DEVELOPMENT OF FORMAL IN INACTIVATED OIL EMULSION ND VACCINE

BY

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DECLARATION

The author hereby declares that the work presented here for the Doctor of Philosophy Degree in Microbiology was carried out under the supervision of Drs. A.A. Ahmad and N.E. Gomwalk. No part of it has been presented for any award or degree and reference to published literature is adequately acknowledged.

Nathaniel Ndamadu Shidali — 7-1/11/88 Date

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CERTIFICATION

This thesis entitled 'Development of formalin inactivated oil emulsion ND vaccine' by Nathaniel Ndamadu Shidali meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

Chairman Supervisory Committee — Date 7/12/88

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DEDICATION

(To my children)

Man is fallible
God is infallible.

God, the Providence
of life and health of
knowledge and understanding
of all reality to eternity.

God, the Almighty
direct the mind a - thought
direct the path a - right
and everything possible.

God, my Lord!
trust and rely on Him
acknowledge and praise Him
"He shall deliver thee"

N.N. Shidali

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ABSTRACT

Eight Newcastle disease virus (NDV) isolates were examined for use in the production of inactivated oil emulsion vaccine (IOEV). The NDV Lasota (lentogenic strain) and NDV Komarov (mesogenic strain) were found most suitable.

A 1:1000 concentration of formalin was optimal for the inactivation of these viruses. The adjuvant effect of palm oil, groundnut oil and liquid paraffin were investigated and liquid paraffin was found to induce the best adjuvant effect. The IOEV produced from the lentogenic and mesogenic viruses consisted of equal volumes of the virus antigens and the oil adjuvant. It was stable and potent for more than 1 year at 22 - 25°C and 4°C and for at least 4 months at 37°c" A 0.4 ml dose IOEV containing 2 xlO^{16} EID_{50} or 640-1280 haemagglutination titre of the virus gave the best serological response and protection in chickens. A schedule consisting of priming the chikens at one day old with $NDVE_1$ before vaccination with IOEV subcutaneously at 3 weeks was developed. Chickens vaccinated with IOEV produced 5 times more HI antibodies when compared with current live vaccines. When the chickens were challenged with 5 virulent isolates from different parts of the country 16 weeks after IOEV vaccination, 98% of them were protected compared to 76% protection in chicken vaccinated with live vaccines. Progeny of TOEV vaccinated parents were found to have enough

protective maternal antibody for upto 3 weeks.

'Egusi[1] oil used in place of the imported Arlacel A as an emulsifier in the production of IOEV gave a more stable emulsion. Birds vaccinated with IOEV containing 'Egusi' oil emulsifier induced serological response and protection similar to those vaccinated with IOEV containing Arlacel A.

TABLE OF CONTENTS

		PAGE
	Title	i
	Declaration	ii
	Certification	iii
	Dedication	iv
	Acknowledgement	v
	Abstract	vii
	Table of Contents	ix
	List of Tables	xiii
	List of Figures	xv
CHAPTER		
1.0.	INTRODUCTION	1
1.1.	Newcastle Disease	1
1.2.	Historical background and spread of Newcastle disease ...	2
1.3.	Economic effects of Newcastle disease	4
1.4.	Newcastle disease in Nigeria ..	5
1.5.	Newcastle disease control measures in Nigeria	6
1.6.	Advantages and Disadvantages of the 3 live ND vaccines	7
1.7.	Objectives of the project	9
2.0.	LITERATURE REVIEW	12
2.1.	Morphology and structure of Newcastle disease virus	12
2.2.	Biological and other activities of Newcastle disease virus ...	12
2.2.1.	Haemagglutinin, Neuraminidase and haemolytic activities	13
2.2.2.	Replication of Newcastle disease virus	14

		PAGE
2.3.	Haemadsorption	15
2.3.1.	Cell fusion	16
2.3.2.	Plaque formation	16
2.4.	Cell and chick embryo culture systems	17
2.5.	Classification of Newcastle disease virus	17
2.6.	Antigenic composition of Newcastle disease virus	20
2.7.	Types of vaccines against Newcastle disease virus	23
2.8.	Inactivated Newcastle disease vaccines	26
2.9.	Advantages of inactivated oil emulsion Newcastle disease vaccine	27
2.10.	Oil adjuvants, emulsifiers and inactivating agents used in preparation of inactivated vaccines	29
2.11.	Serological method for measuring the antibody level induced by inactivated oil emulsion Newcastle disease vaccine	32
3.0.	MATERIALS AND METHODS	34
3.1.	Materials	34
3.2.	Methods	38
3.2.1.	Cultivation of viruses	38
3.2.2.	Titration of viruses - Haemagglutination and Embryo infective dose fifty percent (EID_{50})	39
3.2.3.	Antigen stability test	42
3.2.4.	Bacterial and Viral purity test ...	43
3.2.5.	Inactivation of viruses with formalin	44
3.2.6.	Rearing of chickens	46

		PAGE
3.2.7.	Preparation of inactivated oil emulsion vaccine	47
3.2.8.	Vaccination of chickens with inactivated oil emulsion vaccine ...	49
3.2.9.	Titration of antibodies - haemagglutination inhibition test	50
3.2.10(a)	Safety and stability test	52
3.2.10(b)	Potency testing	52
3.2.11.	Vaccination of larger number of chickens using schedule 2 regimen ...	54
3.2.12.	Maternal antibody experiment	57
3.2.13.	Substitution of Arlacel A with 'Egusi' oil	57
3.2.14.	Field trials with inactivated oil emulsion vaccine	61
4.0.	RESULTS	63
4.1.	Cultivation and titration of viruses	63
4.1.2.	Antigen stability	63
4.2.	Sterility of the vaccine candidate viruses	65
4.2.1.	Optimal concentration of formalin for inactivation of viruses	65
4.3.	Selection of oil adjuvant for the preparation of inactivated oil emulsion vaccine	68
4.4.	Reculturing of virus strains for preparation of emulsion	68
4.4.1.	Viability of the antigen after formalin inactivation	71
4.5.	Safety and stability of the vaccine	71
4.6.	Potency of the vaccine	73
4.6.1.	Challenge with wild viruses 16 weeks after vaccination	73

		PAGE
4.7.	Vaccination of larger number of birds with IOEV using Schedule 2 regimen	77
4.7.1.	Challange trial with wild viruses	77
4.8.	Result of Maternal antibody experiment	82
4.9.	Stability of inactivated oil emulsion vaccine containing 'Egusi' oil emulsifier	82
4.10.	Potency of inactivated oil emulsion vaccine containing 'Egusi' oil emulsifier	82
4.11.	Field trial with inactivated oil emulsion vaccine	87
5.0.	DISCUSSION	91
6.0.	REFERENCES	104

LIST OF TABLES

		PAGE
Table 3.1.	Protocol of the cultivation of viruses	40
Table 3.2.	Concentration of formalin	45
Table 3.3.	Challenge trials of Schedule 2 regimen	56
Table 3.4.	Challenge trials with IOEV containing 'Egusi' oil emulsifier	60
Table 3.5.	Protocol of field trial	62
Table 4.1.	Haemagglutination and embryo infective dosefifty percent titres of vaccine and wild strains of Newcastle disease virus	64
Table 4.2.	The haemagglutinin stability of the viruses at $4^{o}C$ and 22-$25^{o}C$	66
Table 4.3.	The effect of formalin concentration on haemagglutinin and growth of NDVL and NDVK	67
Table 4.4.	Toxicity of formalin concentration in one day old chickens	69
Table 4.5.	The effect of temperature on the stability of IOEV	72
Table 4.6.	Statistical analysis of antibody response by different schedules of IOEV compared to the live vaccines ..	75
Table 4.7.	Mortality of chickens vaccinated with different units of IOEV	76
Table 4.8.	The percentage protection induced by different units of IOEV after challenge with wild viruses	78
Table 4.9.	Statistical analysis of antibody response by 2 different doses of IOEV	80
Table 4.10.	The percentage protection induced by 2 different doses of IOEV	81
Table 4.11.	Maternal antibodies of progency of IOEV vaccinated and live vaccine vaccinated parents	83

