl of a broad range molecular weight marker (BioRad Lab. 2000 Alfred Nobel Dr., Hercules, CA, USA) ranging from 6.5 to 200 KDa were loaded on the stacking gels leaving a free lane in between.

  - After the running buffer was added (the running buffer contains an amount of 15 grams of Tris base dissolved with 72 grams of glycine and 5 grams of SDS in 1 L of distilled water, the pH of the buffer was adjusted to 8.3 using 6 N HCl), the gel was run at 200 V and 120 mA for about 45 minutes or until all the marker bands are separated. Gels were kept at 4°C in distilled
water or in transfer buffer to be used later on in Western Immunoblotting.

- In case you don’t want to progress to Western Immunoblotting, the gel could be stained with 0.1% Coomassie blue {an amount of 0.5 grams of Coomassie Blue R-250 powder (Bio-Rad Lab. 2000 Alfred Noble Dr. Hercules, CA, USA) was mixed with 200 ml of methanol (E. Merck, Darmstadt, Germany) and 50 ml of glacial acetic acid (Mallinckrodt Chemical Works, St. Louis, MO, USA) in 250 ml distilled water} with continuous shaking and then distained with a distainer solution (an amount of 400 ml of methanol and 100 ml of glacial acetic acid was mixed with 500 ml of distilled water) to remove the background (Barbour et al. 1989).

c. Western Immunoblotting for Detection of Specific Antibodies to Vaccine Antigens of IBV and NDV

The serum samples from each of two farms, before and after vaccination, were pooled separately. From each farm, 100 µl of each of ten individual serum samples were added to 1ml glycerol to form a total volume of 2 ml, hence a ratio of 1:1.

Nitrocellulose membrane (NCM), two pieces of blotting filter and two scotch-brite scouring pads were soaked in transfer buffer (an amount of 6.06 grams of Tris-base and 28.8 grams of glycine were added to 400 ml of methanol and made to 2000 ml with distilled water, no acid or base was added and the pH was 8.4, the solution was stored at 4°C), before sandwiching, for 30 minutes. The gel and the NCM were sandwiched between the two pieces of prewetted blotting filter, which were sandwiched between two prewetted scotch-brite scouring pads. The sandwich was placed in a hinged holder and immersed in transfer buffer contained in the transblot cell (BioRad Lab. 2000 Alfred Nobel Dr., Hercules, CA, USA). The electrophoretic transfer time was
one hour at 100 V and 25 mA.

The NCM containing the peptides transferred from SDS-PAGE gel were immersed in Ponceau stain {an amount of 0.5 grams of Ponceau S Stain (Sigma, St. Louis, MO, USA) was mixed with 7.5 grams of trichloroacetic acid and 7.5 grams of sulfosalicylic acid in 250 ml distilled water} for 5 minutes with continuous shaking at 60 rpm. The NCM was then rinsed with distilled water for 2-3 times to resolve the transferred polypeptides. The molecular weight marker was cut and preserved at 4 °C in distilled water. The NCM were immersed in Tris-Buffer Saline (TBS) for 10 minutes shake at 60 rpm (TBS was prepared by dissolving 19.38g of Tris-base and 233.8g of NaCl in 4 liters of distilled water, pH was then adjusted to 7.5 using 6N HCl). Blocking of the active sites was done by immersing the NCM in 5% gelatin-TBS for one hour at 37°C in a water bath with continuous shaking. The NCM was washed twice with TTBS (TTBS was prepared by mixing one liter of TBS with 0.5 ml of Tween-20) for 5 minutes shake per each wash.

SPAFAS IBV Mass, IBV Ark, NDV positive control sera and negative control sera (Charles River SPAFAS Laboratory products, USA) were diluted with 1% gelatin in Tris-Tween-Buffer Saline (TTBS) to 1:100. On the other hand, pooled serum samples from each of the two farms, before and after vaccination, were also diluted with 1% gelatin-TTBS to 1:100. The NCM lanes were cut carefully, so that each lane containing peptides of IBV and NDV will be reacted separately with specific positive control sera, negative control sera, and pooled sera collected before and after vaccination to allow judgment on the presence of specific antibodies to protective antigens of IBV and NDV. These reactions take place after incubating the cut NCM lanes containing the antigens with their specific control and pooled sera for two hours at 37°C in a water bath with continuous shaking. Unbound antibodies were removed by washing the NCM twice
with TTBS for 5 minutes shake per wash. A conjugate of anti-chicken IgG (H+L) chains made in goats and labeled with peroxidase enzyme (Sigma, St. Louis, USA) was diluted to 1:1000 in 1% gelatin-TTBS and was added to the NCM and incubated at 37°C in a water bath with continuous shaking for 30 minutes.

The NCM were washed twice with TTBS for a 5 minutes shake per wash.

NCM were further washed twice in TBS for 5 minutes shake per wash then dried over a filter paper. A 3,3’-Diaminobenzidine (DAB) Peroxidase Substrate Solution (Sigma, St. Louis, USA) was added to the NCM for 30 minutes at 37°C in a water bath with continuous shaking to obtain bands of brown color. NCM were then rinsed with distilled water 2-3 times and dried over a filter paper then photographed with GelDoc 2000 System (BioRad Lab. 2000 Alfred Nobel Dr., Hercules, CA, USA).

d. Western Immunoblotting for Reacting Antigens of ND Clone Vaccine with NDV Sera

For further molecular detection of antibody response to ND polypeptides, an ND clone vaccine (Nobilis R, product of Intervet International B.V., Boxmeer- Holland) was prepared for reaction against the NDV positive and negative antisera and the farm serum samples.

This vaccine was suspended in 2 ml distilled water then distributed over aliquots and stored in the freezer. The Protein Assay using Bio-Rad reagents determined the protein concentration. The protein level in the ND clone sample was 36.8 mg/ml.

The same procedure of SDS-PAGE followed by Western Immunoblotting was conducted on the ND clone vaccine where the samples were diluted 1:2 in the SDS reducing sample buffer.

After blocking the active sites on the NCM, the NCM lanes containing the
NDV peptides were cut and reacted separately with the NDV positive and negative control sera and the pooled serum samples (before and after vaccination). However, the molecular weight standards included in this test were the Kaleidoscope Prestained Standards (BioRad Lab. 2000 Alfred Nobel Dr., Hercules, CA, USA) ranging from 6.5 to 200 KDa.

e. Western Immunoblotting for Reacting Antigens of IB H120 & IB 4-91 Vaccines with IBV Sera

Both IB H120 and IB 4-91 (Nobilis\textsuperscript{R}, product of Intervet International B.V., Boxmeer- Holland) were further used to detect the antibody response to IBV by reacting these two vaccines with the IBV positive and negative control sera and the pooled farm serum samples.

Both vaccines were suspended each in 2 ml distilled water then distributed over aliquots and stored in the freezer. The Protein Assay using Bio-Rad reagents determined the protein concentration. The protein levels were 20.35 and 20.1 mg/ml for IB H120 and IB 4-91 respectively.

The same procedure of SDS-PAGE followed by Western Immunoblotting was conducted on both vaccines where the samples were diluted 1:2 in the SDS reducing sample buffer.

After blocking the active sites on the NCM, the NCM lanes containing the IBV peptides were cut and reacted separately with the IBV positive and negative control sera and the pooled serum samples (before and after vaccination). The molecular weight standards included in this test were the Kaleidoscope Prestained Standards (BioRad Lab. 2000 Alfred Nobel Dr., Hercules, CA, USA) ranging from 6.5 to 200 KDa.
f. Quantitating the Antibodies Reacting to Protective Polypeptides

The antibodies specific to IBV and NDV formed as bands on the NCM were quantitatively measured by reading the optical densities of the bands formed, before and after vaccination, using a computerized program known as Quantity One 1-D Analysis Software (BioRad Lab. 2000 Alfred Nobel Dr., Hercules, CA, USA).

