THE EFFECT OF VARIOUS HOMOEOPATHIC POTENCIES OF THE ANTIGENIC COMPONENT OF A NEWCASTLE DISEASE VACCINE ON THE IMMUNE RESPONSE OF BROILER CHICKENS TO THE NEWCASTLE DISEASE VACCINE.

by
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To my parents, Dirk and Kotie Mostert,
for their endless love, support and motivation.

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ABSTRACT

Two hundred and ten (210) commercially produced Hubbard x Peterson broiler chickens were randomly divided into 5 groups of 42 chickens when they were 14 days old. Each group was divided into two replications. Two separate rooms of a poultry house were used to house the chickens, and each room housed one replication of each group. Group 1 was not vaccinated and acted as an indicator of extraneous Newcastle disease. Group 2, 3, and 4 received 12C, 30C and 200C potencies of the antigenic component of the vaccine respectively. Homoeopathic remedies were administered in the drinking water from day 14 to day 42. Group 5 was the vaccinated control group and received alcohol in the same concentrations as the groups receiving treatment from day 14 to day 42. On day 14 the birds were vaccinated subcutaneously in the retronuchal area with 0.5ml of a killed oil emulsion vaccine. Haemagglutination Inhibition tests were performed to quantify the immune response to the vaccine on blood samples taken from all the birds on days 14, 28 and 42. Three way factorial analyses with groups, rooms and days as factors was carried out using proc GLM. The daily administration of homoeopathic dilutions of the antigenic component of the Newcastle

disease vaccine after the birds were vaccinated, did not have a significant influence on the antibody mediated immune response to this vaccine.

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DEFENITIONS OF TERMS

Adjuvant: a substance which, when administered with a drug or antigen, enhances its pharmacologic effect or its antigenicity

Antigen: any substance capable of producing a specific immune response and of reacting with the products of that response

Broiler: chicken raised to slaughter at six weeks, when its body weight is approximately 1.8kg

Emulsion: a mixture of two immiscible liquids, one being dispersed throughout the other in small droplets

***In vitro*:** in an artificial environment

***In vivo*:** within the living body

Mortality: refers to the death of birds

Panzootic: occurring pandemically among animals

PBS: Phosphate Based Saline

Physiological saline: an isotonic aqueous solution of NaCl for temporarily maintaining living cells

Potency: the stage of altered remedial activity to which a drug has been taken by means of a measured process of deconcentration, with succussion, or by trituration, of the medicinal substance, which is thus brought to a state of diminutive or infinitesimal subdivision

Poultry: domestic fowls

Titre: the quantity of a substance requited to react with or to correspond to a given amount of another substance

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CHAPTER 1

INTRODUCTION

Newcastle disease virus has long been known as one of the most diverse and deadly avian pathogens (Peeples 1988). The outbreak of Newcastle disease in 1993/1994 in South Africa was severely damaging to the poultry industry and has served to remind us that sudden outbreaks are always possible (Fourie and Coetzee 1997). It is therefore important to maintain a high level of immunity to Newcastle disease in a flock by effective vaccination.

Serological testing quantifies the active immunity the bird has produced in response to a vaccine by measuring the number of antibodies that are formed in response to vaccine (Le Roux 1995). The more antibodies produced against the vaccine, the better the protection against the disease (Nakamura *et al.* 1994). The Haemagglutination Inhibition (HI) test is a standard test that is widely used to quantify the antibody response against Newcastle disease in chickens (Spradbrow *et al.*(1988); Jagne *et al.* (1991), Stone (1997) and Roy *et al.*(1998)). The most desirable features of the HI test is its simplicity and economy; material and

technical requirements for the test are minimal, and micro methods facilitate manual or semiautomatic processing of large numbers of samples (Brugh *et al.* 1977). The results obtained by this test are a good predictor of the protection of chickens against this disease (Allan and Gough 1974).

Different approaches have been used in the attempt to improve the immune response to Newcastle disease vaccines.

Franchini *et al.* (1991) studied the effect of vitamin E on the immune response in chickens by partially replacing the mineral oil component of inactivated emulsified vaccines against Newcastle disease with different amounts of vitamin E. Four hundred chickens were used which were divided into 6 groups of 67 birds each. Five levels of vitamin E were used and one control group. The immune response was monitored by the HI test. Results were analyzed by using the Newman-Keuls Test. It was found that vitamin E increased the immune response in chickens to the Newcastle disease virus when the vitamin E component did not exceed 30% of the oil phase.

Stone (1997) tested animal, vegetable and synthetic oils as potential replacements of mineral oil in Newcastle disease oil emulsion vaccines. Several groups of chickens including a control group were used. Because

there were only 10 birds per group one can draw only cautious conclusions of this study. The immune response was monitored by the HI test. Their results were analyzed using the analysis of variance method. The different oil adjuvants did not improve the immune response of these chickens significantly.

Homoeopaths claim that homoeopathically prescribed medicines "stimulate the body's own immune and defense systems to initiate the healing process" (Ulman 1991). Furthermore Bastide (1994) is of the opinion that immunological models are very useful in demonstrating the activity of homoeopathic remedies.

Moss *et al.* (1982) tested the effects of *Belladonna* 8X, *Pyrogenium* 6C, *Staphylococcin* 6C, *Hepar sulphuricum* 8X and *Silicea* 8X on the movement of human leukocytes and guinea-pig macrophages in vitro. In their study homoeopathic remedies were shown to significantly inhibit and facilitate the movement of macrophages and leukocytes. Both of these effects might be advantageous since facilitation speeds the movement towards the site of infection and inhibition is required to keep the cells there.

Superoxide is the fundamental product of oxidative metabolism in all phagocytes when their membranes interact with specific particulate or soluble agents. Adhesion is a function of leukocytes that allows them to migrate outside the blood vessels onto various surfaces. The influences of homoeopathic remedies on these functional parameters were explored in two *in vitro* studies. These studies found that various homoeopathic remedies influenced superoxide release and adhesion to serum- coated plastic surfaces. (Chirumbolo *et al.* 1993, 1997.)

Studies have been conducted to show the influence of homoeopathic remedies on the humoral immune system. Youbicier-Simo *et al.* (1993) did an *in vivo* study where chick embryos were surgically bursectomized at 80 hours of incubation and then administered dilutions of bursin or saline *in ovo*. All the birds were repeatedly immunized with porcine thyroglobulin. It was found that *in ovo* administration of low doses of bursin (100fg, 100pg, 5×10^{-27}g) led to high antibody production in bursectomized birds after repeated immunization.

The effect of high dilutions of antigen on specific humoral immune response has been studied. Mice were pretreated with 6C (10^{-15} M), 7C (10^{-17}M) and 15C (10^{-33}M) dilutions of keyhole-limpet-hemocyanin (KLH) for

three weeks. The mice were then immunized using 1 mg of KLH associated with incomplete Freud's adjuvant in the foot pads. Following immunization an increase in the primary IgM response was observed in almost all the preconditioned groups. A significant increase in the KLH specific IgG response in the KLH – treated groups could also be found. (Weissman *et al.* 1991, 1992. Cited in Bastide 1994.) 12C ($1x10^{-24}$) is the lowest centesimal potency where Avogadro's number ($6.023x10^{-23}$) is exceeded and from here on in the potency range not a single molecule of the original vaccine is expected to remain (Gaier 1991:47).

The only dilution of KLH above Avogadro's number used Weissman's above mentioned experiment was the 15C ($1x10^{-30}$). It is possible that the presence of antigen could have influenced the results obtained by the 6C ($1x10^{-12}$) and 7C ($1x10^{-14}$) dilutions.

The purpose of this *in vivo* study is to investigate the influence of 12C, 30C and 200C potencies of the antigenic component of the Newcastle disease vaccine on the immune response of broiler chickens to this vaccine.

CHAPTER 2

REVIEW OF THE RELATED LITERATURE

2.1 THE POULTRY INDUSTRY

Poultry meat accounts for slightly more than a quarter of the world's meat consumption. This share is increasing as consumption of poultry meat is rising more quickly than consumption of other meats. Worldwide demand for poultry products has increased substantially in both developed and developing countries and is now about 50 million tons per year. (Monnikhof 1997.) The International Finance Corporation forecast that the world demand for poultry should grow by more than 5% per annum during the period 1995 to the year 2000 and that the highest growth would occur in developing countries. This would result in an increase of around 15 million tons on world poultry consumption between 1995 and the year 2000. (Monnikhof 1997.) In South Africa chicken meat has increased its relative share in the meat market from just under 13% in 1969 to almost 44% in 1994 (Liebenberg and Reid 1996).

2.1.1 THE BROILER INDUSTRY

The broiler is one of the most efficient converters of plant protein into meat, reaching a live mass of 1.8 kg at 42 days with a feed conversion ratio

of less than 2 kg of feed per kilo of live mass gained (Fourie and Coetzee 1997). The broiler industry is classed as intensive. Broilers are kept in high population density houses (19 birds / m^2 at Rainbow chickens), and all food is supplied in regular quantity and quality. In such conditions disease prevention and control is very important. (Julian 1995: 526.) Broilers are raised for slaughter at six weeks of age, when their body weight is approximately 1.8 kg (Bell and North 1990: 453). Having such a short life span, the birds do not have time to recover from disease outbreak. Disease prevention is therefore crucial.

2.2 IMMUNE SYSTEM OF THE CHICKEN

Immunity has both acquired (specific) and innate (nonspecific) components. Innate immunity refers to naturally occurring defense mechanisms that protect an animal from disease. As the name implies, this resistance does not exhibit specificity. There are a number of innate defense mechanisms that provide protection against a variety of stressors. Defense barriers comprising innate immunity include anatomic, physiological and phagocytic components in addition to inflammation.

Specific immunity reflects the presence of a functional immune system capable of recognizing and selectively eliminating foreign material. Unlike

innate immunity, specificity, diversity, memory and self-recognition are displayed. "Acquired" and "innate" immune systems interact, since macrophages (of the innate system) are involved in the activation of the acquired system. (Newman 1996.)