PAGE

Table 4.12.	Effect of temperature on the stability of IOEV containing 'Egusi' oil emulsifier	84
Table 4.13.	Statistical analysis of antibody response to IOEV containing 'Egusi' oil emulsifier	86
Table 4.14.	Challenge trials after vaccination with IOEV containing 'Egusi' oil emulsifier 16 weeks after vaccination	88
Table 4.15.	Field trials with IOEV in 3 categories of birds	89

LIST OF FIGURES

		PAGE
Figure 1:	The comparison of the antibody GMT of the 3 oil adjuvants containing Lasota antigen	70
Figure 2:	The comparison of the antibody GMT induced by different schedules of IOEV	74
Figure 3:	The comparison of the HI antibody GMT of IOEV at different doses with those of the 3 live vaccines	79
Figure 4:	Antibody response to IOEV containing 'Egusi' oil emulsifier	85

CHAPTER 1

INTRODUCTION

1.1. Newcastle Disease

Newcastle Disease (ND) is a very lethal and contagious viral disease found in domestic poultry and other wild birds. It is characterised by the involvement of the respiratory, gastrointestinal and central nervous systems. Apart from chickens, some of the avian species found to be susceptible to ND are duck, turkey, pheasant, guineafowl, parrot, owl, pigeon, patridge, canary and eagle. Humans exposed to ND virus may occasionally develop severe conjunctivitis which may lead to lasting impairement of vision. Most of such human infections have occured among laboratory workers handling the virus in research or vaccine production laboratories (Hofstad et al 1984).

Five clinical forms of ND are known. These are Doyle's, Beach's, Beaudette's, Hitchner's and avirulent forms (Beard and Hanson, 1984). The Doyle's form was first recognised in 1926 (Doyle, 1927) and it is an acute lethal infection of all ages of chickens, characterised by haemorrhagic lesion of the intestinal tract. It is often referred to as the Asiatic ND or the Viscerotropic Velogenic Newcastle disease (VVND). The Beach's form (Beach, 1944) is characterised by the lesions of the respiratory tract and the nervous system, while the Beaudette's form is similar to Beach's form except that it is limited to young chickens (Beau-

dette et al. 1949). Hitchner and Johnson in 1948 described the Hitchner's form as a mild or inapparent respiratory infection of chickens of all ages with low mortality rate. The avirulent ND on the other hand lacks clinical signs and is detected only by virus isolation and presence of specific antibodies (Lancaster, 1981). The five forms of ND are caused by ND viruses that are morphologically and antigenically similar (Beard and Hanson, 1984).

1.2. Historical Background and Spread of Newcastle Disease

According to Shope (1964) the historical information concerning ND is inadequate to determine the origin, development and perpetuation of the disease. Kraneveld (1926) gave the first description of ND in this manner "In March 1926, the Veterinary Medical Laboratory received a number of diseased chickens from Batavia (now Djakarta) on the Island of Java, Indonesia, which has fallen victim to a disease appearing in that city for the first time. It is now known across the entire Island of Java and in some part of the outer regions." Picard (1928) later used the term "Pseudo-fowl pest" to describe the disease observed by Kraneveld and stated that "in several native villages literally no single fowl was left." He also stated that "it was impossible to ascertain from where this epidemical disease, with symptoms hitherto unseen, originated."

About the same time and the year when ND was reported by Kraneveld from Indonesia, the disease

also occured at Newcastle-upon-Tyne England in the spring of 1926 (Doyle, 1927). However, Doyle (1927) showed by confirmed laboratory experimentation that the disease "is caused by a filter passing virus and is characterised by a difficulty in respiration and a high mortality." The causative agent thus received its name as the Newcastle Disease Virus (NDV).

The disease originally described by Kraneveld and Doyle had not reached the Western Hemisphere by that date (Beaudette, 1943; Brandly et al. 1946). However, they explained that a rather benign avian disease known as pneumoencephalitis appeared in California, U.S.A. Beach, (1944) later recognised the disease as a form of Newcastle Disease.

Levine (1964) documented the occurence of ND in many countries and stated that Venezuela in 1949-1950 and Mexico in 1950 both witnessed virulent ND outbreaks with 100% mortality in adult poultry. Beard and Hanson (1984) explained that before 1971 the initial Doyle's and Beach's form of ND has been reported in all nations of the world and that the viscerotropic velogenic ND outbreak of 1971 in California, U.S.A., was similar to Doyle's form.

The Organisation for African Unity (OAU) Inter African Bureau for Animal Resources in 1982 published the geographical distribution of ND in Africa. It showed that the disease is wide spread in East and West African sub-regions particularly the entire countries

of Nigeria and Ghana (OAU/STRC, 1982).

1.3. Economic Effects of Newcastle Disease

The economic losses due to ND has been tremendous all over the world. Cockrill (1971) summarised the economic losses due to ND outbreaks in few countries and stated that viscerotropic veologenic ND outbreaks of 1971 to 1974 caused the U.S.A. the loss of over 11,000,000 head of poultry at a total cost of $56,000,000 U.S. dollars. The loss from the British ND outbreak of 1970-71 was estimated to be £20,000,000 (pounds sterling) (Gordon, 1971). May Chew and Liow (1974) gave over $34,000,000 U.S. dollars as loss from poultry mortality in one of the ND outbreaks in Singapore. Newcastle disease spread rapidly and continuously in Japan in 1965 to 1968 (Nobuto, 1968). The government budget for the control of the disease in the fiscal year 1966-1967 was 150 million Yen, about, 18% of the total appropriation for the control of animal infectious disease (Nobuto, 1968). In the Philippines, ND was considered the most serious disease of chickens in 1969 and caused a mortality which represented a loss of 4.4 million pesos (Novilla and Novarro, 1969). In Canada the five outbreaks of ND between 1971 and 1973 caused the loss of 250,000 head of poultry (Lancaster, 1974). With this background it is obvious that the incidence of ND in any country needs serious control measures so as to avert the devastating economic losses due to the disease.

1.4. Newcastle Disease in Nigeria

The first confirmed outbreak of ND in Nigeria was in 1953 (Hill et al. 1953). Since then the disease has been Nigeria's most dreaded disease of the poultry causing untold economic losses through death and reduction in egg production to the farmers (Adu et al. 1985). On the average between 200 to 250 outbreaks are reported annually in the country (Nawathe, 1987). Nawathe et al., (1985) characterised thirteen isolates of the virus from field outbreaks at the National Veterinary Research Laboratory and they were all found to be velogenic strains. Similar characterization was done at Vom in 1985 involving samples from different parts of the country and they were all found to be velogenic strains (Adu et al. 1985).

Apparently normal local chickens have been found to harbour highly virulent strains of ND in Nigeria (Fatunbi and Adene, 1979; Majiyagbe and Newathe, 1981). Onunkwo and Momoh (1981) isolated and characterised viscerotropic velogenic Newcastle Disease (VVND) virus from a local parrot. Okoh (1979) earlier reported the incidence of ND in falcons destined for exportation to Europe from Kano.

Newcastle Disease Virus is therefore endemic in Nigeria. It is estimated that the poultry population in Nigeria (local and exotic) is 200 million (Nawathe, 1986). Although there is no available data on the annual economic loss of these chickens due to frequent ND outbreaks, it is believed that the loss will compare

favourably with those from other parts of the world or probably higher. The need for a more serious control measure can therefore not be over emphasized.

1.5. Newcastle Disease Control Measures in Nigeria

There are two approaches to the control of this disease. The first approach is to prevent contact between infected and non-infected susceptible chickens through quarantine, slaughter, provision of clean water and food, and the restriction of people into chicken houses. This measure is not enough as there is continuous field challenge from wild bird carriers and wind-borne spread of the virus.