The procedure works by selecting a random area equivalent to 5 mm² of a specific polypeptide and then dragging this same area to the other polypeptide that needs to be compared. The program will measure the optical density with respect to the chosen area (OD/mm²) and will calculate the mean optical density (OD). In order for us to obtain statistical analysis of the quantitative immune responses to each polypeptide, three random areas were selected from each polypeptide formed after reacting pooled sera (N=10) of layers from farms before vaccination and were compared to other equal and random three areas of the same polypeptide of farms after vaccination.

The One Way Analysis of Variance (ANOVA-1) using the SPSS version 10 carried out analysis of variance of the quantitative immune responses (before and after vaccination).
CHAPTER IV
RESULTS AND DISCUSSION

A. Experiment 1: Anti-Inflammatory Effect of *Origanum ehrenbergii* Essential Oil against *Staphylococcus aureus* Skin Infection in Rabbits

1. Antimicrobial Activity of Essential Oil from *Origanum ehrenbergii* Harvest of Three Different Locations

The antimicrobial effect of *Origanum ehrenbergii* essential oil on *Staphylococcus aureus* from three different locations in Lebanon was determined. Two different concentrations of the essential oil were used for each harvested location namely, 1/100 and 1/200 and the diameter of inhibition zone was measured after 24 hours of incubation at 37°C. According to Table 6, the greatest diameters of inhibition zones were achieved by using a dilution of 1/100 (location 3) where the mean diameter of inhibition zone was 1.3 cm. However, when pure essential oil was used without any dilution, the largest diameter of inhibition zone (6.25 cm) was observed in essential oil harvested from location 2. An inhibition zone of 10 mm is considered to be the limit zone of being an efficient antibiotic (Alma *et al.* 2003); hence our results prove that *Origanum ehrenbergii* has effective antibacterial effects.

Table 6. The effect of different harvest-locations of *Origanum ehrenbergii* essential oil on *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>Dilution*</th>
<th>Oil location</th>
<th>1/100</th>
<th>1/200</th>
<th>1/100</th>
<th>1/200</th>
<th>1/100</th>
<th>1/200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter of inhibition zone after 24 hrs incubation (duplicates)</td>
<td></td>
<td>0.9 cm</td>
<td>0.0 cm</td>
<td>1.15 cm</td>
<td>0.0 cm</td>
<td>1.2 cm</td>
<td>0.0 cm</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>0.45 cm</td>
<td>0.0 cm</td>
<td>1.03 cm</td>
<td>0.0 cm</td>
<td>1.3 cm</td>
<td>0.55 cm</td>
</tr>
<tr>
<td>Pure oil</td>
<td></td>
<td>5.5 cm</td>
<td>6.25 cm</td>
<td>5.0 cm</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Dilution of essential oil in 40 % DMSO.
Since our interest in this study is to assess the effect of essential oil incorporated with DMSO and water (40 %), then we selected oil harvested from location 3 with a dilution of 1/100.

2. Impact of DMSO Level on Antimicrobial Activity of Dispersed Essential Oil in Aqueous Medium

In order to determine the optimum level of DMSO that must be incorporated with the essential oil, several levels of DMSO dilutions were to be tested. Table 7 shows the DMSO levels ranging from 1 to 5 % while the essential oil concentration was constant at 1 %. The greatest diameters of inhibition zones (average = 0.6 cm) after 24 hours incubation at 37°C were achieved by using a dilution of 1/100 essential oil in 5 % DMSO in water. Table 8 shows the DMSO levels ranging from 5 to 40 % while the essential oil concentration was constant at 1 %. The greatest diameters of inhibition zones (average = 0.95 cm) after 24 hours incubation at 37°C were achieved by using a dilution of 1/100 essential oil in 40 % DMSO in water.

In a study conducted by Nakamura et al. (1999), they tested the activity of Ocimum gratissimum essential oil in inhibiting Staphylococcus aureus in vitro. They incorporated the essential oil with DMSO at two levels (24 and 48 %) and the results showed better antimicrobial activity for the essential oil diluted in 48 % DMSO by forming a diameter of inhibition zone of 21 mm. Our results are almost in agreement with those of Nakamura et al. (1999) with a slight difference in DMSO levels and a variation in inhibition zones probably because of the difference in species between the two essential oils, and possibly due to difference in Staphylococcus aureus strains used in both studies.
Table 7. In-vitro analysis of *Origanum ehrenbergii* essential oil dispersed in different percentages of DMSO in water, and their effect on inhibiting *Staphylococcus aureus* growth

<table>
<thead>
<tr>
<th>DMSO level</th>
<th>1%</th>
<th>2%</th>
<th>3%</th>
<th>4%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Essential Oil*</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>DMSO</td>
<td>1 µl</td>
<td>2 µl</td>
<td>3 µl</td>
<td>4 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>D.W.</td>
<td>98 µl</td>
<td>97 µl</td>
<td>96 µl</td>
<td>95 µl</td>
<td>94 µl</td>
</tr>
<tr>
<td>Diameter of inhibition zone after 24 hours incubation (duplicates)</td>
<td>0.8 cm</td>
<td>0.8 cm</td>
<td>0.8 cm</td>
<td>0.8 cm</td>
<td>0.4 cm</td>
</tr>
<tr>
<td>Average</td>
<td>0.4 cm</td>
<td>0.4 cm</td>
<td>0.4 cm</td>
<td>0.4 cm</td>
<td>0.6 cm</td>
</tr>
</tbody>
</table>

* The essential oil used here is from location 3 which previously gave the best results among the three locations by the Kirby-Bauer method (Table 6).

Table 8. *In-vitro* Analysis of *Origanum ehrenbergii* essential oil dispersed in different percentages of DMSO in water, and their effect on inhibiting *Staphylococcus aureus* growth

<table>
<thead>
<tr>
<th>DMSO level</th>
<th>5%</th>
<th>10%</th>
<th>20%</th>
<th>30%</th>
<th>40%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Essential Oil*</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>DMSO</td>
<td>25 µl</td>
<td>50 µl</td>
<td>99 µl</td>
<td>149 µl</td>
<td>198 µl</td>
</tr>
<tr>
<td>D.W.</td>
<td>470 µl</td>
<td>445 µl</td>
<td>396 µl</td>
<td>346 µl</td>
<td>297 µl</td>
</tr>
<tr>
<td>Diameter of inhibition zone after 24 hours incubation (duplicates)</td>
<td>0.9 cm</td>
<td>0.8 cm</td>
<td>0.9 cm</td>
<td>0.9 cm</td>
<td>0.9 cm</td>
</tr>
<tr>
<td>Average</td>
<td>0.9 cm</td>
<td>0.8 cm</td>
<td>0.9 cm</td>
<td>0.45 cm</td>
<td>0.95 cm</td>
</tr>
<tr>
<td>Pure Oil</td>
<td></td>
<td></td>
<td></td>
<td>3.7 cm</td>
<td></td>
</tr>
</tbody>
</table>

* The essential oil used here is from location 3 which previously gave the best results among the three locations (Table 6).

3. **Optimized Antimicrobial Activity of Essential Oil and DMSO Dilution against Different Staphylococcus aureus Densities**

After determining the best essential oil location among the three available sources along with the optimum concentration of essential oil and DMSO levels, different dilutions of *Staphylococcus aureus* were to be tested to determine the
appropriate inoculum count (CFU/ml). For this purpose, different dilutions of *Staphylococcus aureus* ranging from $10^3$ to $10^6$ CFU/ml were tested in vitro. Table 9 shows that the greatest diameter of inhibition zone (after 24 hours of incubation at 37ºC) that was achieved by the essential oil (used in 1/100 dilution in 40% DMSO in water) against *Staphylococcus aureus* standardized at $10^5$ CFU/ml (mean diameter = 1.03 cm).