2.2.1 ANATOMICAL DEFENSE MECHANISMS

The first line of defense in a bird is the skin that forms a physical barrier to the entry of most microorganisms. Most bacteria and many viral and fungal agents are susceptible to low concentrations of organic acids produced by sebaceous and sweat glands. Sebum maintains the pH of the skin between 3 and 5, which inhibits many microorganisms. The respiratory, urinary and gastrointestinal tracts are covered by mucous membranes and a number of nonspecific defense mechanisms prevent entry of pathogens into the body. Saliva, tears and mucous secretions contain antibacterial and antiviral substances, which neutralize or wash away potential infectious agents. The epithelial cells of mucous membranes secrete viscous mucus, which entraps foreign microorganisms. The mucous membrane of the respiratory and gastrointestinal tract is lined with cilia, which move foreign material to the exterior of the body. (Newman 1996.)

2.2.2 PHYSIOLOGICAL DEFENCE MECHANISMS

Temperature, pH, oxygen and soluble proteins such as lysozyme, lactoferrin, lactoperoxidase, interferon and complement are all involved in immunity. Gastric acidity prevents many potential pathogens from causing infection. In addition, the presence of bile salts in the intestinal tract inhibits many microorganisms by disrupting bacterial membranes. The resident microflora in the gastrointestinal tract also play a protective role by competitive exclusion. (Newman 1996.)

2.2.3 SPECIFIC DEFENCE MECHANISMS

The specific defense mechanisms which are the cell-mediated and antibody mediated immune systems are highly specific defense mechanisms designed to destroy specific pathogens or toxins. (Solomon *et al.* 1990: 792 - 796) Specific immunity is the product of the lymphocyte system. Most of the lymphocytes in normal lymphoid tissue look alike when studied under the microscope, but these cells are distinctly divided into two major populations. One of the populations is responsible for forming the activated lymphocytes that provide cell-mediated immunity and the other for forming the antibodies that provide humoral immunity. Lymphocytes do not originate primordially in the lymphoid tissue but, instead, are transported to this tissue by way of the preprocessing areas of the

thymus and the bursa of Fabricius. When a specific antigen comes in contact with the T and B lymphocytes in the lymphoid tissue, these cells become activated and reproduce wildly forming tremendous numbers of clone lymphocytes. Each clone lymphocyte is specific to the original antigen that sensitized the original lymphocyte. (Guyton 1992: 261 - 263.)

2.2.4 T-CELL- OR CELL MEDIATED IMMUNITY

Once the T lymphocyte has been activated and many clones have been formed these sensitized T-cells are released into the lymph and carried into the blood, then circulate through all the tissue fluids and back into the lymph. These cells can circulate for months or even years.

T lymphocyte memory cells are formed so that on subsequent exposure to the same antigen, the release of activated T cells occurs far more rapidly and much more powerfully than in the first response. Unlike B-cells, the receptor of T-cells only recognizes antigen bound to cell surfaces and exists only as a cell surface molecule and not in a secreted form.

T-cells are classified into three major groups:

- The Helper T-cells (CD4 cells): These cells usually constitutes more than three-quarters of the T-cells. These cells serve as the major regulator of virtually all immune functions by forming lymphokines that act on other cells of the immune system.

- Cytotoxic T-cells (CD8 cells): The receptor proteins on the surfaces of the cytotoxic cells

cause them to bind tightly to those organisms or cells that contain their binding-specific antigen. After binding, the cytotoxic T cell secretes perforins that punch large holes in the membrane of the attacked cell, and releases cytotoxic substances into the attacked cell.

- Suppressor T-cells: These cells are capable of suppressing the functions of other T-cells to keep them from causing excessive immune reactions that might be severely damaging to the body. (Guyton 1992: 261 - 267.)

2.2.5 HUMORAL- OR ANTIBODY MEDIATED IMMUNITY

Prior to the exposure to a specific antigen, the clones of B-lymphocytes remain dormant in the lymphoid tissue. Upon entry of a foreign antigen, the lymphoid tissue macrophages engulf the antigen, process it and display it on its cell membrane. Here a specific T-cell recognizes the digested antigen. The T- cell is activated and then activates a B-cell. Those B-lymphocytes specific for the antigen immediately enlarge and take on the appearance of lymphoblasts. Some of the lymphoblasts then further differentiate to form plasma cells. The plasma cell replicates at a rate of approximately once every 10 hours for about nine divisions. The mature

plasma cell then produces gamma globulin antibodies at an extremely rapid rate. The antibodies are secreted into the lymph and are carried to the circulating blood. This process carries on for days or weeks until death of the plasma cells. (Glick 1997: 485 - 487.)

The antibody mediated immune response may be thymic dependent (TD) or thymic independent (TI). The thymic independent immune response is divided into TI-type I and TI-type II. The majority of antigens is TD and depends on T, B and accessory cells for their response.

The two types of TI antigens are differentiated by the cells that are needed to transfer an immune response to the respective antigens. TI type I requires bursal and splenic cells, while TI-type II requires splenic cells only.

The antibody response in birds consists of primary and secondary responses. Each of these responses is characterized by four phases: a latent phase, immuno-progressive increase in circulating antibody, a peak antibody phase and a regression phase, or decline of antibody titre. During the primary response, IgM class antibody prevails and IgG class antibodies prevail during the secondary response and is thus the important antigen

class for longer term immunity e.g. after vaccination. (Glick 1997: 485 - 487.)

2.2.5.1 ANTIBODIES

Antibodies (or immunoglobulins) are glycoprotiens consisting of two heavy polypeptide chains and two light polypeptide chains held together by disulphide linkages. The antibody binding site is the variable end of each chain, and is the part that enables the antibody to recognize a specific antigen. There are three main types of antibodies in birds that are so similar to their mammalian counterparts that they go by the same names. They are IgM, IgG, and IgA. (Glick 1997: 485 - 487.)

IgM functions as the antibody that characterizes blood type and stimulates macrophages and activates the compliment system together with IgG. IgA represents the predominant immunoglobulin in external secretions such as saliva, tears, yolk and mucous in the respiratory, gastrointestinal and genito-urinary tracts and is mainly involved in the local immune response. IgG is the most prevalent antibody, found mostly in plasma but also in other body fluids. IgG contributes to immunity against many blood-borne pathogens. (Solomon *et al.* 1990: 803)

2.2.5.2 MECAHNISM OF ACTION OF ANTIBODIES

Antibodies protect the body against invading agents by direct attack on the invader and by activation of the complement system. Direct action of antibodies can inactivate the antigen in several ways:

- Agglutination: multiple large particles with antigens on their surfaces, such as red blood cells or bacteria, are bound together in a clump
- Precipitation: the molecular complex of soluble antigen and antibody becomes so large that it is rendered insoluble and precipitates, and
- Neutralization: antibodies cover the toxic site of the antigenic agent.

The indirect protection of the body by antibodies is by the complement system. "Complement" is a collective term for about 20 different proteins (mostly inactive enzyme precursors), which are normally present in plasma and tissue fluid. The complement system is a cascade of events that can be activated by specific or nonspecific immunological mechanisms. The binding of an antibody to an antigen activates the first protein of the chain and sets of a sequence of actions where successively increasing quantities of enzymes are activated in each successive stage of the system. Multiple end products are formed and several of these cause important effects that help prevent damage by the invading organism or toxin. (Guyton 1992: 261 - 267.)

2.2.5.3 THE BURSA OF FABRICIUS

The bursa of Fabricius is a small pouch-like structure with a folded inner surface located near the cloaca. During the latter part of fetal life the bursa of Fabricius preprocesses the B-lymphocytes and prepares them to manufacture antibodies. The bursa of Fabricius plays an important role in the production of circulating antibodies because it helps control antibody formation. The most rapid period of bursal growth occurs during the first two to three weeks posthatch. In general it exhibits a plateau growth period between three and eight weeks and then regresses. (Glick 1997: 484.)

2.3 NEWCASTLE DISEASE

The first recognized outbreaks of Newcastle disease occurred in 1926, in Java, Indonesia, and in Newcastle-upon-Tyne, England. Disease outbreaks, similar to what we now know as Newcastle disease, were reported before 1926 in Central Europe and Korea from 1924 onwards. The name, Newcastle disease, was coined as a temporary measure to avoid a descriptive name that might be confused with other diseases.

Three panzootics of Newcastle disease have occurred since the first recognition of the disease. The first represented the initial outbreak of

the disease and appears to have arisen in Southeast Asia and took over 30 years to spread worldwide. Isolated outbreaks such as in England in 1926 were chance introductions ahead of mainstream. The second panzootic appears to have begun in the Middle East in the late 1960s. This panzootic spread much more quickly and reached most countries by 1973. The third panzootic broke out in the Middle East in the late 1970s and primarily affected pigeons and doves. (Alexander 1997: 541-543.)

2.3.1 CAUSE

Newcastle disease is caused by a group of closely related viruses that form the avian paramyxovirus type 1 (PMV-1) serotype. Considerable antigenic variation exists between different strains of Newcastle disease. (Alexander 1990: 123.)

2.3.2 CLINICAL SIGNS

The different Newcastle disease strains cause disease with different signs and with different severity. Based on indices such as mean death time, intracerebral pathogenicity index and intravenous pathogenicity index, Newcastle disease viruses have been placed in five different pathotypes:

a) Viscerotropic velogenic - a very virulent strain affecting mainly the respiratory and digestive systems of the body.

b) Neurotropic velogenic - a very virulent strain affecting mainly the nervous system and respiratory systems.

c) Mesogenic - moderately virulent strain causing respiratory and sometimes nervous signs.

d) Lentogenic - causing mild or inapparent respiratory infection.

e) Asymptomatic enteric Newcastle disease virus- cause inapparent enteric infection. (Michael 1997.)

The clinical manifestation of the disease may be greatly influenced by additional factors such as immune status of the birds, age, coinfection with other organisms, environmental stress, social stress, route of exposure and the virus dose. Because of the varying clinical manifestations of Newcastle disease, no signs of the disease, or lesion produced by the disease, may be regarded as pathognomonic.

The highly virulent viruses may produce acute infections in young birds where the first sign can be sudden death. In older birds, shell-less or soft-shelled eggs, followed by complete cessation of egg laying, may be an early sign. Signs such as depression, prostration, increased respiration, diarrhoea, muscular tremors and oedema of the head and nervous system may occur. Mortality may reach 100% in flocks of fully susceptible

chickens. The moderately virulent viruses usually cause severe respiratory disease, followed by nervous signs, with mortality up to 50% or more. The viruses of low virulence may cause no disease or mild respiratory disease for a short time in chickens. Even inapparent infection may result in loss of weight gain in broilers. (Alexander 1990:123.)