The second approach, which is the repeated vaccinations of the chickens so as to maintain high level of immunity is therefore the most essential. Three live vaccines are currently in use in Nigeria. These are the Komarov (mesogenic), the Intraocular (lentogenic) and the Lasota (lentogenic). David-West (1972) reviewed that "the Komarov strain of NDV was first isolated from a natural outbreak of ND in Israel" and that "the production of NDV (K) vaccine started at Vom in 1953." He further stated that "vaccination of laying hens with NDV(K) produces a marked drop in egg production for a period of up to six weeks. The vaccine was also unsuitable for the protection of young susceptible chicks and sometimes caused severe reactions in poultry flock." This led to heavy govern-

ment investment on the development of two new improved vaccines.

Crowther (1964) developed the Cyprus originated (B1) Intraocular vaccine at Vom to protect baby chicks before the administration of the Komarov vaccine. Although very mild for young chickens, its immunity lasts only 3-4 weeks and the virus can easily be neutralised by maternal immunity (Nawathe, 1987).

The Lasota strain of the vaccines was developed also at Vom by Owolodun in 1968. It is given to chickens in drinking water at 3-4 weeks between the Intraocular and the Komarov vaccinations. It provides solid immunity for more than eight weeks and has the highest spreading potential among the three live vaccines.

1.6. The advantages and disadvantages of the 3 live ND Vaccines

The 3 live ND vaccines (NDVB1, NDVL and NDVR) have the following advantages:

(i) They induce faster immune response in chickens.

(ii) They are easy and cheaper to apply to chickens. The Lasota vaccine is applied through drinking water while the intraocular vaccine is applied through the eyes. These techniques do not need expensive labour.

(iii) Since the virus is live, it continues to replicate within the body of the chicken inducing both humoral and cell mediated immunity without the need of an adjuvant.

However, they have the following problems:

(i) The annual average outbreaks of ND is still very high. It is about 200 to 250 (Nawathe, 1987).

(ii) Even among vaccinated flock, outbreaks of ND have been experienced (Okoye and Shoyinka, 1983; Adu et al. 1985).

(iii) Tropical high temperatures of the field associated with lack of cold chain facilities contribute significantly in inactivating the live virus, leading to loss of vaccine potency.

(iv) The production of the live vaccines involves the use of heavy lyophilizing machines, capping machines, millions of vials, caps and rubber stoppers. These are not available locally and their importation is very expensive. The maintenance of the heavy machines depends largely on foreign experts.

(v) The NDV(K) is unsuitable for young chickens and laying parent stock (David-West, 1972). The Intraocular vaccine, which can be given at very early age, faces the problem of being neutralised by maternal antibody (Nawathe, 1987).

(vi) The frequent outbreaks of ND even in the vaccinated flock has been associated with possible antigenic differences between

the vaccine strains and indgenous field strains or that the live vaccines are possibly not potent enough (Adu et al. 1985).

1.7. The objective of the project

The objective of this project is to develop inactivated oil emulsion ND vaccine capable of solving all or most of the above problems posed by the current live vaccines. To attain this objective the following experiments will be carried out:

1. Selection of a vaccine candidate virus from locally available ND virus strains in Nigeria by virus culture in embryonated eggs, determination of their pathogenicity and infectivity in embryonated eggs and determination of their haemagglutinin titres in embryonated eggs.
2. Determination of the sterility of the virus strains so selected.
3. Investigation of the possibility of using formalin instead of the rare carcinogenic Beta-propiolactone as an inactivating agent for the inactivation of the vaccine candidate virus.
4. Investigation of the possibility of using Palm oil, Groundnut oil or Liquid paraffin as oil adjuvants and to determine the most suitable in preparing Inactivated oil emulsion ND vaccine.

5. Preparation of anantigen from the ND virus using the most suitable inactivating agent from No. 3.
6. Preparation of an adjuvant using the most suitable oil from No. 4 and an emulsifier, Arlacel A., attempting the use of 'Egusi' oil as an alternative emulcifier to Arlacel A.
7. Preparation of a stable Inactivated oil emulsion ND vaccine from 5 and 6 and the testing of its sterility.
8. Testing the safety and potency of the Inactivated oil emulsion ND vaccine using the vaccine alone and/or its combinations with any of the live vaccines in chickens. This will be done through sero-monitoring and challenges using locally available virulent strains of ND virus.
9. Comparing the potency of the new vaccine and its combinations with the live vaccines to that of the 3 live vaccines currently in use with a view to select the best for protection of chickens in the country.
10. Vaccination of a large number of chickens with the new vaccine in order to re-evaluate it potency.
11. Determination of the stability and shelf life of the vaccine at different temperatures.

12. Carrying out some field trials with the new vaccine.

CHAPTER 2

LITERATURE REVIEW

2.1. Morphology and Structure of *Newcastle disease virus*

The virion of NDV ranges from 100-300nm in size. It consists of an internal component surrounded by an external component known as envelope (Beard and Hanson, 1984). The internal component, known as G antigen or soluble antigen, consists of a single strand of RNA surrounded by structural proteins to form the helical nucleocapsid of 17-18nm wide with a central hollow axis of 4-5nm in diameter (Alexander, 1982). The ether sensitive and osmotically deformable lipoprotein envelope (external component) is of host origin and consists of regular numerous spikes 80A^{O} long and 1-1.5nm wide (Cruickshank, 1964).

Alexander and Allan (1973) described Newcastle disease (ND) virus populations under electron microscope as "pleomorphic" and stated that "individual virions have no rigid structure and frequently appear disrupted on the grid." They gave the chemical composition by weight of purified virions as "67% protein, 1% ribonucleic acid, 24% lipid, 7% carbohydrate."

2.2. Biological and Other activities of Newcastle disease virus

Newcastle disease virus possesses a number of biological activities by which it may be distinguished

qualitatively or quantitatively from other paramyxoviruses. Some of these properties are reviewed below.

2.2.1. Haemagglutinin, Neuraminidase and Haemolytic activities

When Newcastle disease (ND) virus and chick red blood cells (RBC) are mixed, aggregation of the RBC ensues as a result of the virus particles adsorbing to specific receptors on the RBC surface to form a lattice net work between the cells (Wells, 1975). This lattice network of cells known as haemagglutination (HA) can be inhibited by specific antisera (Burnet, 1942). The ability of ND virus to agglutinate erythrocytes is associated with its haemagglutinin which has specific receptors on the RBC (Beard and Hanson, 1984). Apart from chick RBC, ND virus would agglitinate amphibian, reptilian and other avian RBC (Lancaster, 1966). Newcastle disease virus will agglutinate human, mouse and guinea pig RBC but only certain strains have the ability to agglutinate catle, goat, sheep, swine and horse RBC (Wells, 1975).

Agglutinated RBC eventually gets disaggregated due to elution of the virus particles from the RBC - receptors (Waterson, 1968). The elution is caused by the enzyme neuraminidase (Ackerman, 1964). The in vivo function of neuraminidase is not known (Becht et al. 1971). However, it has been suggested that since nasal secretion is a natural substrate for paramyxovirus neuraminidase, the enzyme may be implicated

in the penetration of the mucus barrier by the virus (Morein and Bergman, 1972).

Newcastle disease has the ability to cause lysis of the RBC it agglutinates (Kilham, 1949). This haemolytic activity was initially associated with a specific haemolytic molecule within the virus structure (Wilson, 1958). Apostolov and Poste (1973) however, revealed that ND virus haemolytic activity was caused by the fusion of the virus envelope with RBC membrane resulting in an area of membrane fregility. Important factors that affect the haemolytic activity of ND virus are freezing and thawing, dialysis, sonic vibration, salt concentration and P^H of the suspending solution and temperature at which the reaction takes place (Bratt and Clavell, 1972; Clavell and Bratt, 1972).

2.2.2. Replication of Newcastle disease virus

The first stage of ND virus replication begins with the adsorption of the virus to the host cell surface, the process of which is mediated by the attachment of the haemagglutinin to the glycoprotein receptors of the cell (Kohn, 1965; Rott and Scholtissek, 1967). After adsorption, the virus envelope and the cell membrane fuse together to extrude the nucleocapsid into the cytoplasm. This method of entry into the host cell is similar to that exhibited by Sendai virus (Apostolov and Almeida, 1972).