In a study conducted by Baydar *et al.* (2004) to evaluate the antibacterial activity of essential oils, the inoculum counts of *Staphylococcus aureus* used in vitro were $10^6$ CFU/ml. However, our in vitro results showed that the inoculum count of $10^5$ CFU/ml gave larger diameters of inhibition zones than the inoculum count of $10^6$ CFU/ml.

All the necessary in vitro analysis was conducted after determining the best oil location (location 3), optimum concentrations of essential oil (1 %) and DMSO levels (40 %), and the appropriate bacterial inoculum counts ($10^5$ CFU/ml). Hence, in vivo analysis will follow the in vitro analysis to evaluate the effect of essential oil treatment on intradermal *Staphylococcus aureus* challenge in rabbits.

### Table 9. The Effect of *Origanum ehrenbergii* Essential Oil on Different *Staphylococcus aureus* Counts

<table>
<thead>
<tr>
<th>Inoculum count (CFU/ml)</th>
<th>$10^3$</th>
<th>$10^4$</th>
<th>$10^5$</th>
<th>$10^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter of inhibition zone after 24 hours incubation (triplicates)</td>
<td>0.7 cm</td>
<td>0.9 cm</td>
<td>1.2 cm</td>
<td>0.8 cm</td>
</tr>
<tr>
<td></td>
<td>0.0 cm</td>
<td>1.0 cm</td>
<td>0.8 cm</td>
<td>0.9 cm</td>
</tr>
<tr>
<td></td>
<td>0.0 cm</td>
<td>0.8 cm</td>
<td>1.1 cm</td>
<td>0.9 cm</td>
</tr>
<tr>
<td>Average</td>
<td>0.23 cm</td>
<td>0.9 cm</td>
<td>1.03 cm</td>
<td>0.87 cm</td>
</tr>
</tbody>
</table>

### 4. Experiment 1

In the first experiment, rabbits were challenged with $10^5$ CFU/ml *Staphylococcus aureus* intradermally, and then each group was treated differently. The
average diameter of inflammation zone was recorded for each group over a period of three days (Table 10), and then the values were plotted on a graph (Figure 2). In Table 10, the largest mean diameter of inflammation zone with a value of 0.79 cm was recorded in Group 4 at Day 1 after challenge. While the lowest mean diameter of inflammation zone with a value of 0.30 cm was observed in Group 3 at Day 3 of challenge.

In a study done by Reinoso et al. (2002), rabbits were intradermally inoculated with 0.1 ml Staphylococcus aureus (10^8 CFU/ml) in saline solution and the macroscopic lesions were evaluated according to their diameter of inflammation. The diameter of inflammation was defined as negative (< 2 mm), small (2 to 5 mm), moderate (6 to 10 mm), and severe (> 10 mm). Histopathological examinations showed inflammation at the surface of the skin with abundant hyperemia expanding into the epidermis, dermis, hypodermis, and the muscle layers. The diameter of inflammation was considered moderate since it was measured to be more than 5 mm.

Table 10. Mean diameter of inflammation zone measured on the rabbit^1 ears challenged^2 with Staphylococcus aureus and treated with Origanum ehrenbergii essential oil over a period of three days

<table>
<thead>
<tr>
<th></th>
<th>Group 1 Non-challenged, non-treated</th>
<th>Group 2 Challenged, non-treated</th>
<th>Group 3 Challenged, treated with 1% essential oil in 40 % DMSO/water</th>
<th>Group 4 Challenged, treated with 40% DMSO/water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>NA</td>
<td>0.6455 cm</td>
<td>0.4945 cm</td>
<td>0.7865 cm</td>
</tr>
<tr>
<td>Day 2</td>
<td>NA</td>
<td>0.5240 cm</td>
<td>0.4365 cm</td>
<td>0.6720 cm</td>
</tr>
<tr>
<td>Day 3</td>
<td>NA</td>
<td>0.3300 cm</td>
<td>0.3030 cm</td>
<td>0.4955 cm</td>
</tr>
</tbody>
</table>

^1 Each ear group had n = 7 rabbits.
^2 Challenge was 10^5 CFU/ml Staphylococcus aureus. The injected amount in the skin was 0.1 ml. NA = not applicable.
In comparison to our study, inflammation at Day 3 after treatment with essential oil (Group 3) is considered to be small (3 mm) since it falls within the range of 2 to 5 mm. According to Easmon and Adlam (1983), there might be a possible relationship between dermatonecrosis and α-hemolysin that is produced in high quantities by *Staphylococcus aureus* causing severe skin lesions in rabbits. Hence, this minimal skin inflammation three days after challenge in the non-treated group (Group 2) might be interpreted by the low levels of α-hemolysin production.

In Figure 2, the diameter of inflammation zone decreased by time in all three groups. However, the diameter of inflammation zone in the rabbits treated with 1 % essential oil in 40 % DMSO/water (Group 3) gave the lowest values compared to those treated with 40 % DMSO/water (Group 4) and the non-treated challenged rabbits.
(Group 2), especially on the first day of challenge. This suggests that the *Origanum ehrenbergii* essential oil had an anti-inflammatory effect against the *Staphylococcus aureus* infection. Nevertheless, on the third day of infection, both rabbits treated with the essential oil and those non-treated were having approximately the same inflammation zone. Thus, we conclude that the challenge with *Staphylococcus aureus* was weak. In this experiment, it is worth noticing that rabbits treated with 40% DMSO/water had the highest diameter of inflammation zone compared to those treated with essential oil or to non-treated challenged rabbits. This suggests that DMSO was increasing the inflammation when it was administered alone. However, when incorporated with the essential oil, DMSO gave better results.

5. Experiment 2

In experiment two, two groups of rabbits (each group had 4 rabbits) were challenged intradermally with *Staphylococcus aureus* (10^5 CFU/ml), one group was treated with essential oil over a period of one day as opposed to three days in the previous experiment. The other group was left untreated as a positive control. After one day of treatment, a homogenate of each cut ear was taken and serial dilutions of 1:1, 1:10, and 1:100 were made in tubes containing PBS. These dilutions were streaked on MSA plates and incubated for 48 hours as mentioned in Materials and Methods to obtain the bacterial count. After 48 hours of incubation, no bacterial growth was obtained on any of the MSA plates. Since neither the treated group nor the non-treated group showed any bacterial growth, then the intradermal *Staphylococcus aureus* challenge was not strong enough to persist under the skin for 24 hours. It is worth noting that the same bacterial stock (10^5 CFU/ml) of *Staphylococcus aureus* showed inhibition to treatment done in vitro (Table 9).
6. Experiment 3

Since both of the previous experiments showed these failures, a different approach had to be attempted. When the bacteria cultured in vitro are transformed to in vivo conditions, the virulence factors in the bacteria are not rapidly reactivated to overcome the innate immune defenses of the host. So the virulence of the bacteria can be enhanced and restored by passing the bacteria through animals (Vahidy et al. 1996). In a study conducted by Peters and Peterson (2002), two in vivo passages were required to restore the induction of cell-mediated immunity to passenger antigens and maximum virulence to these antigens. Hence in vivo passages helping in increasing the virulence of *Staphylococcus aureus* were followed in Experiment 3.