2.3.3 SPREAD

The mode of transmission is dependent on the organs in which the virus multiplies. Birds showing respiratory infection shed virus in aerosols of mucous, which may be inhaled by susceptible birds. Viruses that are mainly restricted to intestinal replication may be transferred by ingestion of contaminated faeces, either directly or in contaminated food or water, or by inhalation of small infectious particles produced from dried faeces.

Man seems to play an important role in the spread of Newcastle disease virus, usually by the movement of live birds, personnel and poultry products (including faeces for fertilizer) from affected premises to susceptible birds. (Alexander 1990: 123)

2.4 NEWCASTLE DISEASE VACCINATION

The serious effects of the second Newcastle disease panzootic on the poultry industry of most countries, led to the development of vaccines and regimes that provided significant protection to poultry. Vaccination against Newcastle disease should ideally result in immunity against infection and replication of the virus. Realistically, Newcastle disease vaccination usually protects the bird from the more serious consequences of the disease, but viral replication and shedding may still occur, even though at a reduced level. (Alexander 1997:560.)

2.4.1 LIVE VACCINES

The objective of live vaccines is to establish a mild infection in the flock. The efficiency of a live virus vaccine depends on its invasiveness and its power to multiply sufficiently within the chicken to set up an adequate immune response (Meulemans 1988). Vaccine virus may spread from birds that have been successfully vaccinated to those that have not. Local immunity is stimulated by infection of live viruses, and protection occurs very soon after vaccination. Two strains of Newcastle disease virus, namely the mesogenic and lentogenic strains, are usually used to manufacture live vaccines. The mesogenic vaccines were employed in the

early years of live Newcastle disease vaccination, but are now only used as secondary vaccines, because of their more virulent nature. The less virulent lentogenic vaccines are now predominant because of their effectiveness and safety when used under field conditions. (Meulemans 1988.) Even in the lentogenic group, there is a range in virulence as was demonstrated by Borland and Allan (1980), who developed a stress index test to assess the potential effects of vaccines on susceptible chickens. The immune response increases as the pathogenicity of the live vaccine increases. Therefore, to obtain the desired level of protection without serious reaction, vaccination programs are needed that involve sequential use of progressively more virulent viruses, or live virus followed by inactivated vaccine. (Alexander 1997:560.)

Live vaccines are mostly applied to the drinking water, because this method is not a labor intensive method, and does not cause respiratory vaccine reactions. Mass application can be done by the spray method where an aerosol is used to spray the vaccine over the chickens. The vaccine is inhaled into the lungs and can cause secondary symptoms in young birds. Careful attention should be paid to droplet size, because too fine droplets will penetrate too deeply into the lungs, and coarse spray will not penetrate deeply enough to produce sufficient reaction. Individual bird

treatments such as intranasal instillation, eyedrop and beak-dipping are often used for lentogenic vaccines. (Travers 1995.)

2.4.2 INACTIVATED VACCINES

Inactivated vaccines are usually produced from infective allantoic fluid treated with β-propiolactone or formalin to kill the virus and then mixed with a carrier adjuvant. Early inactivated vaccines used aluminum hydroxide adjuvants but the development of oil emulsion-based vaccines proved a major advancement. Different oil-emulsion vaccines vary in their formulation of emulsifiers, antigen and water-to-oil ratios; most now use mineral oil. (Lovell 1996.)

Oil emulsion vaccines are composed of oil, water (containing the antigen) and surfactants. The aqueous phase is dispersed into small globules. This is the internal or discontinuous phase. The oil surrounds the aqueous phase and is called the continuous or external phase. The surfactants are added to both the oil and the antigen to help emulsify the two liquids and provide stability. The oil and its ingredients serve two purposes. One is to stimulate the immune system to produce a greater immune response. The other is to allow a slow release of antigen to stimulate the immune system over a long period. With a single injection of an oil emulsion vaccine, oil can still be seen histologically twenty eight-weeks post infection. (Lovell 1996.)

Various seed viruses used in the production of the oil-emulsion vaccines include Ulster 2C, B1, La Sota, Roakin, and several virulent viruses.

One or more other antigens may be incorporated into the emulsion with Newcastle disease virus, and bivalent or polyvalent vaccines may include infectious bronchitis virus, infectious bursal disease virus, egg drop syndrome virus, and reovirus. Inactivated vaccines are administered by injection, either intramuscularly or subcutaneously.

Oil emulsion vaccines should be stored between 2°C and 7°C. Heating and freezing should be avoided. Failure to properly store oil emulsion vaccines can lead to product failure due to broken emulsion. An oil emulsion can have four distinct appearances:

a) Normal: an oil emulsion should appear as a homogeneous white to slight off-white liquid. The off-white appearance is usually noticed when there is a high antigen content or with tissue culture derived antigen.

b) Creaming: this is due to excess oil floating on the top of the emulsion. The vaccine will have two layers, a light white or clear top layer above the white appearance of the oil emulsion. This is a normal process that appears sporadically during shipment. If the vaccine is gently shaken, the normal appearance will return and creaming will probably not occur again.

c) Antigen settling: Settling of antigen usually occurs with high antigen content vaccines. The small globules of water and antigen surrounded by

oil are heavier than the oil alone. Therefore, the water globules settle towards the bottom. The vaccine is white from top to bottom with a gradual increase in darkness toward the bottom. This is not a broken emulsion. The vaccine can be gently shaken and used.

d) Broken emulsion: A broken emulsion occurs when the aqueous and oil phases separate from each other. The vaccine will have two well defined layers. There will be the normal white emulsion, with a tea coloured water layer at the bottom. If a broken emulsion is shaken, it will appear normal but within a few hours the two phases will separate again. When an emulsion breaks the vaccine should not be used. If a broken emulsion is injected, the immune response will be high but of short duration. (Lovell 1996)

Inactivated vaccines are far easier to store than live vaccines. They are expensive to produce and to apply because of the labor needed for their application. Inactivated oil-emulsion vaccines are not as adversely affected by maternal immunity as live vaccines. The major advantages of inactivated vaccine are the very low level of adverse reactions in vaccinated birds, the ability to use them in situations unsuited for live vaccines, especially if complicating pathogens are present, and the extremely high levels of protective antibodies of long duration that can be achieved. (Alexander 1997:561)

2.4.3 VACCINATION PROGRAMES

Vaccination programes vary considerably from area to area, according to local pattern of disease, maternal immunity of the young chicks, availability of labor, climatic conditions, expected life of the flock, past vaccination history and cost.

Vaccination of laying hens always requires more than one dose of vaccine to maintain immunity through their lives. Because the life span of a broiler is so short and the immune system is immature vaccination programs have to be carefully planned. (Travers 1995.)

2.5 IMMUNITY TO NEWCASTLE DISEASE VIRUS

2.5.1 CELL MEDIATED IMMUNITY

Cell-mediated immunity appears to be the first immunological response that can be demonstrated following Newcastle disease vaccination, and has been detected as early as day two and three after infection with live vaccine strains (Timms and Alexander 1977). This explains the early protection against challenge that has been recorded in vaccinated birds before a measurable antibody response is seen (Gough and Alexander 1973). Cell-mediated responses are greater to live vaccines than to dead

vaccines. The importance of the cell-mediated immunity in protection conferred by vaccines is not clear, and strong secondary response to challenge similar to the antibody response does not occur (Timms and Alexander 1977).

2.5.2 HUMORAL IMMUNITY

Antibodies capable of protecting the host are usually detectable within 6-10 days after infection or vaccination. Peak response is about 3-4 weeks after exposure to the virus. Decline of antibody titre varies with the titre achieved but is much slower than their development. Haemagglutination Inhibition antibodies may remain detectable for up to 1 year in birds recovered from infection with mesogenic viruses or after a series of vaccinations. Reinfection or immunization some weeks after the titre begins to decline, produces a secondary response. (Alexander 1997:560.)

2.5.3 LOCAL IMMUNITY

Antibodies appear in some secretions of the upper respiratory and intestinal tracts of chickens at about the time humoral antibodies can be first detected. In the upper respiratory tract, the immunoglobulins appear to be chiefly IgA with some IgG. Similar excretions occur in the Harderian gland following ocular, but not parenteral, infection. Effective

local immunity was demonstrated when it was found that birds might be susceptible to infection at one site but protected at another. (Parry and Aitken 1977.)

2.6 FACTORS THAT INFLUENCE THE IMMUNE RESPONSE TO VACCINES.

2.6.1 MATERNALLY DERIVED IMMUNITY

Hens with antibodies to Newcastle disease virus will pass this on to their progeny via the egg yolk. Levels of antibody in day old chicks will be directly related to titres in the parent. (Chu and Rizk 1975.) Chicks are only devoid of maternally derived antibodies at about 41 days of age (Chu and Rizk, 1975), although the majority of birds have lost measurable maternal antibodies by 28 days of age (Thomson 1998). Maternal immunity is protective and, thus, must be taken into account when timing primary vaccination of chicks because it may prevent the mild infection that must be produced in order for a live vaccine to work. Maternal immunity has a marked effect on the pathogenicity of live vaccine viruses and strongly affects the immune response of chicks from vaccinated hens. (Chu and Rizk, 1975.) Box (1965) has shown that maternal antibody can interfere with the immune response to inactivated vaccines.

2.6.2 AGE OF CHICKENS AT VACCINATION

Chu and Rizk (1975) determined at what age the immune mechanism to Newcastle disease is fully developed in chicks. Leghorn chickens from non-vaccinated hens were vaccinated intramuscularly with inactivated virus to eliminate the complications of further viral multiplication. The chicks were vaccinated at different ages, namely, 2 days, 10 days, 4 weeks, 10 weeks and 8 months with 0.5ml of the inactivated virus regardless of age and weight. Blood samples were taken from the chickens before vaccination and at 11 and 21 days after vaccination. The HI of individual serum samples was determined. It was found that the antibody production increased with age. The best immune response took place when the birds were vaccinated at 32 weeks of age. This agrees with the finding of Wolfe *et al.* (1957) that the ability of chickens to produce precipitating antibody against bovine serum albumin did not reach full maturity until they were 22 weeks of age. There was a great deal of individual variation of the immune response of chickens in each group of the above mentioned experiment (Chu and Rizk 1975). It was found that not all birds in a group responds with equally high antibodies to the vaccine that was given. This shows that a number of birds are not responding optimally to the vaccine, and there is room for improvement of their immune response to the vaccine.