The ND virions possess RNA dependent RNA polymerase which synthesises complementary RNA to the viral RNA (Huang *et al*, 1971). The complementary RNA produced are heterogenous in size (18-22S and 35S) and some are the same size with the virion RNA (Bratt and Robinson, 1967; Blair and Robinson, 1968; Kaverin and Varich, 1971). Some of the complementary RNA act as mRNA for the synthesis of viral structural proteins, while others become viral RNA (Kingbury, 1970). After the assembly of the structural proteins and the viral RNA to form nucleocapsid, the nucleocapsid become aligned under modified cell membrane of the host from where they are slowly budded off as complete virus particles by a process that is the reverse of entry (Wells, 1975).

2.3. Haemadsorption

The first evidence of CPE seen in ND virus the ability of the infected cells to adsorb RBC (haemadsorption) to their surface (Wells Marcus (1962) has shown that haemadsorp' be demonstrated within 4 hours of HeLa ce with ND virus and suggested that this modification was brought about by newl haemagglutinin into the cell membran (1964); Reeve and Alexander (1970) h the amount of haemodsorption exhibit was broadly related to its virulence

(1971) have shown that haemadsorption can be inhibited by specific antibodies.

2.3.1. Cell fusion

One of the CPE of ND virus in chick embryo cells is the formation of polykaryocytes or syncytium which has been associated with one of the envelope glycoproteins, the Fusion (F) glucoprotein (Nagai et al. 1976). Although strains of ND virus vary in their ability to induce cell fusion, the amount of such fusion and the size of the polykaryocytes formed are directly related to the virulence of the infecting virus strain (Reeve and Alexander, 1970; Reeve and Poste, 1971). Cell fusion can be inhibited by the inhibitors of protein synthesis if added within 3 hours of infection (Reeve et al. 1972).

2.3.2. Plaque formation

When ND virus is inoculated into a susceptible confluent cell monolayer, discrete lesions (plaques) can be seen after 24-72 hours, particularly after staining with neutral red. The neutral red is adsorbed into the lysosome of the active cells resulting in the normal cells staining red and the dead or dying cells remaining colourless (Wells, 1975). After studying 41 isolates of ND virus, Daniel and Hanson (1968) showed that most virulent strains produced the largest plaques and avirulent strains produce small or no plaques. Two basic types of colours (clear and red)

and several size classes ranging from 0.5 - 4mm diameters are some of the characteristics of plaques induced by ND viruses (Schloer and Hanson, 1968b).

2.4. Cell and chick embryo culture systems

Newcastle disease virus is capable of producing CPE in many cell culture systems. Many types of primary cells particularly of avian origin and certain cell lines of mammalian origin are readily infected by ND virus (Beard and Hanson, 1984). Among the cells that are commonly used are chick embryo fibroblast, BKH21, HEP-2 and bovine cell line MDBK (Wells, 1975).

All strains of ND virus induce infection of chicken embryos. Several routes of inoculation, amnio-allantoic, chorioallantoic, yold sac and intravenous are employed (Beard and Hanson, 1984). This chicken embryo system which is more economical and produces larger quantities of viruses is employed not only in ND virus vaccine production (Allan et al. 1978) but also in ND virus subpassages to study its subpopulation behaviours (McMillan et al. 1986).

2.5. Classification of Newcastle disease virus

Lwoff et al. (1962) proposed that "viruses should be classified by the following characteristics:

(a) nucleic acid, RNA or DNA.

(b) symmetry of nucleocapsid.

(c) the diameter of nucleocapsid.

(d) presence or absence of envelope surrounding the nucleocapsid."

The name 'myxovirus' (Andrews et al. 1955) was then accepted to include the influenzaviruses A, B and C, ND viruses and mumps viruses which not only have affinity for mucus membranes but share the above characteristics except the nucleocapsid diameter. However, Waterson (1962) grouped the myxoviruses into two: (i) the influenza group with nucleocapsid diameter of 9-10nm and (ii) the ND virus group with nucleocapsid diameter of 17-18nm which included ND viruses, mumps, parainfluenza viruses, measles, rinderpest and distemper viruses.

Waterson and Almeida (1966) suggested a more significant characteristics than the nucleocapsid size in grouping myxoviruses. They proposed 'true myxoviruses' for myxoviruses possessing neuraminidase and 'pseudomyxoviruses' for those without neuraminidase. Wildly (1971), on the other hand, divided the myxoviruses into two genera on the basis of nucleocapsid diameters with the generic names of 'Orthomyxovirus', for the influenza group and 'paramyxovirus' for the ND virus group.

Fenner (1976) using the recommendation of the International Committe of Taxonomy of Viruses (WHO, 1971) improved on Wildly's classification by introducing two families to myxoviruses: the Orthomyxoviridae' (formerly Orthomyxovirus genus) and 'Paramyxoviridae' (formerly paramyxovirus genus). The Paramyxoviridae family was then divided into three genera: Paramyxovirus

(ND virus group), Morbillivirus (measles virus) and Pneumoviruses (respiratory syncytial viruses and other related agents). The Paramyxovirus genus therefore consists of the ND viruses and other avian paramyxoviruses, parainfluenza viruses 1-5, and Mumps Viruses.

As the number of isolations of avian paramyxoviruses increased, Tumova et al. (1979b) suggested a method of serogrouping then based on serological differences like haemagglutination inhibition (Alexander, 1980), serum neutralization test (Tumova et al. 1979a) and neuraminidase inhibition test (Kessler et al. 1979). This suggestion, which has been generally accepted, subgroup the avian paramyxoviruses into seven distinct serotypes, namely PMV-1 to MPV-7 of which ND virus is PMV-1.

The ND virus strains of PMV-1 have been further classified according to their virulence or pathogenicity in chickens. Allan et al. (1978) and Hanson (1980) used mean death time in hours of inoculated eggs, intracerebral pathogenicity index (ICPI) in one day old inoculated chickens, intravenous pathogenicity index (IVPI) in six weeks old inoculated chickens and Plaque morphology tests to designate strains of ND virus (PMV-1) into velogenic, mesogenic and lentogenic strains. Strains with the highest virulence such as the Milano, Herts and viscerotropic velogenic ND viruses (VVND) were classified as velogenic while mild strains such as Komarov and avirulent strains

such as B1 and Lasota were classified as mesogenic and lentogenic respectively (Beard and Hanson, 1984).

In summary, ND viruses belong to the Paramyxoviridae family of the paramyxoviruses genus of the PMV-1 serotype and have velogenic, mesogenic and lentogenic strains depending on their virulence.

It is important to note that "the word 'strain" when applied to NDV system is used to mean a stable well characterised line of virus and does not imply serologically distinct types" (Alexander and Allan, 1973).

2.6. Antigenic composition of Newcastle disease virus

The nucleocapsid and the envelope of ND virus are antigenic. The nucleocapsid (NP) contains seven glycoproteins which are often referred to as G or soluble antigen (Beard and Hanson, 1984). Two other glycoproteins, the Haemagglutinin - Neuraminidase (HN) and the Fusion (F) are associated with the envelope. These form part of the pattern of projections or spikes (80A^{o} long) that stimulate the host to produce haemagglutinin inhibiting and virus neutralizing antibodies (Rott, 1964). The HN glycoprotein has both haemagglutinating and neuraminidase activities which are responsible for attachment of virus to host cell receptors and receptor destroying activity, respectively (Scheid and Choppin, 1973; Choppin and Compans, 1975). The F glycoprotein is responsible for fusion with and

penetration through the host cell membrane (Nagai et al. 1976).