In Experiment 3, two groups of rabbits were challenged intradermally with *Staphylococcus aureus* that has been subjected to 3 in vivo passages to increase its virulence. One group was treated with 1 % essential oil in 40 % DMSO and the other group was left untreated as mentioned in Materials and Methods. Results showed that there was a significant difference in the mean weight of the ear lesion between the rabbits treated with the essential oil having a mean weight of 1.24 g as compared to those left untreated having a mean weight of 2.72 g (P<0.05). This suggests that the essential oil played a role in reducing the inflammatory response (Table 11).

Usually, an inflammatory response is induced by infection with *Staphylococcus aureus* and it is associated with swelling and redness, and then the polymorphonuclear leukocytes will follow by attracting and accumulating. In case the infection continues and spreads deeper, it will reach the subcutaneous tissue forming an abscess. This abscess leads to painful swelling that is known as boil (Todar 2004). In our case, the application of the essential oil might have played a role in diminishing the abscess and decreasing the accumulation of white blood cells. Thus a reduction in the mean weight
of the ear lesion was observed as compared to those left untreated.

Table 11. Effect of *Origanum ehrenbergii* essential oil on the weight and bacterial count of lesions in rabbit ears challenged with *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean weight of ear lesion (Grams)</th>
<th><em>S. aureus</em> CFU/gram of ear lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 hrs</td>
</tr>
<tr>
<td><em>Origanum ehrenbergii</em>(^1)</td>
<td>1.24(^b)</td>
<td>1.64*10(^7)</td>
</tr>
<tr>
<td>Control</td>
<td>2.72(^a)</td>
<td>2.60*10(^6)</td>
</tr>
</tbody>
</table>

\(^{a-b}\) Means with different alphabetical superscripts in columns are significantly different (P<0.05).
\(^1\) The essential oil was used at 1% in 40% DMSO/water at the rate of 2 times with 4 hours intervals.

In this same experiment, the bacterial count of the ear lesion for both groups was determined after 24 and 48 hours of incubation (Table 11). However, results showed no significant differences in the bacterial counts. After 24 hours of incubation, the bacterial counts in the group treated with essential oil (1.64 x 10\(^7\) CFU/ml) were higher than those of the non-treated group (2.60 x 10\(^7\) CFU/ml). While after 48 hours of incubation, the bacterial counts were almost equal in both groups (2.58 x 10\(^6\) CFU/ml). These results suggest that after 24 hours of incubation, the essential oil had no effect on the bacterial counts of *Staphylococcus aureus*. While after 48 hours of incubation, the essential oil reduced the bacteria counts but not to a high extent whereby the final count was almost equal to the control group. This failure could be contributed to the ability of *Staphylococcus aureus* to coagulate the plasma (Blair 1939; 1962; Bayliss and Hall 1965) forming a protection around *Staphylococcus aureus* cells thus preventing the essential oil to have its antimicrobial effect against this pathogen. It is worth noting that this essential oil has a high antimicrobial effect against *Staphylococcus aureus* in vitro.
as mentioned earlier and this failure to clear the infection in the rabbit is most likely due to the inability of essential oil to reach to the *Staphylococcus aureus* cells in skin.

B. Experiment 2: Serological Profiling and Molecular Detection of Viral Protective Polypeptides by Sera of Sentinel Free-Range Layers

1. **Serology for Disease Diagnosis**

   Serological profiling was used in evaluating the outbreak titers of free-range chicken layers. Table 12 represents the immune responses to Infectious Bronchitis Virus (IBV), Newcastle Disease Virus (NDV), Avian Leukosis Virus (ALV), *Mycoplasma gallisepticum* (MG), and Infectious Bursal Disease Virus (IBDV) using the Enzyme-Linked Immunosorbant Assay. While the immune response to Egg Drop Syndrome (EDS) is represented in Table 12 using the Hemagglutination-Inhibition (HI) test. The immune responses in Table 12 are categorized according to the different regions in Jezzine area. Each region; namely the north, south, east, west, and the central region of Jezzine is represented by two farms. Nine serum samples were randomly collected from each farm for analysis of the above six diseases. Previous workers depended on serological profiling to uncover exposure of poultry to specific disease antigens (Cooper *et al.* 1989; Gast and Beard 1990; Keck *et al.* 1993; McBride *et al.* 1991; Schelling *et al.* 1999; Wunderwald and Hoop 2002).

   The ELISA titers specific to IBV showed significant differences in humoral antibody levels between the different regions in Jezzine. The highest relative titers for IBV were observed in the south and the east followed by the north, and the lowest relative titers were observed in the west and the central region of Jezzine.

   The ELISA titers specific to NDV also showed significant differences in humoral antibody levels between the different regions in Jezzine. The highest relative
Titters for NDV were observed in the south and the central region followed by the north and the west, and the lowest relative titters were observed in the eastern region of Jezzine.

Table 12. Outbreak mean titters from the different regions in Jezzine area for six different viruses

<table>
<thead>
<tr>
<th>Farm Regions</th>
<th>No. of Farms</th>
<th>Samples/Farm</th>
<th>Means</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IBV</td>
</tr>
<tr>
<td>North</td>
<td>2</td>
<td>9</td>
<td>9988.11\textsuperscript{ab}</td>
</tr>
<tr>
<td>South</td>
<td>2</td>
<td>9</td>
<td>11986.67\textsuperscript{a}</td>
</tr>
<tr>
<td>East</td>
<td>2</td>
<td>9</td>
<td>11831.94\textsuperscript{a}</td>
</tr>
<tr>
<td>West</td>
<td>2</td>
<td>9</td>
<td>7799.17\textsuperscript{b}</td>
</tr>
<tr>
<td>Central</td>
<td>2</td>
<td>9</td>
<td>7707.33\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a-b-c} Means with different alphabetical superscripts in columns are significantly different (P<0.05).

No significance was observed in the column of ALV (P>0.05).

The ELISA titters specific to ALV showed no significant differences in humoral antibody levels between the different regions of Jezzine. In addition, the mean titer values among the different regions are relatively low with respect to the titer values of the other diseases.

The ELISA titters specific to MG showed significant differences in humoral antibody levels between the different regions in Jezzine. The highest relative titters for MG were observed in the central region followed by both the south and the west after which came the north while the eastern region gave the lowest relative titters for MG.

The ELISA titters specific to IBDV showed a slightly significant difference in humoral antibody level between the north, south, west, and central regions of Jezzine as
compared to the eastern region which gave a relatively lower titer for IBDV.

The Hemagglutination-Inhibition (HI) test used for evaluating the outbreak titers for EDS showed significant differences in humoral antibody levels between the different regions in Jezzine. The highest relative titers for EDS were observed in the south followed by the central region, then the west and finally both the north and the east with lowest relative titers for EDS.

Among the six viruses that were serologically evaluated for their immune response with respect to the different regions, the south region seemed in many instances to have the highest titers to most etiologic agents studied (Table 12). Farms situated in the southern area of Jezzine are geographically closer to the southern borders of Lebanon; hence they are most likely subjected to aerosol-transmitted viruses coming from the poultry farms in Israel. Future investigations should emphasize the search for transboundary diseases to help in formulating regional projects for control of the spread of such identified diseases (FAO 1997).

2. Serology for Vaccine Evaluation

Serological profiling was used in evaluating a trivalent oil emulsion vaccine against three viruses, namely NDV, IBV, and EDS. The humoral antibody response for the three viruses (IBV, NDV, and EDS) was measured before the trivalent vaccine was administered and 21 days after vaccination. Previous literature suggests that the immune response to killed vaccines will show its significance after 21 days of the administration. Hence paired serum samples are recommended for serological profiling whereby the first sampling occurs at the time of vaccination (acute) and the convalescent sampling 3 weeks later (Gillingham 2003). Table 13 represents the immune response to IBV and NDV using the ELISA, while the immune response to
EDS is represented in the same table using the HI test. The immune responses to each virus were measured twice, before and after vaccination (Table 13). Two farms were included, each farm containing 9 samples to be analyzed.