2.6.3 VIRAL DOSE

Toro *et al.* (1997) vaccinated 23-day-old male leghorn chickens by ocular installation. Three groups of twenty birds were used and each group received a different dose of infectious bronchitis virus vaccine. It was demonstrated that larger doses of Infectious Bronchitis virus induced a larger antibody response in chickens.

Gross (1978) investigated the effect of various doses of antigen on the antibody response of chickens. Different doses of sheep red blood cells were administered intravenously to 6-to-7-week-old birds. Plasma was collected 6 days after antigen administration. It was found that higher doses of Antigen caused larger antibody responses.

2.6.4 IMMUNOSUPPRESSIVE DISEASE

Immunosuppressive diseases of chickens include the viral diseases, infectious bursal disease and avian infectious anaemia and the lymphoproliferative diseases: Marek's disease, lymphoid leukosis and reticuloendotheliosis. Some reo and adenovirus strains can cause extensive lymphoid depletion and have been shown to be immunosuppressive. Exposure to infectious bursal disease and avian infectious anaemia at an early age will significantly compromise the

humoral and local immune response to Newcastle and Marek's disease vaccines. (Lombardi 1993.)

2.6.5 SEX OF BIRD

Franchini *et al.* (1991) found that the influence of sex on antibody response to the Newcastle disease vaccine used in his study seemed to be correlated with days post vaccination. Higher Haemagglutination Inhibition titres were observed in females than in males during the later samplings (58 and 84 days post vaccination). The sex difference in titres are related to a similar sex difference in plasma protein levels, due to the fact that sex hormones, especially oestrogens, favour higher serum globulin levels in females (Sturkie and Newman, 1951).

2.6.6 GENETIC STRAIN OF THE BIRD

Van der Zijpp (1981) studied the effect of genetic origin on the immune response of chickens. White Plymoth Rocks, White Leghorns and Warren chickens were injected with sheep red blood cells at day 34 of age. Blood samples were taken on the day of injection and 3, 7 and 14 days later. Haemagglutinin Assay was used to measure antibody response. Differences among genetic groups resulted in significant differences for total antibody titres.

2.6.7 NUTRITIONAL STATE OF THE BIRD

Nutritional status may affect immunocompetence of the bird and consequently its resistance to infectious agents. Chronic, severe nutrient deficiency impairs immunocompetence as a result of insufficient availability of that nutrient for its structural or metabolic function in the cells of the immune system. Poultry are rarely subjected to chronic nutrient deficiencies under commercial conditions, and for most nutrients, levels that optimize growth are also adequate for optimal immunocompetence. (Klasing and Halquist 1992.) Exceptions may include vitamins A and E and methionine (Tsiagbe *et al.* 1986). Dietary manipulations of some nutrients result in immunoregulatory consequenses due to the paritcipation of the nutrient or its products in cellular communication. (Klasing and Halquist 1992.) An example of this is the role of nonessential dietary polyunsaturated fatty acids in modifying the generation of eicosenoid second messengers during and immune response. It has been found that dietary n-3 fatty acids enhance the antibody response of pullets to sheep red blood cell vaccinations but suppress rates of lymphocyte mitogenesis after mitogen stimulation. (Fritsche *et al.* 1990.)

2.7 SEROLOGY

Post-vaccinal serology can be used to confirm successful application of vaccine and an adequate immune response by the bird. Antibodies to Newcastle disease virus may be detected in poultry sera by a variety of tests including single radial immunodiffusion (Chu *et al.* 1982), single radial hemolysis, agar gel precipitin, virus neutralization (VN) in chick embryos , plaque neutralization, Enzyme-linked immunosorbent assays (ELISA) and Haemagglutination Inhibition (HI) (Alexander 1997:555). Several reports have described ELISAs for Newcastle disease (Miers *et al.*1983, Snyder *et al.* 1983, Wilson *et al.* 1984 and Adiar *et al.* 1989). These authors concluded that the ELISAs for detecting Newcastle disease antibodies was reproducible, showed high specificity and sensitivity, enabled distinction between protective and non protective antibody levels, and correlated well with the HI test (Adiar *et al.* 1989, Brown *et al.* 1990 and Cvelic-Cabrilo *et al.* 1992).

2.8 HAEMAGGLUTINATION INHIBITION (HI) TEST

HI is a convenient and economical serologic tool that has been applied extensively to the control of several avian diseases caused by haemagglutinating viruses, including Newcastle disease and infectious

bronchitis, by measuring vaccine response and evidence of past infection (Beard 1989:192). The nucleic acids of haemagglutinating viruses encode surface proteins that agglutinate the red cells of a variety of species. This knowledge is used as an aid in the identification of haemagglutinating viruses. Specific antiviral antibodies can combine with these haemagglutinating viruses and "neutralize" their ability to cause haemagglutination. In the HI test, the bird's serum is incubated with known haemagglutinating viral antigen (inactivated Newcastle disease virus) and then the mixture is exposed to red cells. The absence of agglutination of the red cells is indicative of a positive test (specific antiviral antibody is present). (Stansfield 1981: 186)

Due to standardization of laboratory equipment and semi-automated micropipettes, the microtiter system that uses test plates has now replaced the older macro system that used test tubes (Thomson 1988).

2.8.1 HAEMAGGLUTINATION INHIBITION MICROTITRE PROCEDURE (Beard 1989: 192 - 194)

2.8.1.1 DETERMINING THE HA VALUE OF THE ANTIGEN

Before the HI test can be performed a haemagglutination (HA) test must be done to standardise the antigen. Serial doubling dilutions of the antigen

(inactivated Newcastle disease virus) are made with a buffer solution from 1:2 through to 1:4096 ($1:2^{12}$) in a round bottomed 96 well micro titre plate. A constant amount of chicken erythrocytes is added to each well including the control containing no antigen. The plate is left at room temperature until a compact button of cells forms in the control well (a negative reaction). Agglutination is indicated by adherence of the red blood cells to the bottom of the well as a film of cells that does not flow when the plate is tilted at 45 degrees. The greatest dilution of antigen that shows complete agglutination contains one haemagglutination unit (1 HA unit).

2.8.1.2 PREPARATION OF THE ERYTHROCYTE SUSPENSION

Blood is drawn from a chicken into a syringe containing an anticoagulant such as 4% sodium citrate (1 part to 4 parts blood) or Alsever's solution (equal volumes). After it is mixed gently the blood is transferred slowly into a large centrifuge tube for washing. An equal amount of phosphate-buffered saline (PBS) at pH 7.0 - 7.2 is added. The suspension is centrifuged at 500 x g for 5 minutes. The supernate is removed and 20 to 30 volumes of PBS is added to the packed cells. The cells are resuspended gently, and the centrifugation step is repeated and the supernate removed. The cells can now be used to prepare the 1 ml suspension by adding 1 ml of packed cells to 100 ml of PBS at pH 7.0 - 7.2.

2.8.1.4 THE HI TEST

	1	2	3	4	5	6	7	8	9	10	11	12
A	⊙	⊙	⊙	●	●	●	●	●	●	●	●	●
B	⊙	⊙	⊙	⊙	⊙	●	●	●	●	●	●	●
C	⊙	⊙	⊙	⊙	●	●	●	●	●	●	●	●
D	⊙	⊙	⊙	●	●	●	●	●	●	●	●	●
E	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	●	●	●
F	⊙	⊙	⊙	●	●	●	●	●	●	●	●	●
G	⊙	⊙	◍	◍	●	●	●	●	◍	●	●	●
H	⊙	⊙	⊙	⊙	⊙	⊙	◍	●	◍	●	●	⊙

Table 2.1. Schematic representation of a HI test plate.

⊙ = Agglutination ◍ = No agglutination

50µl of test sera is added to the first well of rows A to G, whilst 50µl of a known positive control serum is added to the first well of row H. 50µl PBS is added to all the wells. Doubling dilutions of the sera are made by using a micropipette set at 50µl volumes. 50µl is taken from the first row of wells containing 50µl serum and 50µl PBS, and transferred into the second row of wells, and mixed well. Then 50µl is taken from the second row of wells and transferred into the third row, etc. In row H this process stops in row 10, leaving wells H11 and H12 with PBS but no serum.

The antigen is diluted in PBS based on the HA activity of the antigen to yield 10 HA units per 50µl ; e.g. if the antigen titre is 1:2560, add one volume of antigen to 256 volumes of PBS.

50µl this diluted antigen is added to each well except the last well of row H (H12). The plates are incubated for 15 minutes at room temperature. 50µl of the 1% chicken erythrocyte suspension is added to all the wells. The plates are left at room temperature until the known positive serum wells exhibit a tight, well circumscribed button of unagglutinated, sediments erythrocytes. Those wells that are HI negative will have wells with a diffuse sheet of agglutinated erythrocytes covering the bottom. The plates are tilted at 45 degrees and tear dropping or flowing of unagglutinated wells will be observed. The HI value is recorded as the highest value at which there was complete inhibition of haemagglutination. The known positive serum control should inhibit haemagglutination within one dilution of the precalculated titre. Agglutination should be observed in the virus control (H11) which contains the Haemagglutinating virus and red cells. No agglutination should be seen in the red cell control (H12) where no antigen was added.

2.8.1.4 FACTORS THAT MAY INFLUENCE THE TEST AND ENDPOINT TITRES

- The virus (strain, number of haemagglutinating units used, passage level, host system used to propagate virus)

- The erythrocytes (species, age and sex of the donor bird, concentration of suspension, differences in individual donors, cell storage)
- The test conditions (temperature of reaction, pH, cations)
- The procedures used.

Although this test was described in the 1940s and has been extensively used in the laboratory for decades, there are still problems concerning sensitivity (ability to recognize a true positive sample) and specificity (ability to recognize a true negative sample) with this procedure.