It has long been thought that the degree of virulence displayed by ND virus strains is determined by its two envelope glycoproteins. Alexander and Allan (1973) comparing the cross haemagglutination - inhibition tests and the neuraminidase - inhibition tests of different strains using monospecific antibodies could not demonstrate any correlation. They stated that "no isolates so far tested are significantly different from the suggested reference strain HERTS 33 or from any other more recent isolates. All vaccine strains examined at the Central Veterinary Laboratory (New Haw, Weybridge, Surrey, England) are capable of giving protection against the whole series of challenge strains which have been collected." Other workers later showed that although antigenic variation occured as a serological phenomenon, the cross protection tests failed to confirm that the in vitro results were significant in field vaccinations (Kaliannan et al. 1975; Martone et al. 1976).

In their further studies of F glycoproteins Nagai et al. (1976) showed that FO (original F glycoprotein) was the precursor of F glycoproteins and that in virulent ND virus strains the proteolytic cleavage of FO to F occured in a wide range of host cell types in cell culture; in avirulent strains the FO was only cleaved in restricted range of cell types. Noninfectious

ND virions were also found to be infectious by treatment with trypsin (Nagai and Klenk, 1977). Similarly extremely avirulent ND strains required proteolytic cleavage of HNO (original HN glycoprotein) for generation and activation of HN whereas the HN glycoproteins did not require cleavage in the more virulent strains (Garten et al. 1980).

To fully understand the nature of these two important antigenic components in relation to the virulence of ND virus strains several workers have started to study them at the molecular level. Jorgensen et al. (1987), McGinnes et al. (1987) and Millar et al. (1988) have constructed cDNA from genome RNA of different strains of ND viruses and have determined the complete nucleotide sequence of their F and HN genes. Similarly, Iorio et al. (1986) and Samson et al. (1988) have used monoclonal antibodies to identify antibody binding sites on the F and HN glucoproteins of certain strains of ND virus. It is hoped that in the very near future the antigenic component of Nd virus in relation to its virulence will be fully understood.

However, as a result of the important role the envelope glycoproteins play in inducing an immune response which results in the production of virus - neutralizing and haemagglutinin - inhibiting antibodies, the different types of vaccines developed against Newcastle disease virus use the glycoproteins as their major antigenic constituent.

2.7. Types of Vaccines against Newcastle disease virus

The following vaccines are either being developed or are in commercial use against Newcastle disease virus:

Live Virus Vaccines.

Subunit Vaccines.

Synthetic and genetically engineered vaccines.

Inactivated oil emulsion vaccine.

The live virus vaccine consists of the live lentogenic and the live mesogenic Newcastle disease virus strains in which the virion's nucleocapsid and the envelope are intact (Allan et al. 1978). The live lentogenic vaccines used the B1 (Hitchner and Johnson, 1948), the F (Asplin, 1952), and the Lasota (Winterfield, et al. 1957) strains. Generally the B1 and F vaccines do not cause nervous disease in day old chicks but may give rise to mild and transitory respiratory symptoms while the Lasota strain may cause post vaccination respiratory symptoms (Allan et al. 1978). Nigeria uses two lentogenic vaccines: the intraocular which is of the B1 strain and which is administered intraocularly (Growther, 1964) and the Lasota strain (Owolodun, 1968) which is administered through drinking water. The mesogenic live vaccines include the Roakin which was isolated in the U.S.A. as a naturally occuring strains (Beaudette et al. 1949); the Kamorov strain which was attenuated from the virulent strain by intracerebral passage in ducklings (Komarov and Goldsmit, 1946), the Hertfordshire strain which was Herts 33

attenuated by serial passage in eggs (Iyer and Dobson, 1940); and the Mukteswar strain which was attenuated from its virulent form by serial passage in chick embryos (Haddow and Idnani, 1946). All mesogenic vaccines are administered parenterally and they are neither recommended for immunization of chicks under 8 weeks old nor are they recommended for adult birds not previously immunized (Allan et al. 1978). Nigeria uses the Komarov mesogenic vaccine (David-West, 1972). The attenuation of virulent Newcastle disease virus to mesogenic strains has also been carried out in cell cultures. Huygelene and Peetermans (1963) adapted Komarov in bovine kidney monolayer cells to obtain the tissue culture komarov. Similarly Vasic (1965) adapted the Mukteswar in pig kidney monolayer cells to obtain the Mukteswar tissue culture strain. In spite of the side reactions associated with the live vaccines they are still the most widely used for the protection of chickens and turkeys against Newcastle disease virus (Kelleher et al. 1987).

The subunit vaccine involves the use of the two envelope glycoproteins excluding the nucleocapsid and the lipid matrix. The glycoproteins which are isolated from the envelope are reconstructed in form of nicellar complexes as vaccines. Morein et al. (1984) prepared a complex designated 'immunostimulating complex' (LSCOM) using a glycoside extracted from the back of a South African tree, Molina saponaria

quillaria. The ISCOM contained HN and F glycoproteins and it was said to induce high antibody production in mice. Other forms of subunit vaccines are glycoproteins prepared in form of miscelles or virosomes (Morein and Simons, 1985) and (DeVries et al. 1988). The subunit vaccines are still at the developmental stage. It is feared however that the molecular weight of the miscelles or ISCOM is quite low compared to whole virus vaccines and may therefore need to be conjugated with larger proteins to induce as much antibody response (Carlson, 1986).

The synthetic vaccine involves the isolation and the sequencing of the antigen responsible for protection, the prediction by computer analysis of the specific epitope of the antigen and the synthesis of the polypeptide (Brown, 1984). The recombinant DNA technique involves the sequencing of the genes responsible for the transcription of the message of the antigen, the cloning of such genes and its insertion into a vector for the eventual expression into protein (Carlson, 1986). Bacteria, yeast and even vaccinia virus have been used as vectors (Smith et al. 1983; Scolnick, 1984; Mackett et al. 1984). Like the subunit vaccine, the synthetic and the recombinant NDA vaccines are still at the developmental stage. They must be conjugated with larger proteins since the polypeptides are too small to be antigenic (Carlson, 1986).

The last type of vaccine is the Inactivated oil emulsion Newcastle disease vaccine which is the subject of this project. It is discussed in the next topic.

2.8. Inactivated Newcastle disease vaccines

Inactivated vaccines are vaccines in which the live microorganisms have been inactivated or killed without damaging the antigenic composition of the microorganisms. In the case of Newcastle disease vaccines the nucleic acid of the virus is inactivated with certain chemical agents leaving, especially and most importantly, the envelope glycoproteins (Weir, 1979). The virus does not multiply in the tissues of the host with inactivated Newcastle disease vaccine as is the case of live Newcastle disease vaccine. Therefore the concentration of the virus which is directly related to the antigenic mass of the vaccine is a major factor in the potency of an inactivated vaccine (Brugh and Siegel, 1978).

Hofstad (1953) and Piercy *et al*. (1964) were among the early workers on inactivated Newcastle disease vaccine. While Hofstad inactivated the virus with formalin, Piercy *et al*. inactivated the virus with betapropiolactone and adjuvanted the vaccine with aluminium hydroxide. These vaccines did not receive wide acceptance. The re-emergence of Newcastle disease as a serious poultry disease of world wide scope in the early 1970s resulted in the marked improvement in the technique of developing inactivated Newcastle disease vaccines (Stone *et al*. 1980). The vaccine

could now be emulsified in oil and it is the oil phase that is responsible for the adjuvant effect of the vaccine (Weir, 1979).

A number of countries have developed inactivated oil emulsion Newcastle disease vaccine using their local strains to control the disease. Such countries are Israel, Britain, United States of America and Malaysia (Zakay-Rones and Levy, 1973; Box and Furminger, 1975; Stone et al. 1981, Ernawati and Ibrahim, 1984).

In Nigeria, the only inactivated oil adjuvant vaccine is the contagious pleuropneumonia vaccine for protection of cattle against pleuropneumonia (Garba and Terry, 1986; Garba et al. 1987). The need for such vaccine to be developed and used against Newcastle disease in Nigerian poultry cannot be over emphasized due to its numerous advantages.