Table 13. Vaccine titers before and after 21 days of vaccination with a trivalent killed vaccine against IBV, NDV, and EDS

<table>
<thead>
<tr>
<th>Vaccination Status</th>
<th>No. of Farms</th>
<th>Samples/ Farm</th>
<th>Means</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IBV</td>
</tr>
<tr>
<td>Before</td>
<td>2</td>
<td>9</td>
<td>9366.17</td>
</tr>
<tr>
<td>After 3 weeks</td>
<td>2</td>
<td>9</td>
<td>11378.33</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td></td>
<td>600.90</td>
</tr>
</tbody>
</table>

No significance was observed in the columns of IBV, NDV, and EDS (P>0.05).

The ELISA titer specific to IBV showed no significant difference in humoral antibody level before and after vaccination, although there was an increase in the titer after vaccination. In the case of NDV, there was a slight increase in the titer after vaccination but there was no significant difference among the values.

The HI test specific to EDS showed no significant difference in humoral antibody level before and after vaccination where there was a small difference in the titer values before and after vaccination.

The immune responses of farms before and after vaccination with a trivalent inactivated vaccine were evaluated (Table 13). With respect to IBV, no significant differences in the titer values were observed; however, there was a 21.5% increase in titer. This increase in titer value after vaccination shows that the birds responded to the vaccine by acquiring more immunity levels. Similarly, with respect to NDV, the birds responded to the vaccine by a slight increase of 4.7% in titer. Nevertheless, this increase
in titer was not considered to be significant. In the case of EDS, a slight decrease of 1.5% in titer was observed after vaccination; still, this decrease was not considered to be significant.

It is worth noting that although there was no significant difference in the humoral antibody level after 3 weeks of vaccination, the farms showed an increase of 10% in egg production and an increase of 13% in feed consumption. This is an indication that the vaccine has enhanced the immunity and the health of the birds that reflected in a positive manner on their egg production and their feed consumption.

3. Vaccine Failure

According to Butcher and Miles (1993), a failure in vaccination occurs when inadequate antibody titers are developed after vaccination and/or the chickens are exposed to field disease outbreaks. A failure in vaccination is most probably due to the vaccine. However, additional factors may play a role in causing a vaccine failure such as:

• High level of maternal antibodies present in young birds that may block the vaccine virus receptors thus preventing them from attaching to the receptor cells and multiplying in them. This process will lead to a decrease in immune response to the vaccine.

• Wide variation in immunological responses among individual birds as a result of stress. Stress usually appears when the bird is subjected to environmental extremes, parasitism, and inadequate nutrition, in addition to other diseases.

• Mechanical failure resulting from improper storage, handling, and administration of the vaccine. To avoid such failure, the manufacturer’s recommendations should be followed carefully.
• Different strains or serotypes present in the vaccine may be different than those required to stimulate protective immunity.

• Missed birds in different areas of the poultry house due to poor vaccine distribution particularly while administering live vaccines by the spray or water route. The live vaccine may be transmitted from vaccinated to unvaccinated birds, but this may result in delayed immunity at the level of the flock. As for killed vaccines, birds that are missed will get no protection since killed vaccines are not transmitted among birds.

• Birds may already have acquired the disease that the vaccine aims to protect against. In such situations, the birds will be diseased since antibody production requires time to reach protective levels.

• Immunosuppressed birds as a result of infection with Marek’s disease, IBDV, or due to high levels of mycotoxins in the diet.

• Poor quality vaccines that might be contaminated or contain low titer levels.

Nevertheless, vaccine failure resulting from problems with the vaccine is rare due to the quality control on the vaccine manufacturing industries.

In our study, there was obviously an insignificant seroconversion after three weeks of vaccination as shown by ELISA and HI (Table 13). This could be due to several factors that are discussed earlier. One of these factors is the immunosuppression caused by IBDV since serological profiling for indicating the outbreak titers revealed high titers for IBDV (Table 12). Another possible reason for vaccine failure could be that the strains present in the vaccine might be different than those used in the coated ELISA plates. This was evident when the antigens of the trivalent vaccine reacted only with the positive Mass sera and showed no reaction with the Ark positive sera (Figure 4). Also birds might already have acquired the disease before being vaccinated; this was
evident by the serological profiling to uncover the disease outbreak for NDV, IBV, and EDS that showed high titers before vaccination (Table 12). A wide variation in immunological responses might also be a possible explanation for a vaccine failure. This wide variation is caused by stress that the birds are subjected to. In our case, the birds were probably subjected to stress caused by environmental extremes since the free-range farms are situated on high elevations where the temperature fluctuates between day and night. In addition, the farms are not tightly constructed to prevent wind from entering the barn and thus causing stress.

4. Quantitative Immune Responses to Polypeptides

The immune responses to the trivalent vaccine were assessed by Western Immunoblotting (Figure 3). After reacting the vaccine antigens with each of two pooled serum samples of the farms (before and after vaccination), the formed bands of sera after vaccination had a higher optical density than the bands formed by the sera collected of farms before vaccination. Furthermore, additional bands were observed from sera of farm flocks after vaccination (60 KDa). This suggests that the vaccine enhanced the development of acquired immunity in the birds with new antibodies that have specificity to certain antigens that were not initially present. In Figure 3, the antigens of NDV showed high competitiveness to antigens of IBV and EDS in the trivalent vaccine due to the fact that antibodies developed in the vaccinated birds were mainly against NDV antigens namely, the 248 and 60 KDa. The NDV antigens could be highly immunogenic or the antigens of NDV might be present in higher quantities compared to IBV and EDS in the trivalent vaccine. This lead to an immune response specific to two proteins that are present in the NDV, namely to the 248 and the 60 KDa proteins. It is reported before that the 248 KDa protein is the large (L) protein that is a
subunit of the paramyxovirus RNA polymerase complex (Takimoto et al. 2000).

According to Lamb et al. (1976), the paramyxovirus L protein is the least abundant among the structural proteins having a molecular weight of more than 200 KDa. This large L protein is responsible for most of the enzymatic activities involved in transcription and viral replication. All the catalytic steps of RNA synthesis, capping and methylation are executed by the paramyxovirus L proteins (Wagner and Rose 1996).

As for the protein with the molecular weight of 60 KDa, it is reported to be the fusion (F) protein. The fusion between the virion envelope and the cellular plasma
membrane is mediated by the F protein (Nagai et al. 1989). For the F protein, the quantity of basic amino acids at the cleavage site will determine the cleavability of the protein. This number of basic amino acids will give the virus its ability to spread and multiply in both animals and cell culture (Römer-Oberdörfer et al. 2003). In his study, Römer-Oberdörfer et al. (2003) showed that the primary determinant responsible for NDV virulence is the basic amino acid sequence present at the cleavage site of the F protein. According to Humhuan et al. (2004), effective protection against paramyxoviruses is significantly contributed by antibodies against the F protein that was determined to have a molecular weight of 60 KDa. It was recommended that the paramyxovirus vaccine should be designed to contain both the HN and the F proteins in order to obtain effective results (Merz et al. 1980). Table 14 shows the quantitative immune responses three weeks after vaccination with the trivalent vaccine compared to the initial antibody level at the time of vaccination. It was detected that the mean optical density (OD) reflecting antibody levels for the large (L) protein was significantly higher after vaccination compared to the initial antibody levels. The mean OD values of antibodies in the sera of Farms 1 and 2 reacting to the 248 KDa large (L) protein of NDV were 4.76 and 4.95 after vaccination compared to sera collected before vaccination that were 3.92 and 3.47, respectively (P<0.05).