The test has some disadvantages. Discrepant results are sometimes observed between laboratories and even between replicate tests within the same laboratory. False negative reactions, often due to the presence of natural agglutinins in the serum, and cross-reactions with related viruses do occur. (Beard 1989: 190)

2.9 HOMOEOPATHY

Homoeopathy is a system of treatment that is based on the law of similars. The law of similars states that a substance that can cause a set of symptoms in a healthy individual, can cure a sick person with a similar set of symptoms. Another fundamental principle of homoeopathy is that it treats the patient as a whole and as an individual. People with the same

condition will often receive different remedies. Mental, emotional and physical symptoms are considered in the choice of a remedy. Homoeopathic remedies are prepared by a process of serial dilution and succussion, often beyond the point where any molecules of the original substance are likely to be present in the dilution. (Sankaran 1992 : 1 - 3)

2.9.1 RESEARCH IN HOMOEOPATHY

The individualised nature of homoeopathy presents a challenge to placebo-controlled studies of the efficacy of homoeopathy. This problem has been solved by three different trial methodologies:

- One disease/ any homoeopathic medicine.
- One disease/ one homoeopathic medicine
- Isopathy. To circumvent the need of individualisation, some researchers have experimented with isopathy. Isopathy is the treatment of the 'same' with 'same' (e.g. treating a person with hay fever triggered by house dust with house dust in potency), as opposed to 'like' with 'like' in homoeopathy (treating a person with hay fever triggered by house dust with *Sabadilla,* which can cause the same symptoms in a healthy individual). (Fisher 1992.)

2.9.2 HOMOEOPATHY AND IMMUNOLOGY

Although the whole organism is understood to respond to homoeopathic remedies, the effect of homoeopathic remedies can be illustrated using immunological models. In addition the effect of highly diluted substances on immunological models can lead to new perspectives in the field of immunology. (Bastide 1994: 27.)

2.9.2.1 *IN VITRO* STUDIES OF THE EFFECT OF HOMOEOPATHIC REMEDIES ON THE CELLULAR IMMUNE SYSTEM

Moss *et al.* (1982) tested the effects of *Belladonna* 8X, *Pyrogenium* 6C, *Staphylococcin* 6C, *Hepar sulphuricum* 8C and *Silicea* 8X on the movement of human leukocytes and guinea-pig macrophages *in vitro*. The remedies were tested to establish whether they could inhibit or facilitate the movement of macrophages or leukocytes. Both of these effects might be advantageous since facilitation speeds their movement towards the site of infection and inhibition is required to keep them there. Five hundred and thirty three tests were done and significant increase or decreases were found in 47 of these tests.

The effect of *Podophyllum peltatum* extract, 4C, 12C and a *Podophyllum* compositum which contained *Podophyllum* 4X, *Podophyllum* 12X, *Ignatia* 5X

Acidum muriaticum 6X, *Acidum muriaticum* 12X and *Mercurius sublimatus corrosivus* 10X was tested on human neutrophil functions *in vitro*. The functional parameters explored in this study were superoxide release and adhesion to serum- coated plastic surfaces. Superoxide is the fundamental product of oxidative metabolism in all phagocytes when their membranes interact with specific particulate or soluble agents. Adhesion is a function of leukocytes that allows them to migrate outside the blood vessels onto various surfaces. *Podophyllum* 4X produced the most significant increase in superoxide release and the most significant inhibition of adhesion functions. (Chirumbolo *et al.* 1997).

Chirumbolo *et al.* (1993) studied the effect of a selection of ten homoeopathic remedies in potencies ranging from 6C to 600C on superoxide production and adhesion function of human neutrophils. A 15 - 30 % inhibition of superoxide production by remedies such as *Sulphur* 6X, *Manganum phosphoricum* 6X and 8X, and *Magnesium phosphoricum* 6X and 8X was found. Negligible differences were detected on adhesion functions of human neutrophils.

2.9.2.2 THE EFFECT OF HOMOEOPATHY ON THE CELLULAR IMMUNE SYSYEM OF HIV AND AIDS PATIENTS

Rastogi *et al.* (1998) found that homoeopathic medicines, when prescribed on an individual basis, have an immune modulating role in HIV infected individuals. The 6 month study consisted of a Double blind Placebo-controlled trial of homoeopathic medicines in HIV/AIDS under two separate schemes. The first scheme investigated patients with asymptomatic HIV infection and the second patients with persistent generalized lymphadenopathy. CD4+ T cells was used as an indicator of the immune status of the patient. The asymptomatic group showed no significant changes in the cell counts. In the group with persistent generalized lymphadenopathy, significant increases in CD4+ T cell counts were observed in the group that received homoeopathic medication compared to the placebo group.

2.9.5 STUDIES ON THE EFFECT OF HOMOEOPATHIC REMEDIES ON THE HUMORAL IMMUNE SYSTEM

Davies (1971) administered a 30C potency of the Hong Kong Influenza virus to 27 healthy volunteers. Venous blood samples were taken before and two weeks after administration of the potency, which was given in three divided doses twelve hours apart. The antibodies were quantified by and

Haemagglutination Inhibition test. It was found that administration of the 30C potency of the influenza virus produced no rise in circulating haemagglutinating antibodies to the virus.

A similar study was conducted by Lewith *et al.* (1989). In this study 108 healthy volunteers were divided into 4 groups. The volunteers received either one or four tablets of dilution of 10^{-60} (30C) of a standard inactivated influenza virus vaccine, or placebo. If they were given four tablets, then these were taken one tablet twice a day over a period of 48 hours. Blood samples were taken before and 28 days after taking the nosode. Haemagglutination Inhibition titres were used as a measurement of antibodies formed against the Influenza virus. The administration of a 30C homoeopathic dilution of influenza vaccine produced no significant or consistent increase in titre against the virus in the treated group.

Weisman *et al.* (1992) demonstrated that specific antibody generation by experimental animals could be modulated by homoeopathic dilutions of the antigen Keyhole-limpet-hemocyanin (KLH). C57B1/6J mice were preconditioned by repeated intraperitoneal injections three times a week during 8 weeks with succussed high dilutions of KLH, thyroglobulin and saline. This was followed by regular immunization with 1μg of KLH per

mouse in incomplete Freund's adjuvant in the foot pads. Serum levels of anti-KLH antibodies were determined by ELISA for each individual mouse sequentially, every two weeks or less. Antigen specificity was checked by ELISA on rabbit serum albumin and/or thyroglobulin coated plates. The results of the experiments showed: a) Preconditioning of mice with repeated injections of highly diluted antigens or saline, induced and IgM response (polyclonal). There was no difference between the various treatments. b) Following immunization, an increase in the KLH-specific IgG response in preconditioned mice preconditioned with 7C and 15C KLH, as well as by thyroglobulin 15C (in one experiment). The authors conclude that highly diluted antigens, even beyond Avogadro's number, may modulate at least the secondary IgG immune response. This study confirms the findings of above mentioned studies by Davies and Lewith, that no specific immune response is induced by highly diluted antigen by itself. The results imply that highly diluted substances can result in significant enhancement of the IgG immune response when given as a form of preconditioning to the vaccine. The researchers note that the results of various experiments diverge in details, and they feel the experiments have to be repeated many more times before confident conclusions can be made about the reproducibility and specificity of the effects of high dilutions of antigens on the humoral immune response.

Youbicier-Simo *et al.* (1993) did an *in vivo* study where chick embryos were surgically bursectomised at 80 hours of incubation and then administered bursin dilutions or saline *in ovo*. The study included sham operated birds, bursectomised birds that received saline solution and bursectomised birds that received dilutions of bursin produced by Dolisos Homeopathic Laboratories. All the birds were repeatedly immunized with porcine thyroglobulin at 21, 30 and 39 days of age. Serum titres of specific antibodies against thyroglobulin were measured on days 20, 29, 38 and 47. The specific antibodies in serum samples were determined by an ELISA technique. Specific antibodies to thyroglobulin remained at very low background levels in the bursectomised birds that received saline solution, whereas the non-bursectomised group produced a strong specific antibody response. This is to be expected because the bursa of Fabricius is involved in the specific antibody response in birds. However *in ovo* administration of low doses of bursin (100fg, 100pg, 5×10^{-27}g) led to high antibody production in bursectomised birds after repeated immunization.

Thymic hormones act on the immune system at the humoral and cellular levels (Doucet-Jaboeuf *et al.* 1984). Among these hormones, thymosin fraction 5 stimulates the humoral response. Some studies however, show

that thymosin suppresses the humoral response. Thymopoetin II similarly has stimulating and inhibiting qualities. These findings led to the investigation into an immuno-regulator effect of high dilutions of thymosin and thymopoetin on the seasonal variations in the immune response of mice. (Doucet-Jaboeuf *et al.* 1984.) Six-week-old male mice were divided in groups of ten. The following concentrations of hormones were tested: 500 pg ml^{-1}, 5 x 10^{-4} pg ml^{-1}, 5 x 10^{-8} pg ml^{-1} and 5 x 10^{-12} pg ml^{-1} for thymopoietin II peptide fragment 29 - 41 and 5 x 10^{-4} pg ml^{-1} and 5 x 10^{-8} pg ml^{-1} for thymosin fraction 5. Control mice received solvent alone. Thymic hormone dilutions or solvent were administered in a volume of 0.2 ml intraperitoneally 15, 13, 11 and 8 days before sacrifice. Four days before sacrifice, the mice were immunized intraperitoneally with 0.2 ml of 10 % sheep red blood cells. The direct plaque forming cell (PFC) technique was used to measure humoral activity. The results were analysed by Student's t-test and Newman-Keuls method of multiple comparisons. It was found that Thymosin fraction 5 caused a decrease in the number of PFC from mice treated in May and June, and an increase in PFC in November and December. This seasonal variation occurred over a period of two consecutive years. Thymopoetin II peptide fragment 29 - 41 caused a similar variation over a one-year period at all four doses tested. The

period of the physiological rhythms of mice treated in this experiment was not changed but the amplitude was increased.

CHAPTER 3

MATERIALS AND METHODS

250 day old, as hatched, Hubbard x Peterson chickens were reared in a brooding room until they were 14 days old. Electrical brooding was used as necessary. On day 14 the birds were randomly divided into groups. This was be done by placing the first chick into group 1, the second chick into group 2, the third chick into group 3 *etc.* and repeating the process until all the chicks were divided into groups.

Room 1	Room 2
Group 3 replication 1	Group 5 replication 2
Group 5 replication 1	Group 2 replication 2
Group 1 replication 1	Group 4 replication 2
Group 4 replication 1	Group 3 replication 2
Group 2 replication 1	Group 1 replication 2

Table 3.1 Distribution of groups in the poultry house.

The experiment consisted of five groups. Each group consisted of 42 birds, which was divided into two replications of 21 birds each. The birds

were housed in two separate rooms of the same poultry house. Each replication was housed in a 2m x 2m pen. (*See table 3.1.*) The trial was conducted over 42 days.