2.9. Advantages of inactivated oil emulsion Newcastle disease vaccines

The fact that inactivated oil emulsion Newcastle disease vaccine, if properly developed, induce higher antibody production and better protective immunity than the live Newcastle disease vaccines has been in no doubt in the minds of those who have used them. Giambrone (1985) reporting in the Canada poultry digest, showed the feeling of the Canadian poultry farmers about the inactivated oil emulsion Newcastle disease vaccine as follows: "the inactivated products have become increasingly popular because they contain no live organisms which may adversely affect growth,

egg production, hatchability and fertility of the breeders or be shed throughout the egg causing clinical disease in the chick. Also the new killed product when preceded with proper combination of primer vaccines can induce higher, long lasting and more uniform antibody titres in chickens than can be attained from continued used of live vaccines."

Eidson et al. (1980) and Eidson et al. (1982) reported that "live vaccines have been shown to contribute to reduced egg production due to stress and other factors. Inactivated vaccines are superior to the repeated use of live virus vaccines."

Partadiredja et al. (1979) reporting on the immunization of broiler breeder chickens with killed oil emulsion vaccine said "antibody response was rapid and high in the broiler breeder chickens receiving 1 or 2 vaccinations of inactivated oil emulsion Newcastle disease vaccine. The antibody titers remain high during the 41 week experimental period."

Brugh (1985) while explaining the adverse effect of the presence of maternal antibody on live Newcastle disease vaccines said that "a combination of live virus vaccine and oil emulsion killed virus vaccines could induce systemic and local protection even in the presence of maternal antibody."

The above advantages will apply to the Nigerian situation when inactivated oil Newcastle disease vaccine

is developed and used in the country. In addition the heavy annual expenditure (in foreign exchange) presently incured in the importation of vials, caps and maintenance of freeze-drying machines will be greatly reduced as oil emulsion vaccines will not be lyophilised; locally manufactured bottles will also be adequate to dispense them. The high tropical temperatures which frequently inactivate the current live vacines in the field rendering them impotent will be eliminated using the inactivated oil emulsion vaccine.

2.10. Oil adjuvants, Emulsifiers and Inactivating agents used in the preparation of inactivated vaccines

The high immune response of inactivated Newcastle disease vaccine is to a large extent dependent on the adjuvant effect from the oil (Stone et al. 1980). However, the actual mechanism of oil adjuvants in the inducement of an immune response to an antigen has not been fully explained. It is generally supposed that the oil surrounds the antigen to form very tiny particles which aids in the slow absorption of the antigen from the site of injection resulting in higher antibody production (Cernik, 1970). Fruend (1956) explained that the activity of the oil adjuvant in antibody production was in three stages: (i) "it enables the antigen to settle at the site of injection so as to secure a gradual and persistent release for antibody stimulation", (ii) "it provides a vehicle

for transport of the emulsified antigen through the lymphatic system to new foci of antibody production - lymphnodes and spleen", (iii) "it enables the formation and accumulation of the mononuclear type cells able to produce antibodies." Wilson and Miles (1955) also explained that the local tissue reaction to some adjuvants probably stimulated the cells to produce antibody or that the adjuvants probably caused more antibody producing centres to form. The tissue reaction to oil adjuvants has been described as similar to the reaction of tissues in inflammation, the only difference being the degree of the inflammation reaction (McKinney and Davenport, 1961). Steiner et al. (1960) and Peck et al. (1964) described tissue reaction to oil adjuvants as starting with acute inflamatory reaction followed by tissue necrosis and finally sterile absess. However, with the refined technique in the use of oil adjuvants, these tissue reactions are now greatly minimised (Hitchner, 1984).

Garba et al. (1987) used liquid paraffin, Fruend's incomplete adjuvant and aluminium hydroxide when developing inactivated oil emulsion mycoplasma vaccine for Nigeria cattle.

The mechanism of emulsifiers in the production of emulsions has been known. Emulsifiers act by emulsifying the oil phase of the emulsion so as to obtain a stable mixture of the oil and the water phase (Halbert et al. 1945). More recently, a highly purified prepara-

tion of mannide mono-oleate called Arlacel A was found to have emulcifying property and was extensively used (Davenport, 1968; Garba et al, 1987). Other emulsifiers that were used in the preparation of emulsions were Drakeol 6 VR oil, Bayol F. Mayolin 248 D, Falba and Tween 80 (Gernik, 1970).

Another important component of an inactivated vaccine is the inactivating agent. The inactivation of pthogenic organisms to render them safe for use in a vaccine, without altering the immunizing ability of the antigen, is essential in producing an effective vaccine. Failure to accomplish this may have been partially responsible for the variation in killed vacines in the pat (Hitchner, 1984). Early attempts to produce inactivated vaccines against Newcastle disease employed a variety of inactivating agents: chloroform, toluol, trichesol, phenol, hydrochloric acid, formalin and ether (Beaudette, 1943). The early attempts were unsuccessful and presumably led to the reputation that killed ND vaccines were ineffective. Recently betapriopiolactone has been shown as an effective agent in killing the virus without altering the antigenicity. It acts very rapidly but it has been shown to be carcinogenic (Allan, 1978). In addition to this disadvantage, it is not easily available in Nigeria. Studies with poliomyelitis virus demonstrated that under controlled conditions a very small concentration (1:4000) of formalin could be used to inactivate

the virus without appreciably damaging the antigenicity (Salk, 1954).

In this project, formalin will be used to inactivate the Newcastle disease virus.

2.11. Serological method for measuring the antibody level induced by inactivated oil emulsion Newcastle disease vaccine

Sera of chickens that have had ND virus or have been vaccinated with ND virus vaccine contain antibodies that inhibit agglutination of chick red blood cells (RBC). The antibody neutralizes the haemagglutinin glycoproteins of the virus responsible for haemagglutination of the erythrocytes; the level of this haemagglutination inhibition (HI) is a direct measure of the HI antibody produced (National Academy of Science, 1971). National Academy of Science (1971) further defined two procedures for HI test: alpha procedure which utilized constant volume of serum and varying dilution of antigen, the beta procedure which utilized varying dilution of serum and constant volume of antigen. The HI titre was determined as the reciprocal of the highest dilution of serum giving complete inhibition. Beard and Wilkes (1973) introduced the microtest procedure of HI test in which micro instead of macro volumes are used. Alan and Gough (1974) advocated the beta procedure as being more reliable using 4HA50 unit of the virus antigen and expressing the HI titre as highest dilution of the serum giving 50% inhibition of haemagglutination.

Beard and Wilke (1973) stated that "haemagglutination-inhibition (HI) test is generally considered to be a reliable, economical, rapid means of measuring the response of poultry to Newcastle vaccination." Thayer et al. (1987) using HI test, solid-phase immunoassay and enzyme linked immunosorbent assay (ELISA) to measure antibodies against Newscastle disease virus and infectious bronchitis virus stated that HI test compared very favourably with solid-phase immunoassay and ELISA test in terms of sensitivity and reliability.

The beta procedure of HI test will be used in this project.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

1. The following viruses were used for the project:

   (i) Newcastle disease (ND) virus B1 (NDV B1).

   (ii) Newcastle disease virus LaSota (NDVL).

   (iii) Newcastle disease virus Komarov (NDVK).

   (iv) Newcastle disease virus isolated from parrot (NDVP).

   (v) Newcastle disease virus isolated from ND outbreak in Ibadan (UI 104).

   (vi) Newcastle disease virus isolated from ND outbreak in Oji River (OJP).

   (vii) Newcastle disease virus Herts 33/50 strain.

   (viii) Newcastle disease virus isolated from ND outbreak in Kano (K1090).

The NDVB1, NDVL (lentogenic strains) and NDVK (mesogenic strain) were obtained from Dr. E.N. Okeke, Head of Virology Division, National Veterinary Research Institute (NVRI), vom. The NDVP, UI 104, K1090 and OJP (velogenic strains) were obtained from Dr. F.D. Adu, Virology Division, NVRI, Vom. The UI 104 isolate was from the Western Zone of Nigeria, the K1090 the Northern Zone of the OJP the Eastern zone. The NDVP represented isolated from wild birds. Since the parrot is a major distributor of ND viruses in Nigeria. Herts 33/50 is a velogenic strain first isolated in Hert-

forshire, England (Iyer and Dobson, 1940). It is commonly used as a challenge virus to test the potency of ND vaccines. It was obtained from Dr. D. Shamaki, Virology Division, NVRI, Vom.