Table 14. Mean optical densities of humoral antibody response induced by a killed trivalent vaccine against the 248 KDa polypeptide of NDV present in the same trivalent vaccine antigens

<table>
<thead>
<tr>
<th>Polypeptide (KDa)</th>
<th>Farm 1 before vaccination</th>
<th>Farm 1 after vaccination</th>
<th>SEM</th>
<th>Farm 2 before vaccination</th>
<th>Farm 2 after vaccination</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>248</td>
<td>3.92</td>
<td>4.76</td>
<td>0.19*</td>
<td>3.47</td>
<td>4.95</td>
<td>0.34*</td>
</tr>
</tbody>
</table>

* Means in a row before the SEM value followed by a star-superscript on the SEM value are significantly different (P<0.05).
In Figure 4, the antigens of the trivalent vaccine reacted only with the positive IBV-Mass strain serum forming three bands, while no reaction was observed with positive IBV-Ark strain, positive NDV, and the negative control sera. The three bands formed on the positive Mass lane had molecular weights of 90, 84, and 51 KD resembling the S1 glycoprotein, S2 glycoprotein, and the phosphorylated nucleocapsid protein of IBV, respectively (Stern and Safton 1984). Both the S1 and S2 glycoproteins belong to the spike (S) glycoprotein of IBV that constitutes the large surface projections on the coronaviruses (Cavanagh et al. 1992; Kant et al. 1992).

Fig. 4. Banding patterns and molecular weight (KDa) of each detected polypeptide band after reacting the antigens of the trivalent vaccine (NDV+IBV+EDS) with positive sera and negative control sera as revealed by Western Immunoblotting. Lane 1 = molecular weight markers (KDa), lane 2 = sample reacted with positive IBV-Mass strain sera, lane 3 = sample reacted with positive IBV-Ark strain sera, lane 4 = sample reacted with positive NDV sera, lane 5 = sample reacted with negative control sera. The 90, 84 and 51 KDa bands represent the S1 spike protein, S2 spike protein and the nucleocapsid protein, respectively, those are present in IBV.
These glycoproteins are responsible for the binding of the virions to the host cell surface leading to cell fusion and then infection (Holmes et al. 1981). However, the S1 glycoprotein has been identified to be the main inducer of protective immune responses since it induces hemagglutination-inhibition (HI) and virus neutralization (VN) antibodies (Cavanagh et al. 1984; Kock et al. 1990; Mockett et al. 1984). As for the nucleocapsid protein that is associated with genomic RNA, this protein plays an essential role in the replication and the structure of IBV (Stern and Sefton 1982; Seo et al. 1997). During replication, large quantities of nucleocapsid proteins are produced. In addition, among the various IBV strains, the nucleocapsid protein has a highly conserved amino acid sequence (Siddel et al. 1983; Williams et al. 1992; Seo et al. 1997). Sneed et al. (1989) showed that in addition to the protective immunity present in the spike protein, still the nucleocapsid protein is highly immunogenic in terms of cytotoxic T lymphocytes (CTL) and antibody levels. This suggests that in order to obtain effective vaccine strategies against IBV, both the spike protein and the nucleocapsid protein should be incorporated together especially since the S1 glycoprotein is known for its continuous antigenic diversity.

Figure 5 shows the banding patterns of detected polypeptides present in the ND clone 30 live vaccines. Three proteins were found to correlate in their molecular weight to proteins present in NDV. These proteins had molecular weights of 248, 60, and 40 KDa representing the large L protein, the fusion F protein, and the matrix M protein (Elango 1989) present in NDV, respectively. The functions of the L protein and the F protein were mentioned earlier. However, the matrix M protein is believed to coordinate the budding process of virus particles after being assembled at the plasma membrane. This coordination done by the M protein involves interactions with the nucleocapsid, the plasma membrane, and the cytoplasm of the viral glycoprotein (Peeples 1991;
Peeples et al. 1992). In addition, the M protein has been proved to play a role in inhibiting the host RNA transcription and its protein synthesis and thus inducing cytopathologic changes (Dunigan et al. 1986; McGowan et al. 1982). In this regard, it is worth noting that NDV is known to have the highest cytopathic effect among the different paramyxoviruses (Hightower and Bratt 1974), probably due to the characteristic provided by the M protein in inhibiting the host protein synthesis.

Fig. 5. Banding patterns and molecular weight (KDa) of each detected polypeptide band of the antigens of ND clone 30 live vaccine. Bands are seen on a nitrocellulose membrane after being transferred from SDS-PAGE and stained with a Ponceau stain. Lanes 1 to 4 are replicates of the same polypeptides of ND clone 30, lane 5 = molecular weight markers (KDa). The 248, 60 and 40 KDa bands represent the large L protein, fusion F protein and the matrix M protein, respectively, those are present in NDV.

In Figure 6, the antigens of the ND clone 30 live vaccine were reacted with the positive and negative NDV sera. Two bands with molecular weights of 60 and 40 KDa representing the F protein and the M protein reacted with the positive NDV sera while
no reaction was observed on the negative NDV sera. In Figure 5, the ND clone 30 live vaccine showed three antigens belonging to NDV. Nevertheless, after reacting the antigens of the live vaccine with the positive NDV sera (Figure 6), only the F protein and the M protein were observed while the L protein was undetected. From these results, we may suspect that the positive NDV sera are specific to two main proteins in NDV, namely the F protein and the M protein.

In Figure 7, the antigens of the ND clone 30 live vaccine were reacted with the pooled serum samples from farms before and after three weeks of being administered
the trivalent vaccine. Sera from both farms before being vaccinated reacted with the F protein and the M protein with molecular weights of 60 and 40 KDa, respectively.

Three weeks after vaccination, sera from both farms reacted to the same proteins but their reaction to the M protein was more obvious. Table 15 shows the quantitative immune response against both the F protein (60 KDa) and the M protein (40 KDa). The mean OD values of antibodies in the sera of Farm 1 and Farm 2 reacting to the F protein were 4.74 and 4.73 after vaccination compared to sera collected before vaccination that were 4.66 and 5.55, respectively.

![Fig. 7. Banding patterns and molecular weight (KDa) of each detected polypeptide band after reacting the antigens of ND clone 30 live vaccine with pooled serum samples from farms given a trivalent vaccine (NDV+IBV+EDS) as revealed by Western Immunoblotting. Lane 1 = antigens of ND clone 30 vaccine reacted with serum samples from Farm 2 three weeks after vaccination, lane 2 = antigens of ND clone 30 vaccine reacted with serum samples from Farm 2 before vaccination, lane 3 = antigens of ND clone 30 vaccine reacted with serum samples from Farm 1 three weeks after vaccination, lane 4 = antigens of ND clone 30 vaccine reacted with serum samples from Farm 1 before vaccination, lane 5 = molecular weight markers (KDa). The 60 and 40 KDa bands represent the fusion F protein and the matrix M protein, respectively, those are present in NDV.](image)
Table 15. Mean optical densities of humoral antibody response induced by a killed trivalent vaccine against different polypeptides present in ND clone 30 vaccine

<table>
<thead>
<tr>
<th>Polypeptide (KDa)</th>
<th>Farm 1 before vaccination</th>
<th>Farm 1 after vaccination</th>
<th>SEM</th>
<th>Farm 2 before vaccination</th>
<th>Farm 2 after vaccination</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>3.71</td>
<td>3.85</td>
<td>0.06</td>
<td>3.46</td>
<td>4.23</td>
<td>0.20*</td>
</tr>
<tr>
<td>60</td>
<td>4.66</td>
<td>4.74</td>
<td>0.05</td>
<td>5.55</td>
<td>4.73</td>
<td>0.20</td>
</tr>
</tbody>
</table>

* Means in a row before the SEM value followed by a star-superscript on the SEM value are significantly different (P<0.05).