3.1 FEEDING AND WATERING

Starter rations was used for the first three weeks and finisher rations was used for weeks four to six. The feed was mixed by Epol Feeds in Pietermaritzburg.

The birds were fed *ad libitum* (Doucet-Jaboeuf *et al.* 1984) from feeder troughs and the water was supplied by a constantly flowing common municipal water supply.

3.2 VACCINATIONS

The vaccine was an emulsion of killed LaSota type Newcastle disease virus with a mineral oil adjuvant (Stone *et al.* 1978). Before use the vaccine was shaken to ensure that the vaccine was consistent throughout the bottle and each bird received an identical dose. The birds were inoculated (0.5 ml per bird) subcutaneously in the retronuchal area (Franchini *et al.* 1991) with an 18-gauge, $\frac{1}{2}$ inch needle. The skin on the back of the neck was lifted up to create a pocket between the skin and neck muscles. The needle was inserted through the skin into this pocket with the needle

pointing toward the bird's body. Care was taken not to inject into the neck muscles or close to the head. (Lovell 1996.) Each bird received a constant amount of antigen.

Groups 1 to 4 were vaccinated on day 14. Group 5 was a non-vaccinated control group and acted as an indicator of natural or extraneous disease.

3.3 TREATMENTS

Group 1 was the vaccinated control group. This group received alcohol in their drinking water at the same concentration as the groups receiving homoeopathic remedies (i.e. 10 ml of 20% alcohol in one litre of water), on Mondays to Fridays from day 15 onwards. Groups 2, 3 and 4 received 12C, 30C and 200C potencies of the vaccine respectively. The remedies were administered on Mondays to Fridays from day 15 onwards. Group 5 did not receive vaccine, homoeopathic remedy or alcohol.

3.4 PREPARATION AND ADMINISTRATION OF THE HOMOEOPATHIC REMEDIES

Homoeopathic medicines were prepared according to method 44 of the German Homoeopathic Pharmacopoeia, up to the potency below that which was needed for the experiment, by Pharma Natura (British Homoeopathic Association 1978: 370-374). The potencies were made up in 20% alcohol.

12C ($1x10^{-24}$) is the lowest potency used because at this dilution Avogadro's number ($6.023x10^{-23}$) is exceeded and from here on in the potency range not a single molecule of the original vaccine is expected to remain (Gaier 1991:47).

10 ml of the potency below what was required was added to 1 litre distilled water in a 1.25 litre bottle and succussed 50 times by hand against a thick book to make the desired potency, as was done by and Searcy et al. (1995) in their study on bovine mastitis and Guajardo *et al.* (1996) in their study on the growth promoting effects of Sulphur 201C in pigs.

Group 1 received a mixture of 10 ml 20% alcohol in 1 litre water and this mixture was not succussed. Before the 1.25 litre bottle was re-used to make up a next potency it was rinsed in cold water at least twice and baked at 180°C for not less than 30 minutes (Pharma Natura company procedures no P12.8 :12)

The common water supply was removed for two hours before administration of the remedy to ensure that all birds drank the remedy (Pollock, 1998:26).

The remedy of the desired potency was then poured into a drinking trough. The birds were allowed to drink from this trough until the 1 litre volume was consumed, in which time it was presumed that all the birds will have

drunk. Then the continuous flow water supply was replaced. The water troughs were labeled and the same trough was used for the same group throughout the experiment. The water troughs were emptied, rinsed and refilled before each administration.

3.5 COLLECTION OF THE DATA

Deaths were recorded daily.

Blood samples were taken on days 14, 28 and 42 from each bird. 1 (one) ml of blood was bled from the big wing vein in the corner of the elbow (Le Roux 1995) on the stipulated days by poultry workers under the researcher's supervision. The serum was collected in serostores. HI tests were performed on all the samples. The Haemagglutination-Inhibition test was performed according to the Beta procedure (Diluted-Serum Constant -Virus) prescribed by the American Association of Avian Pathologists (Beard 1989). Titres were expressed as log to the base 2 (log_2) of the highest dilution of serum that caused complete inhibition of haemagglutination (Box *et al.* 1988). The tests were performed by Rainbow Veterinary Laboratories, Hammarsdale, and the researcher was actively involved in the testing.

The researcher and poultry house personnel under the researcher's supervision, collected the data.

3.6 EVALUATION OF THE DATA

The objective was to determine whether any significant difference existed between the four groups of chickens with reference to the respective treatment given to each individual group. Means tables were drawn up and 3 way factorial analysis with groups, days and rooms as factors was carried out using procedure GLM (SAS Institute Inc. 1987).

CHAPTER 4

RESULTS

GROUP	VACCINATED?	TREATMENT
1	Yes	Placebo
2	Yes	12C
3	Yes	30C
4	Yes	200C
5	No	No treatment

Table 4.1. Key to groups and treatments

DUNCAN GROUPING		MEANS	GROUPS
	A	1.7857	1
B	A	1.6190	3
B	C	1.2857	4
	C	1.0952	2
	C	1.0476	5

Table 4.2. Duncan Multiple Range Analysis for mean $\log_2$ HI results of groups on day 14 (maternally derived immunity). Means with the same letter are not significantly different.

On day 14 (before commencement of the trial) levels of maternally derived antibodies diffed significantly between the groups (Table 4.2) even though the chickens were divided randomly into groups.

DUNCAN GROUPING	MEANS	GROUPS
A	2.5952	3
A	2.3659	2
A	2.3095	1
A	2.0476	4
B	0.6667	5

Table 4.3. Duncan Multiple Range Analysis for mean $\log_2$ HI results of groups on day 28. Means with the same letter are not significantly different.

On day 28, two weeks after vaccination, group 5, the non-vaccinated control group has a significantly lower HI titre than the vaccinated groups (Table 4.3.). There is no significant difference between the vaccinated groups that received homoeopathic dilutions of the vaccine and group 1 that received only alcohol.

DUNCAN GROUPING	MEANS	GROUPS
A	3.1026	4
A	2.9737	2
A	2.8718	3
A	2.7073	1
B	1.9250	5

Table 4.4. Duncan Multiple Range Analysis for mean $\log_2$ HI results of groups on day 42. Means with the same letter are not significantly different.

The results of the non-vaccinated control group is influenced by a mild Newcastle disease virus that affected this group in the cage that was housed in room 2 (see Graph 4.1). As was noticed at 28 days, at 42 days, the vaccinated groups do not differ significantly from one another (Table 4.4).

SOURCE	DF	TYPE III SS	MEAN SQUARE	F VALUE	PR > F
Groups	3	4.00189034	1.33399345	0.94	0.4194
Rooms	1	0.67552506	0.67552506	0.48	0.4898
Groups*Rooms	3	6.00399870	2.00133290	1.42	0.2375
Days	2	177.83897721	88.91948861	62.89	0.0001 *
Groups*Days	6	17.74038765	2.95673127	2.09	0.0530
Rooms*Days	2	0.39630833	0.19815416	0.14	0.8693
Groups*Rooms*Days	6	6.29586845	1.04931141	0.74	0.6160

Table 4.5. Multifactorial analysis of variance.

*indicates a significant interaction between groups ($P < 0.05$).

Note that the groups and days value shows a tendency towards significance. This is due to the slight difference in profile of day 1 as compared to the other days. (See graph 4.1.)

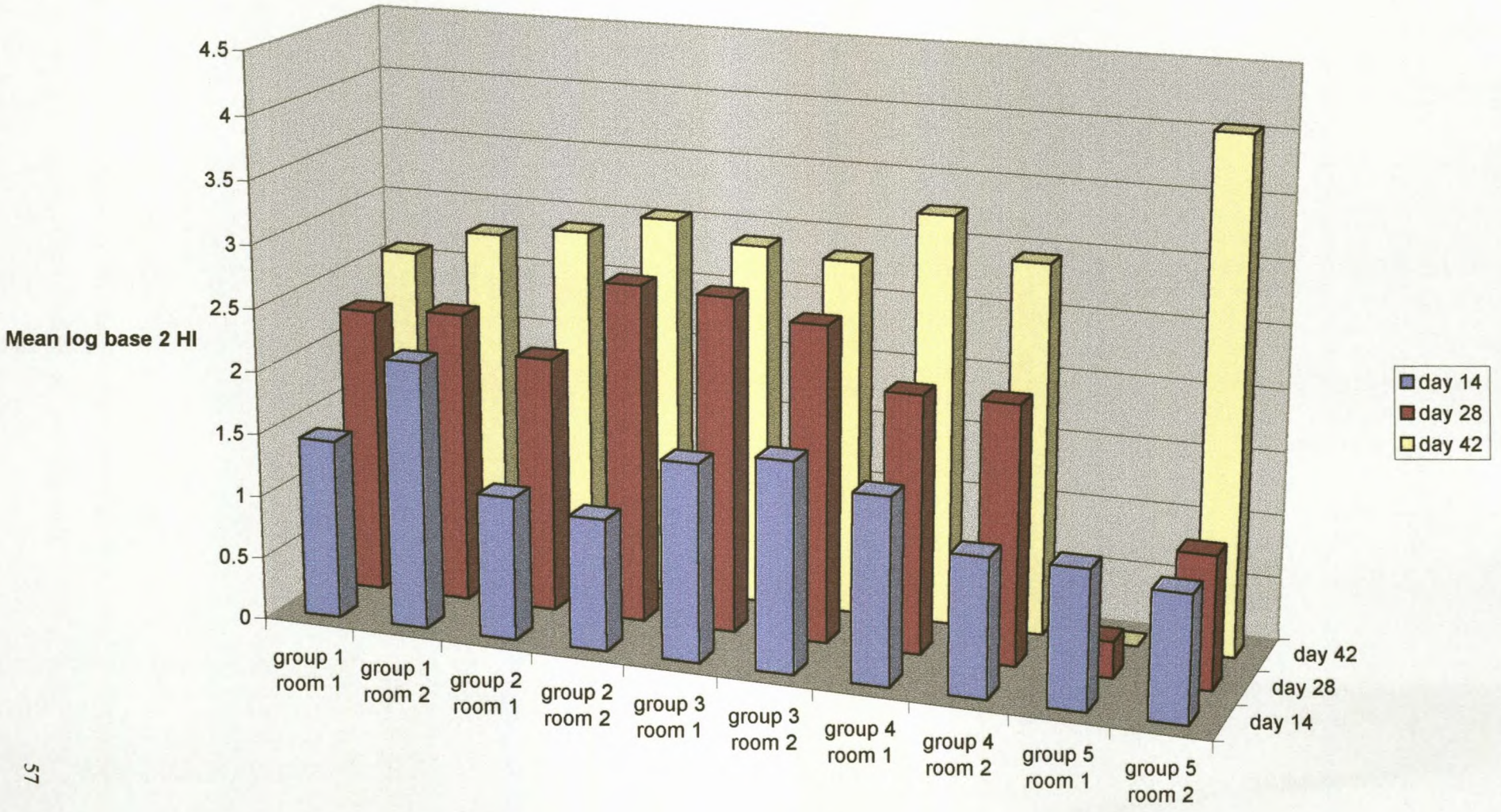
There is a significant difference between the mean HI values of the different days. Duncan multiple range tests were performed to establish which days were significantly different from one another (Table 4.6).