2. The chickens used for the project were of three species:
   (i) White leghorn.
   (ii) Babcock.
   (iii) High breed between white leghorn and Babcock.

   The species were obtained from Mallam Y. Shuaibu, Head of Poultry Division, through the permission of the Director, NVRI, Vom.

3. The embryonated chicken eggs for virus cultivation were 10 days old and were mixtures of white and brown shelled eggs. They were obtained from Mallam Y. Shuaibu, Head of Poultry Division, through the permission of the Director, NVRI, Vom. The yolk, albumin, allanto-amniotie fluids of each batch of eggs were examined by HA test to ensure that the eggs were free from ND virus infection.

4. The chicken red blood cells (RBC) for haemagglutination (HA) and haemagglutination inhibition (HI) tests were collected by wing vein puncture of white leghorn adult cocks kept for bleeding purposes at the Virology Division, NVRI, Vom

5. Microtitre system used for the tests were:
   (a) round-bottom microtitre plates containing 96 reaction wells (Flow laboratories, Irvine Scotland, U.K.).
   (b) microtitre diluters of 25ul and microtitre droppers of 25ul (Cooke laboratory product, 900 slaters Lane Alexandria, Virginia, U.S.A).
6. Syringes and needles:
   These were obtained from Becton, Dickinson and Company, Rutherford, N.J. 07070 U.S.A. They were syringes of 20ml, 2ml, 1ml and needles of 26 gauge and 14 guage.
7. Egg incubator set at $37^{o}C$.
8. Waterbath set at $56^{o}C$ for heat inactivation of serum samples.
9. Refrigerator set at $4^{o}C$.
10. Deep freezer ($-20^{o}C$) for storing lyophilized virus isolates.
11. Bleeding tubes for serum collection.
12. Automixer for emulsification, obtained from vaccine production section, NVRI, Vom.
13. Bottles (50ml) for dispensing vaccine (Bottle manufacturing Company, Kaduna, Kaduna State, Nigeria).
14. ND virus antigen (Dr. E.N. Okeke, Virology Division, NVRI, Vom).
15. ND virus specific antiserum (Dr. E.N. Okeke, Virology Division, NVRI, Vom).

16. Reagents:

(a) Palm oil was obtained from National Institute for Oil Palm Research (NIFOR) Benin, Nigeria.

(b) Groundnut oil was obtained from Kano State Oil and Allied Products Limited, Kano, Nigeria.

(c) Liquid paraffin (Virology Division, NVRI, vom).

(d) Arlacel A (Sigma Chemical Company, P.O. Box 14508, St. Lous MO63178, U.S.A.).

(e) 'Egusi' seed was purchased from the Bukuru market, near Jos, Plateau State.

The oil was prepared in the laboratory as described under the "methods."

17. Solutions:

(a) Phosphate buffered saling P^H 7.4

Nacl	8g
Kcl	0.2g
Na_2 HPO4.$2H_2O$	1.2g
KH_2 PO4	0.2g
Deionized water	1 litre

(b) Vaseline wax mixture

Vaseline	1g
Wax MPT 54.5^{o}-57^{o}C	3g
Phenol	few crystal

The mixture was heated to melting point.

(c) Formalin (Analar, 40% formaldehydre BDH chemicals, U.K.)

(d) Alcohol (70%).

(e) Deionized water (NVRI, Vom).

18. Two pairs of curved scissors.

19. Two egg punch.

20. Bacteriological media.

    (a) Blood agar.

    (b) Mc Conkey agar.

    (c) Cooked meat medium.

21. Chick fibroblast cell monolayer.

22. Live Newcastle disease (ND) vaccine (Dr. E.N. Okeke, Virology Division, NVRI, Vom).

    (a) NDV intraocular or B1 Batch 36 of 3/86 and Batch 57 of 3/87.

    (b) NDV Lasota Batch 80 of 3/86 and Batch 3 of 5/87.

    (c) NDV Komarov Batch 15 of 3/86 and Batch 41 of 4/87.

    These were obtained by persmission of the Director, NVRI, through Dr. E.N. Okeke, Head of Virology Division, NVRI, Vom.

3.2. Methods

3.2.1. Cultivation of viruses

Ten day old embryonated chicken eggs were candled to select the viable ones and their air sacs marked with a pencil. About 3mm below each air sac an area of less vasculation was marked with an "X" to indicate the site through which the virus would be inoculated into the allantoic cavity. Each egg was disinfected with 70% alcohol after which the marked area was punctured using an egg punch.

Each virus strain was diluted to 10^{-3} in sterile phosphate buffered saline (PBS) P^H 7.4 containing 100 I.U. penicillin and 100mg streptomycin per millilitre. With sterile 1ml tuberculin syringe and 26 guage needle, 0.1ml of the diluted virus suspension was inoculated into the allantoic cavity. Each strain was inoculated into 25 eggs while 5 control eggs received PBS as shown below (Table 3.1). Each egg was sealed with molten wax at the site of inoculation and incubated at 37°C. Earlier the incubation period for each strain was determined by incoulating the eggs as described, harvesting the allantoic fluid (AF) from 5 eggs after every 24 hours and finding the number of hours giving the highest HA titire by each strain. The optimum incubation period for each strain was thus determined.

All eggs were candled every 24 hours and any dead embryo within 24 hours was discarded. Dead embryos after 24 hours were removed and stored at 4°C. At the end of the incubation periods the eggs were kept at 4°C for 24 hours before conducting the haemagglutination test (HA).

3.2.2. Titration of viruses - Haemagglutination and Embryo infective dose fifty percent (EID_{50})

Freshly collected chick red blood cells (RBC) were washed 3 times in PBS P^H 7.4. and 10% and 1% solution made from the packed cells in PBS. Each chilled embryo was cut open at the airsac with a pair

Table 3.1: Protocol of the cultivation of viruses

Virus strain	Number of egg inoculated	Control eggs inoculated with PBS	Period of incubation at $37^{o}C$
NDVK	25	5	48 hours
NDVL	25	5	72 hours
NDVB1	25	5	96 hours
NDVP	25	5	72 hours
UI 104	25	5	48 hours
K1090	25	5	48 hours
OJP	25	5	48 hours
Herts 33/50	25	5	48 hours

of sterile curved scissors. A sterile wire loop full of the allantoic fluid (AF) was mixed with a wire loop full of 10% chick RBC on a perspex tile and observed for haemagglutination. All allantoic fluid from each strain showing positive HA were collected asceptically into a sterile bottle, placed on ice.

An aliquot of each of the virus strain was diluted and in PBS P^H 7.4 from 1:10 to 1:2560 in a microtitre plate in 0.025ml volume. To each dilution of the virus 0.025ml of 1% chick RBC was added. A positive control consisting of ND antigen was similarly set up. The negative control well received PBS and chick RBC only. The test was done in triplicate and left at room temperature for 30-45 minutes. The titre of the virus was taken as the reciprocal of the highest dilution of the AF showing complete haemagglutination.

To ensure that each strain was specifically Newcastle disease virus, the following neutralization test was carried out. To 1.0ml of each strain of the ND virus, 1.0ml of specific anti ND virus serum was added and left for 30 minutes at room temperature. The virus-serum mixture was inoculated allantoically into embryonated eggs as described earlier. After incubation and chilling, the allantoic fluids were collected and the haemagglutination titration was carried out as described above. The negative HA titre demonstrated by each of the strains confirmed them as ND virus strains.