For the M protein, the mean OD values of antibodies in the sera of Farm 1 and Farm 2 were 3.85 and 4.23 after vaccination compared to sera collected before vaccination that were 3.71 and 3.46, respectively. However, for the F protein, no significant change in quantitative immune response was observed three weeks after the vaccination with the trivalent vaccine as compared to the initial antibody level specific to the F protein at the time of vaccination (Table 15). The following data suggest that the trivalent vaccine has boosted the immunity of the birds with antibodies specific to the M protein present in NDV. No significant quantitative change was observed for the F protein probably because they initially had high quantitative levels (mean OD = 5.55) before being vaccinated.

Figure 8 shows the antigens of two IBV live vaccines, IB H120 and IB 4-91, after being reacted with positive IBV-Mass strain sera and negative IBV sera. It is interesting to notice that although the two vaccines contain different strains of IBV; however, both of them had the same banding patterns on the positive Mass lane. This suggests that the two vaccines contain similar polypeptides, but they may differ in their epitopes. The polypeptides of either IB H120 or IB 4-91 that reacted with the positive IBV-Mass strain sera had molecular weights of 155, 90, 51, 36, and 28 KDa.
corresponding to the S protein, S1 glycoprotein, nucleocapsid protein, and the two membrane proteins GP36 and GP28, respectively. The role of the Spike protein, S1 protein, and the nucleocapsid protein has already been mentioned. As for GP36 and GP28, these heterogeneous membrane proteins have the same 23 KDa core polypeptide but they only differ in the type and number of asparagine-linked oligosaccharides (Stern and Sefton 1982). These membrane glycoproteins protrude slightly outside the virions interacting with the inner viral membrane through the nucleocapsid and the nucleate assembly (Sturman et al. 1980). Thus the IBV membrane glycoproteins might play a role in spanning the virion envelope.

Fig. 8. Banding pattern and molecular weight (KDa) of each detected polypeptide band after reacting the antigens of two live vaccines, IB H120 and IB 4-91, with positive IBV-Mass strain sera and negative IBV sera as revealed by Western Immunoblotting. Lane 1 = antigens of IB 4-91 vaccine reacted with negative IBV sera, lane 2 = antigens of IB H120 vaccine reacted with negative IBV sera, lane 3 = antigens of IB 4-91 vaccine reacted with positive IBV-Mass strain sera, lane 4 = antigens of IB H120 vaccine reacted with positive IBV-Mass strain sera, lane 5 = molecular weight markers (KDa). The 155, 90, 51, 36 and 28 KDa bands represent the spike S protein, S1 spike protein, nucleocapsid protein, glycoprotein 36 and glycoprotein 28, respectively, those are present in IBV.
Figure 9 shows the antigens of two IBV live vaccines, IB H120 and IB 4-91, after being reacted with positive IBV-Ark strain sera and negative IBV sera. Almost the same results were obtained as compared to those in Figure 8. This suggests that both antibodies specific to IBV-Mass strain and IBV-Ark strains react to the similar polypeptides present in both IBV vaccines, namely the IB H120 and IB 4-91.

**Fig. 9.** Banding pattern of each detected polypeptide band after reacting the antigens of two live vaccines, IB H120 and IB 4-91, with positive IBV-Ark strain sera and negative IBV sera as revealed by Western Immunoblotting. Lane 1 = antigens of IB 4-91 vaccine reacted with negative IBV sera, lane 2 = antigens of IB H120 vaccine reacted with negative IBV sera, lane 3 = antigens of IB 4-91 vaccine reacted with positive IBV-Ark strain sera, lane 4 = antigens of IB H120 vaccine reacted with positive IBV-Ark strain sera, lane 5 = molecular weight markers (KDa). The 155, 90, 51, 36 and 28 KDa bands represent the spike S protein, S1 spike protein, nucleocapsid protein, glycoprotein 36 and glycoprotein 28, respectively, those are present in IBV.

In Figure 10, the antigens of both IB H120 and IB 4-91 live vaccines were reacted with the pooled serum samples from Farm 1 before and after three weeks of
being administered the trivalent vaccine. The banding pattern of sera from Farm1 before and after vaccination was the same. In addition, Farm 1 reacted to the same polypeptides present in both vaccines; namely, the 155 KDa spike protein, the 90 KDa S1 protein, the 51 KDa nucleocapsid protein, and the two membrane proteins GP36 and GP28. The quantitative immune response of the antibodies in sera of Farm 1 showed significant values only for the nucleocapsid protein (51 KDa) after reacting the sera with the antigens of IB H120 (Table 16).

![Figure 10](image)

Fig. 10. Banding pattern of each detected polypeptide band after reacting the antigens of two live vaccines, IB H120 and IB 4-91, with pooled serum samples of Farm 1 given a trivalent vaccine (NDV+IBV+EDS) as revealed by Western Immunoblotting. Lane 1 = antigens of IB 4-91 vaccine reacted with serum samples of Farm 1 three weeks after vaccination, lane 2 = antigens of IB H120 vaccine reacted with serum samples of Farm 1 three weeks after vaccination, lane 3 = antigens of IB 4-91 vaccine reacted with serum samples of Farm 1 before vaccination, lane 4 = antigens of IB H120 vaccine reacted with serum samples of Farm 1 before vaccination, lane 5 = molecular weight markers (KDa). The 155, 90, 51, 36 and 28 KDa bands represent the spike S protein, S1 spike protein, nucleocapsid protein, glycoprotein 36 and glycoprotein 28, respectively, those are present in IBV.
Table 16. Mean optical densities of humoral antibody response induced by a killed trivalent vaccine against different polypeptides present in IB H120 and IB 4-91 vaccines

<table>
<thead>
<tr>
<th>Polypeptide (KDa)</th>
<th>Farm 1 reacted with IB H120 before vaccination</th>
<th>Farm 1 reacted with IB H120 after vaccination</th>
<th>SEM</th>
<th>Farm 1 reacted with IB 4-91 before vaccination</th>
<th>Farm 1 reacted with IB 4-91 after vaccination</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>4.57</td>
<td>4.35</td>
<td>0.06</td>
<td>4.59</td>
<td>4.21</td>
<td>0.09*</td>
</tr>
<tr>
<td>36</td>
<td>4.27</td>
<td>4.32</td>
<td>0.02</td>
<td>4.34</td>
<td>4.04</td>
<td>0.07*</td>
</tr>
<tr>
<td>51</td>
<td>4.22</td>
<td>4.45</td>
<td>0.06*</td>
<td>4.40</td>
<td>3.88</td>
<td>0.12*</td>
</tr>
<tr>
<td>90</td>
<td>4.46</td>
<td>4.56</td>
<td>0.08</td>
<td>4.45</td>
<td>4.03</td>
<td>0.11*</td>
</tr>
<tr>
<td>155</td>
<td>4.11</td>
<td>4.12</td>
<td>0.04</td>
<td>4.23</td>
<td>3.92</td>
<td>0.08*</td>
</tr>
</tbody>
</table>

* Means in a row before the SEM value followed by a star-superscript on the SEM value are significantly different (P<0.05).

The mean OD values of antibodies reacting to the 51 KDa nucleocapsid protein of IBV was 4.45 when the sera was collected after vaccination compared to sera collected before vaccination (4.22) (P<0.05). However, the antibodies in the sera from Farm 1 that reacted with the antigens of IB 4-91 showed an unexpected significant decrease in mean OD values when collected after vaccination in comparison to that before vaccination (Table 16).