DUNCAN GROUPING	MEAN	DAYS
A	2.9108	14
B	2.3293	28
C	1.4464	42

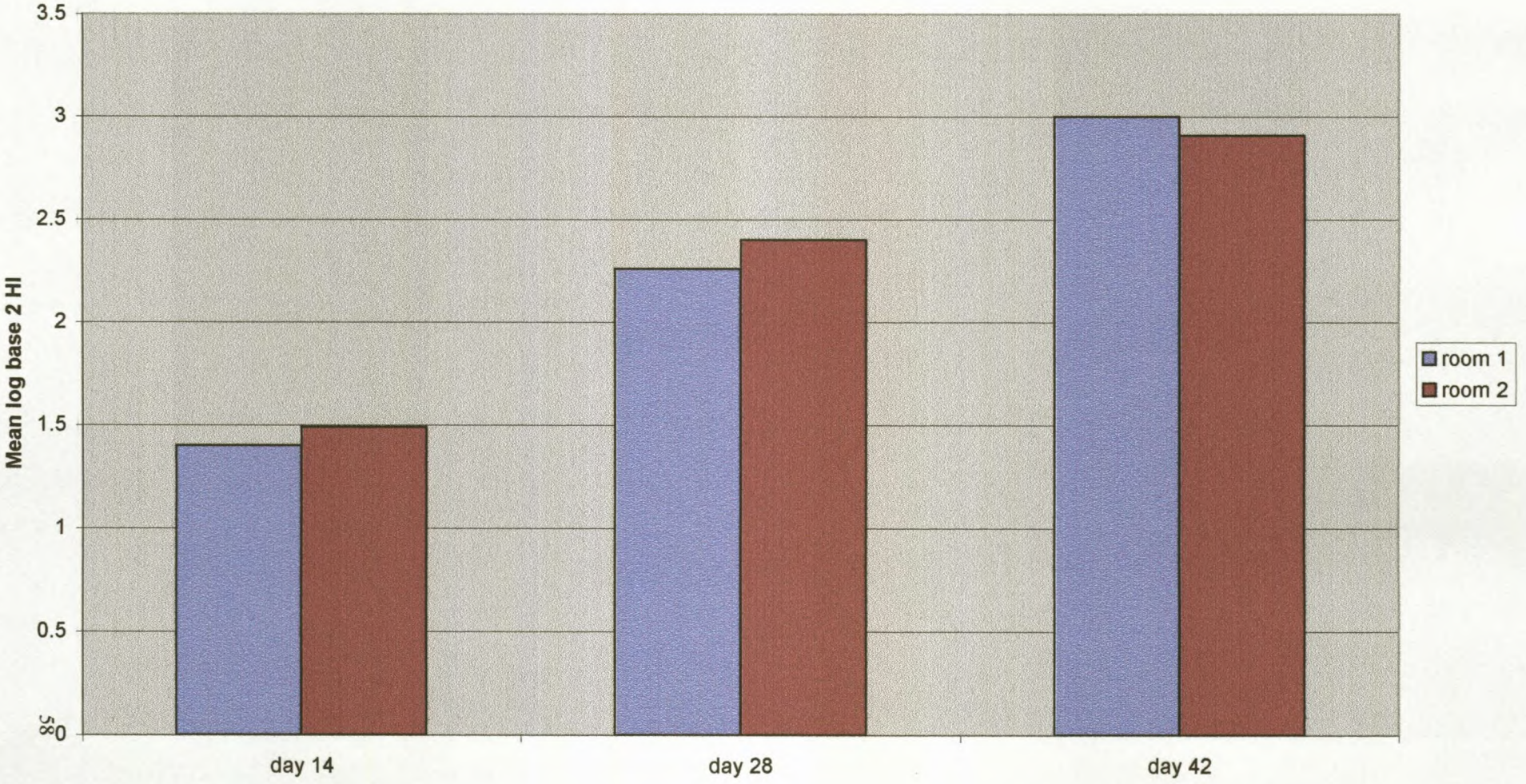
Table 4.6. Multiple Range Analysis on mean $\log_2$ HI results of days, to establish which days were significantly different from one another. Means with the same letter are not significantly different.

These results show that the HI levels increased significantly from day 14 to 28 and from day 28 to day 42, which means the birds were responding to the vaccine.

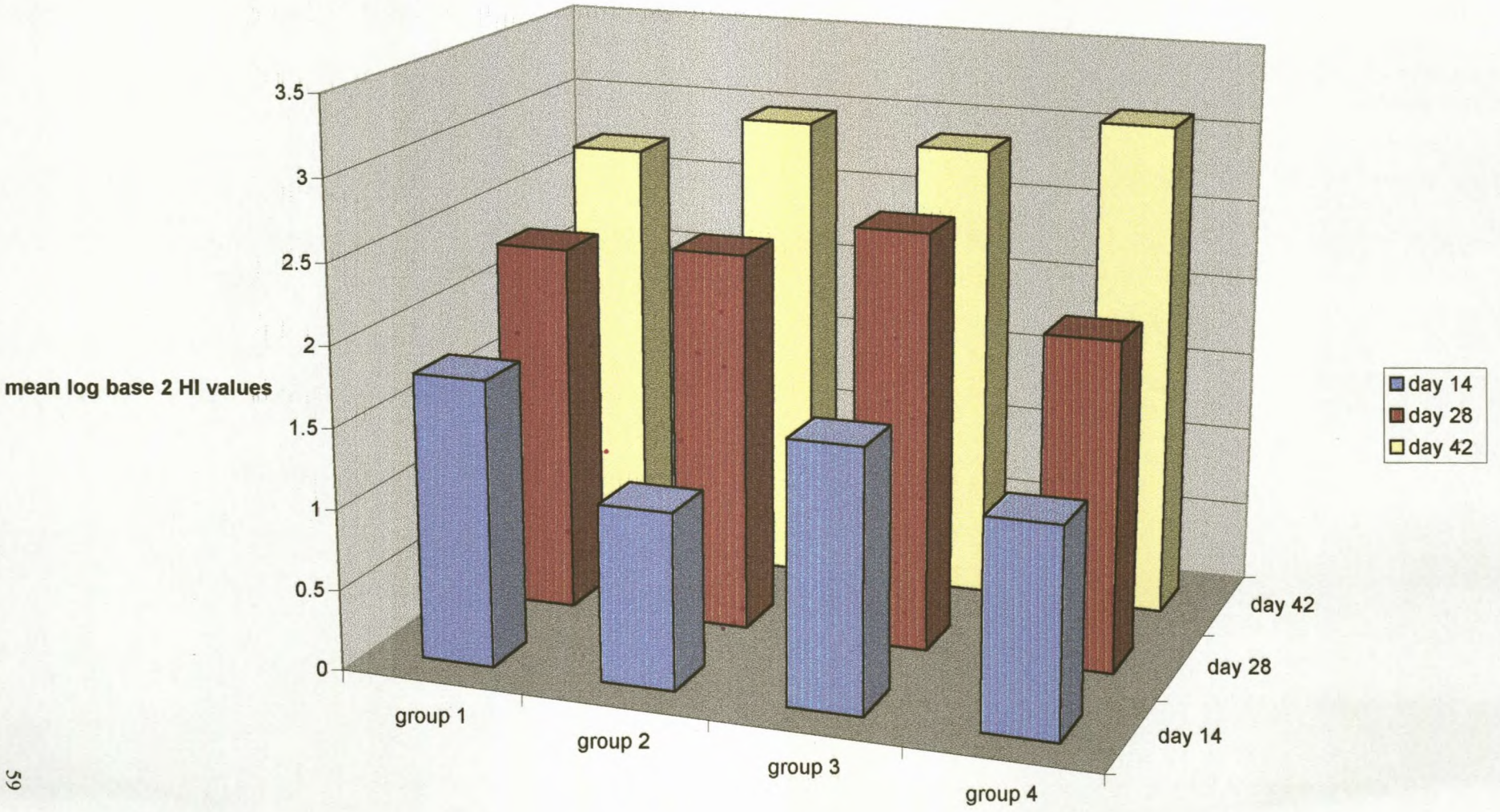
Graph 4.1. Interaction of means between groups, rooms and days.



Graph 4.2. Comparison of the mean log base 2 HI levels of the vaccinated birds housed in rooms 1 and 2.



Graph 4.3. Comparison of mean log base 2 HI levels of the vaccinated groups.



CHAPTER 5

DISCUSSION

The aim of this study was to determine the effect of homoeopathic dilutions, of the antigenic component of the Newcastle disease virus, on the immune response of broiler chickens to a killed oil emulsion vaccine. Blood samples were taken before vaccination (day 14) and two and four weeks after vaccination (days 28 and 42 respectively) with a killed oil emulsion Newcastle disease vaccine. The immune response was measured by Haemagglutination Inhibition tests, which were performed on all the blood samples.

There was a significant difference in maternally derived immunity in the groups on day 14 (see table 4.2), even though the birds were randomly divided. This indicates that the parent stock had varying degrees of immunity against Newcastle disease (Box 1965). This difference becomes insignificant because the maternal immunity slowly decreases to statistically insignificant levels by day 28 (see HI levels of group 5 room one on graph 4.1.). By day 28, there was a significant difference between

the non-vaccinated group and the vaccinated groups (Table 4.3). There was no difference between the vaccinated groups that received homoeopathic remedies, and the vaccinated group that received placebo (Table 4.3). The same trend can be observed on day 42 (Table 4.4). Again there was a significant difference between the non-vaccinated group and the vaccinated groups (in spite of the influence of the high values of group 5 room 2, that can be observed in graph 4.1), and no significant difference between the vaccinated groups receiving homoeopathic remedies and placebo. There appears to be no significant benefit in administering homoeopathic dilutions of the antigenic component of the Newcastle disease vaccine in conjunction with the orthodox vaccine.