In Figure 11, the antigens of both IB H120 and IB 4-91 live vaccines were reacted with the pooled serum samples from Farm 2 before and after three weeks of being administered the trivalent vaccine. Before vaccination, the pooled serum samples from Farm 2 reacted to two polypeptides present in both IB H120 and IB 4-91, namely the nucleocapsid protein and the membrane glycoprotein GP28 with molecular weights of 51 and 28 KDa, respectively. Three weeks after vaccination, the pooled serum samples from Farm 2 reacted to the same two proteins, while an additional protein with a molecular weight of 36 KDa was recognized by new acquired antibodies, representing the membrane glycoprotein GP36.
The banding pattern of each detected polypeptide band after reacting the antigens of two live vaccines, IB H120 and IB 4-91, with pooled serum samples of Farm 2 given a trivalent vaccine (NDV+IBV+EDS) as revealed by Western Immunoblotting. Lane 1 = antigens of IB 4-91 vaccine reacted with serum samples of Farm 2 three weeks after vaccination, lane 2 = antigens of IB H120 vaccine reacted with serum samples of Farm 2 three weeks after vaccination, lane 3 = antigens of IB 4-91 vaccine reacted with serum samples of Farm 2 before vaccination, lane 4 = antigens of IB H120 vaccine reacted with serum samples of Farm 2 before vaccination, lane 5 = molecular weight markers (KDa). The 51, 36 and 28 KDa bands represent the nucleocapsid, glycoprotein 36 and glycoprotein 28 proteins, respectively, those are present in IBV.

The quantitative immune response of the antibodies in the sera of Farm 2 showed significant increase to the membrane glycoprotein GP28 with a molecular weight of 28 KDa, when the sera was collected 3 weeks post vaccination and when reacted to the antigens of both IB H120 and IB 4-91 (Table 17). The mean OD values of antibodies in sera reacting to the 28 KDa membrane glycoprotein of IBV was 3.21 and 3.12 in the sera collected after vaccination compared to sera collected before vaccination namely, 2.81 and 2.84 for IB H120 and IB 4-91, respectively (P<0.05). As for the nucleocapsid protein, the quantitative immune response of the antibodies in the
sera of Farm 2 showed no significant values knowing that the mean OD values increased from 3.11 and 3.25 before vaccination to 3.76 and 3.78 after vaccination, when reacting the sera to IB H120 and IB 4-91, respectively (Table 17). These results suggest that the trivalent vaccine has boosted the immunity levels to the membrane glycoprotein GP28; also the trivalent vaccine enhanced the immune system by forming antibodies against an additional protein, namely the membrane glycoprotein GP36.

Table 17. Mean optical densities of humoral antibody response induced by a killed trivalent vaccine against different polypeptides present in IB H120 and IB 4-91 vaccines

<table>
<thead>
<tr>
<th>Polypeptide (KDa)</th>
<th>Farm 2 reacted with IB H120 before vaccination</th>
<th>Farm 2 reacted with IB H120 after vaccination</th>
<th>SEM</th>
<th>Farm 2 reacted with IB 4-91 before vaccination</th>
<th>Farm 2 reacted with IB 4-91 after vaccination</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>2.81</td>
<td>3.21</td>
<td>0.09*</td>
<td>2.84</td>
<td>3.12</td>
<td>0.07*</td>
</tr>
<tr>
<td>51</td>
<td>3.11</td>
<td>3.76</td>
<td>0.19</td>
<td>3.25</td>
<td>3.78</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* Means in a row before the SEM value followed by a star-superscript on the SEM value are significantly different (P<0.05).

On the other hand, the Western-immunoblotting experiments did reveal in many instances, an induction of quantitative increase in antibodies specific to more polypeptides of the viruses after 3 weeks of the vaccination compared to those present at the vaccination time. This could be summarized in appearance of new antibodies to the F protein (60 KDa) in the case of NDV, and the appearance of new antibodies to the membrane glycoprotein GP36 (36 KDa) in the case of IBV three weeks after vaccination compared to at vaccination time. In addition, there were more quantitative levels of antibodies to the large (L) protein (248 KDa) and the matrix (M) protein (40 KDa) in NDV and to the nucleocapsid protein (51 KDa) and the membrane glycoprotein
GP28 (28 KDa) in the case of IBV three weeks after vaccination compared to levels at the time of vaccination. These positive results on induction of better immunity to protective antigens could cause a better field protection to the IBV and NDV viruses. Future investigations should correlate such improvements in antibody induction specific to certain polypeptides to protection against controlled challenge by NDV and IBV in free-range poultry.
CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Two experiments were conducted in this study; the first was to assess the anti-inflammatory effect of *Origanum ehrenbergii* essential oil on skin lesions of rabbits challenged intradermally with *Staphylococcus aureus*. The second experiment was conducted on free-range poultry farms in which serological profiling was followed in order to determine the disease outbreaks and to evaluate the birds’ humoral immune responses 3 weeks after being administered an inactivated trivalent vaccine. Furthermore, the immune responses to the trivalent vaccine were both quantitatively and qualitatively assessed by reacting the pooled farm sera with antigens of the trivalent vaccine and antigens of different live vaccine strains by Western Immunoblotting.

A. Experiment 1: Anti-inflammatory Effect of *Origanum ehrenbergii* Essential Oil against *Staphylococcus aureus* Skin Infection in Rabbits

Based on the results and discussions (Chapter IV), the following conclusions and recommendations can be made:

- In vitro analysis of 1 % *Origanum ehrenbergii* essential oil diluted in 40 % DMSO/water showed an inhibition zone of 10.3 mm when tested against *Staphylococcus aureus* \(10^5\) CFU/ml.
- Intradermal challenge with *Staphylococcus aureus* \(10^5\) CFU/ml was not strong enough to persist under the skin for more than 3 days.
- Two in vivo passages of *Staphylococcus aureus* in the rabbit ears were able to increase its pathogenicity.
- Topical treatment with *Origanum ehrenbergii* essential oil was able to
reduce the mean weight of the ear lesions in rabbits while it failed in reducing the bacterial counts in the ear lesions.

- Our data shows that the *Origanum ehrenbergii* essential oil has an antibacterial effect when tested in vitro, but in vivo treatments with *Origanum ehrenbergii* essential oil on ear lesions challenged with *Staphylococcus aureus* failed to show antibacterial effects. This suggests that the essential oil was not reaching the site of infection probably due to the coagulation of plasma formed by *Staphylococcus aureus*. Further studies should be made on the antibacterial effect of *Origanum ehrenbergii* essential oil on in vivo *Staphylococcus aureus* challenge.

**B. Experiment 2: Serological Profiling and Molecular Detection of Viral Protective Polypeptides by Sera of Sentinel Free-Range Layers**

Based on the results and discussions (Chapter IV), the following conclusions and recommendations can be made:

- Serological profiling for disease diagnosis showed high outbreak titers for IBV, NDV, EDS, MG and IBDV.

- Serological profiling for vaccine evaluation failed to show significant changes in humoral immune responses for IBV, NDV and EDS. Reasons for this failure could be attributed to the immunosupression caused by IBDV or the difference in strains between the antigens present in the vaccine and the antigens coating the ELISA plates. This suggests that serological profiling for vaccine evaluation using the ELISA system is not as accurate as the molecular detection of serum antibodies specific to protective polypeptides of viruses.

- Although the ELISA system showed no significant differences in humoral immune responses after 3 weeks of vaccination, reacting the pooled serum samples of
the farms with the antigens of different vaccine strains showed an improvement in both quantitative and qualitative immune response at 3 weeks post vaccination in comparison to that before the vaccination.

- Future investigations should correlate such improvements in antibody induction specific to certain polypeptides to protection against controlled challenge by NDV and IBV in free-range poultry.
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