The HI values of the replication of the non-vaccinated control group housed in room 2 of the poultry house, showed a steady increase in HI levels, with higher levels on day 42 than any other group (Graph 4.1). The presence of Newcastle disease antibodies in the serum of a non-vaccinated bird indicates that the bird has been infected with Newcastle disease virus. It was concluded that the birds in room 2 were exposed to Newcastle disease virus, and that this virus infected some of the birds. The antibody levels of the non-vaccinated control housed in room 1 decreased to zero on day 42 (Graph 4.1). This indicates that room 1 was

not infected by Newcastle disease. On day 28, the HI levels of group 5 in room 2 does not differ significantly from the levels of group 5 housed in room 1, although group 5 in room 2 shows no decrease in HI titres from day 14 (Graph 4.1). From this information, it is suspected that Newcastle disease virus was introduced into room 2 just before day 28. The results obtained from the vaccinated birds in room 2 on day 42 did not differ significantly from the vaccinated birds in room one. (Graph 4.2). It therefore appears as if the virus had no effect on the vaccinated birds. This is an indication that the relatively low levels of antibodies that these birds showed against Newcastle disease, was protective against the virus. The more virulent a virus is, the greater mortality rates caused by the virus. The moderate mortality rate in the infected cage (3 birds out of 21 birds between day 14 and 42) indicate that the Newcastle disease virus that infected the birds was a not a very virulent strain. A virulent virus strain can cause up to 100% mortality (Alexander 1997:549). The virus could have been a mild strain used for live vaccination that was carried over to this room of the poultry house by a poultry worker that had contact with birds vaccinated with a live vaccine.

The immune titres produced by the oil emulsion vaccine were generally low. Four weeks after vaccination average titres obtained in the vaccinated groups barely reached $\log_2 3$, compared to titres varying between $\log_2 5$ and $\log_2 7$ obtained by Stone *et al.* (1971) four weeks after primary vaccination of one day old chicks with oil emulsion vaccines.

Phillips (1973) stated that field challenge by Newcastle disease will not kill chickens whose HI levels are $\log_2 5$ or more, nor will it affect egg production if the levels are $\log_2 7$ or above. The titres obtained in this trial (two and four weeks post vaccination) were according to this information not high enough to protect against egg loss or mortality if the chickens were to be challenged with a virulent virus.

A few factors could have contributed to the low antibody titres obtained in this trial.

Box (1965) found that vaccinating 14-day-old birds carrying high levels of maternally derived immunity may cause an overall lowering of the immune response and may be accompanied by a possible failure to respond to the vaccine. Chicks with a high level of maternally derived immunity were not fully responsive to vaccination until after three weeks old (Box 1965). It is now generally accepted that the older the chicks are at the time of vaccination the better the immune response. This is due firstly to the

progressive enhancement of their immune mechanism and secondly to the gradual disappearance of their maternal immunity. (Chu and Rizk 1975.) Chu and Rizk (1975) confirmed the studies of Wolfe *et al.* (1957) that antibody optimum antibody response was not reached until the birds were 22 weeks old. At two weeks of age, the immature immune system responds more slowly.

The primary antibody response to killed oil emulsion vaccines is slower than the response to live vaccines (Box and Furminger 1975). The primary antibody response to an oil emulsion vaccine takes about 3 weeks, and the fact that the last blood results in this trial were taken 4 weeks after vaccination may well have contributed to the low immune response to the vaccine in this trial. Under commercial conditions the birds are first vaccinated with a live vaccine to provide fast immunity which is not long lasting. Subsequently, an oil emulsion vaccine is administered, which provides long term protection with a high secondary antibody response. The birds in this trial were not primed with a live vaccine, because there was a non-vaccinated control group incorporated into the design of the trial, to act as an indicator of extraneous infection with Newcastle disease. If the birds were vaccinated with a live vaccine, the live vaccine would have contaminated the non-vaccinated control group.

The trial was run under field conditions where the birds could have been subclinically infected by Infectious Bronchitis virus, Marek's disease or Chicken Anaemia Agent, all of which are known to inhibit immune responses to Newcastle disease vaccination (Lombardi 1993). A faulty batch of vaccine could have caused the low immune responses. Although an experienced person administered the vaccine, and birds with obvious vaccine leakage were eliminated from the trial, inadequate vaccination technique could have contributed to the low immune response to the vaccine. If the low results were due to faulty administration, it could be argued that some birds would have received the correct dose and would have responded with high antibody titres, which was not the case. It can be speculated that the low immune response of the chickens to the vaccine could have influenced the result of this trial, although there was still room for the homoeopathic remedies to improve the immune response.

Weisman *et al.* (1992) demonstrated that the specific antibody response of experimental mice to an antigen, could be modulated by homoeopathic dilutions of the antigen that were administered before vaccination. In this trial, no significant change in the immune response to an oil- emulsion-Newcastle-disease-vaccine was observed when administering homoeopathic dilutions of the Newcastle disease virus antigen subsequent to vaccination.

From the results obtained from Weisman's study, it seems more beneficial to administer homoeopathic dilutions of the antigen before vaccination, than after vaccination.

CHAPER 6

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

Twelve, fifteen and two hundred centissimal homoeopathic dilutions of the antigenic component of the Newcastle disease virus was found to be ineffective in increasing the antibody mediated immune response of broiler chickens to the killed oil emulsion vaccine.

6.2 RECOMMENDATIONS

If similar trials are to be conducted in future, it may be better to eliminate the interference caused by maternally derived immunity. This can be done by using birds that come from a parent stock with no Newcastle disease antibodies, or by vaccinating birds only at three and a half to four weeks of age, at which point Box (1965) found that maternal immunity has decreased to such low levels that the immune response to oil emulsion vaccines are not influenced.

In this field trial, the birds could have been subclinically infected by diseases which are known to inhibit immune responses to Newcastle disease vaccination e.g. Infectious Bronchitis, Marek's disease or Chicken Anaemia Agent (Lombardi 1993). Similar trials could be conducted in biological isolators where exposure to disease agents are minimised. The use of such facilities would dramatically increase the cost of such trials.

A further recommendation is that individually numbered birds and blood samples are used so that information on individual birds can be ascertained. By doing this, birds with no response due to, faulty vaccination for example, can be excluded from the trial.

In this study killed virus was used as an antigen. Further studies could investigate the effect of potencies of antigen on the immune response to live vaccines.

Weisman *et al.* (1992) demonstrated that specific antibody generation by experimental mice could be modulated by homoeopathic dilutions of the antigen KLH. The mice were preconditioned by repeated intraperitoneal injections (three times a week over 8 weeks) with succussed high dilutions of KLH (7C and 15C) and saline. This was followed by regular immunization

with KLH in Freud's adjuvant. It was found that, following immunization, there was a significant increase in the KLH-specific antibody response in mice preconditioned with KLH in homoeopathic dilutions.

In this trial, no significant change in the immune response to an oil-emulsion-Newcastle-disease-vaccine was observed when administering homoeopathic dilutions of the Newcastle disease virus antigen subsequent to vaccination. From the results obtained from Weisman's study (mentioned above), it seems more beneficial to administer homoeopathic dilutions of the antigen before vaccination, than after vaccination. It would be interesting to investigate the influence of route of administration (i.e. intraperitoneal injection v.s. oral) on the effect of the homoeopathic dilutions.

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APPENDIX

RAW DATA

GROUP	ROOM	INDIVIDUAL LOG_2 HI VALUES BEFORE VACCINATION ON DAY 14																				
1	1	0	0	0	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	3	3	3
1	2	0	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	4	4	4	4
2	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2	2	2	3	3
2	2	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2
3	1	0	0	0	0	1	1	1	1	2	2	2	2	2	2	2	2	2	2	3	3	3
3	2	0	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	3	3	3
4	1	0	0	0	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	3	3
4	2	0	0	0	0	0	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2
5	1	0	0	0	0	0	0	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2
5	2	0	0	0	0	0	0	0	1	1	1	1	1	1	1	2	2	2	2	2	2	2

GROUP	ROOM	INDIVIDUAL LOG_2 HI VALUES ON DAY 28, TWO WEEKS POST VACCINATION																				
1	1	0	1	1	1	1	2	2	2	2	2	2	2	2	2	3	3	4	4	4	4	4
1	2	0	1	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	5
2	1	0	0	0	0	1	1	1	1	1	2	2	2	2	2	3	3	4	4	4	6	6
2	2	0	0	1	1	2	2	2	2	3	3	3	3	3	3	4	4	4	4	4	5	5
3	1	1	1	1	1	1	2	2	2	2	2	2	2	3	3	4	4	4	4	4	5	6
3	2	1	1	1	2	2	2	2	2	2	2	2	3	3	3	3	3	3	4	4	4	4
4	1	0	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	3	3	3	4	6
4	2	0	1	1	1	1	1	1	1	2	2	2	2	2	2	2	3	3	4	4	4	4
5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	3
5	2	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	2	3	4	4	5	1

GROUP	ROOM	INDIVIDUAL LOG_2 HI VALUES ON DAY 42, FOUR WEEKS POST VACCINATION																				
1	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3	3	4	4	4	4	5	
1	2	0	0	1	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	5
2	1	0	2	2	2	2	2	2	2	3	3	4	4	4	4	4	4	4	4			
2	2	0	1	1	2	2	2	3	3	3	3	3	3	4	4	4	4	4	5	5	5	
3	1	0	1	1	2	2	2	2	2	3	3	3	3	3	3	4	4	4	5	5	6	
3	2	0	1	2	2	2	2	3	3	3	3	3	3	3	3	4	4	4	4	5		
4	1	0	1	1	1	1	2	2	3	3	3	3	3	4	4	4	4	4	4	5		
4	2	0	1	2	2	2	2	3	3	3	3	3	3	3	4	4	4	4	4	4	5	
5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
5	2	0	1	2	2	3	3	4	4	4	4	5	5	5	5	5	5	6	7	7		