Ten day old embronating chicken eggs were candled, disinfected and prepared for inoculation as described earlier. Ten-fold dilution of the virus from 10^{-1} to 10^{-18} was carried for each strains in sterile PBS P^H 7.4. Beginning from 10^{-5} to 10^{-18}, each dilution was inoculated into 5 eggs at 0.1ml per egg. The eggs were sealed and incubated as described earlier. The eggs were chilled at the end of the incubation period, disinfected, cut open and the allantoic fluid tested for haemagglutination. The number of eggs showing positive and negative HA in each dilution was recorded. Using spearman Karber formular modified by Allan (1978) $m=\bar{x} + \frac{1}{2}d - d\frac{(Eri)}{n}$, the EID_{50} of each virus strain was determined.

m	=	end point.
x	=	last titration dilution
d	=	incremental step between dilution.
$\sum ri$	=	sum of uninfected eggs.
n	=	number of eggs per dilution.

3.2.3. Antigen Stability test

An aliquot of allantoic fluid of each ND virus strain was taken. This was divided into two parts: One part was kept at 4^oC for 2 weeks while the other part was left at room temperature for 2 weeks. The haemagglutination titres before and after the exposure to these conditions were then determined. The strain

with the highest HAa titre and therefore the most stable haemagglutinin antigen was then determined.

The NDVL and NDVK with the highest HA titres, EID_{50} end point and very stable antigen were selected as the vaccine candidate viruses. All the virus strains were lyophilized and stored at $-20^{o}C$.

3.2.4. Bacterial and Viral Purity test

An aliquot of each of the viral strain was taken and cultured in Blood agar (BA) McConkey agar (MCA) and cooked meat medium (cmm). the inoculated BA, MCA and CMM were incubated at $37^{o}C$ for 5 days. To ensure that there was no other virus present in the allantoic fluids an aliquot of the fluids was treated with equal volume of specific anti ND virus serum for 30 minutes to neutralize the ND virus. The allantoic fluid-serum micture was then inoculated allantoically into 10-day embryonating eggs (10 in number) as described in 3.2.1. The inoculated eggs were incubated at $37^{o}C$ for 5 days. Five embryonating, uninoculted eggs and 5 ND virus inoculated eggs were also incubated along as controls. The AF of the 3 inoculations were harvested and examined for HA. The two treated AF and the 2 controls were also inoculated unto chick fibroblast monolayer cells and examined for 7 days for cytopathic effect. Samples of the virus strains were also sent to Bacteriology Division of NVRI for mycoplasma test by the method of Yoder and Hofstad (1964).

3.2.5. Inactivation of viruses with formalin

From the NDVL and NDVK allantoic fluids, 22.5ml was dispensed into 5 sterile universal bottles at each 4.5ml per bottle as shown on table 3.2. to bottle one 0.5ml of formalin was added. A log dilution of the formalin was then carried out from 10^{-1} to 10^{-4} as shown on table 3.2. The bottles were shaken at intervals of 1 hour for 8 hours after which they were left at room temperature over-night. The total inactivation period at room temperature was 24 hours. Each dilution was titrated by HA test to determine the minimum concentration at which the formalin did not damage the haemagglutinin antigen. To determine the minimum concentration required for complete inactivation of the viruses, each dilution was cultured allantoically in 5 embryonated eggs at 0.4ml per egg. Their allantoic fluids were tested by spot HA after incubation period of 72 hours at $37^{o}C$. The minimum concentration of the formalin which completely inactivated the viruses without loss of HA titre of the viruses was selected for the preparation of the inactivated oil emulsion vaccine.

Table 3.2: Concentration of formalin.

Bottle number	1	2	3	4	5
Allantoic fluid (ml)	4.5	4.5	4.5	4.5	4.5
Formalin (ml)	0.5	→ .5	→ .5	→ .5	-
Concentration of formalin in the allantoic fluid	10^{-1}	10^{-2}	10^{-3}	10^{-4}	0

3.2.6. Rearing of chickens

The experimental chicken house was built of concrete blocks with cemented floors. The house was partitioned such that cross contamination of viruses from room to room was minimised. In addition it had large windows covered with wire gauze for adequate ventilation and for preventing wild birds, reptiles, cats etc. from getting into the house. The chickens were reared on deep litres hence the floors were covered with saw-dust which was changed every 2 weeks. At the door of each room was a container of disinfectant for deeping the feet before entering the rooms. The room space was about 1 square foot per adult bird.

The chickens which were collected at one day old were kept under brooders to provide warmth for 7 weeks. From one day old to 7 weeks they were fed with chick mash. From 8 - 18 weeks they fed on growers mash and after 18 weeks they were fed with layers mash. They were provided with fresh clean water once a day.

Random visits to the chicken house by many people was prevented while clean environment was maintained throughout the period of the experiment both within the around the chicken house. Regular checks by a veterinarian was carried out to ensure the health status of the chickens.

3.2.7. Preparation of inactivated oil emulsion vaccine

(a) Selection of oil adjuvant. Palm oil and groundnut oil were first heated gently for 15 minutes to about 70°C. The liquid paraffin and Arlacel A (emulsifier) were filter sterilized using sterile sietz filter of 0.45u and 0.22u. The 4 oils were then cultured on Blood agar (BA), McKonkey agar (MCA) and Cooked meat medium (CMM) at 37°C for 48 hours to check their sterility. A mixture of each of the 3 oil adjuvants containing 10% Arlacel A was prepared. Five mililitres of each oil was then homogenized mechanically with 5.0ml of live Lasota virus suspension, in a sterile universal bottle, to form an emulsion.

Three week old susceptible chickens were inoculated subcutaneously as shown below:

(i) Palm oil adjuvant containing Lasota antigen, 5 chickens at 0.4 ml per chicken.

(ii) Groundnut oil adjuvant containing Lasota antigen, 5 chickens at 0.4ml per chicken.

(iii) Liquid paraffin adjuvant containing Lasota antigen, 5 chickens at 0.4ml per chicken.

(iv) Five uninoculated chickens as controls.

Blood (0.5ml) was removed from each chicken every two weeks for 8 weeks. Sera extracted were tested for haemagglutination inhibition.

(b) Preparation of emulsion

The NDVL and NDVK were recultured in 10 day embryonated eggs (30 in number) and their allantoic fluids harvested as described in 3.2.1. The titre of the viruses and their infectivity were determined as described in 3.2.2. Using the 10^{-3} formalin concentration the virus strains were inactivated at room temperature for 24 hours as described in 3.2.5. Equal volumes of the inactivated NDVL and NDVK were mixed to obtain a total volume of 500 ml of the antigen.

Liquid paraffin and Arlacel A were filter sterilized and their sterility checked as described in 3.2.7 (a). Five hundred mililitres of the sterile liquid paraffin containing 10% steril Arlacel A were first homogenized in a sterile homogenizer for 3-5 minutes after which the mixture was transferred to a sterile 1 litre jar. With a sterile 20ml tuberculin syringe and a long hypodermic needle 15.0ml antigen was withdrawn at a time and injected forcefully into the adjuvant phase. The procedure was continued until the 500 ml antigen was completely injected into the adjuvant phase. The mixture of the antigen and the adjuvant was then homogenized in a sterile automixer until an emulsion was formed. To test for the formation of an emulsion a drop of the mixture was allowed to fall unto cold clean water in a beaker. The emulsion should not spread over the surface of the water but

remains as a discrete drop (Halbert, 1978). The inactivated oil emulsion vaccine (IOEV) was dispensed in sterile bottles in 50ml amount.

3.2.8. Vaccination of chicken with inactivated oil emulsion vaccine

The chickens were first tagged on the wing with tag-numbers for easy identification. Each chicken was bled to extract the pre-vaccination serum. The skin of the thigh and the skin of the fold of the neck are the common sites for subcutaneous vaccination in chickens. For the inactivated oil emulsion vaccine (IOEV) vaccination, the fold of the neck was used. The site was first disinfected with 70% alcohol. With sterile 2ml tuberculin syringe and 26 gauge hypodermic needle the IOEV was withdrawn asceptically. Lifting the skin of the neck-fold with 2 fingers carefully, 0.4ml of the IOEV was introduced gradually under the skin. The site of inoculation of each chicken was examined daily for 2 weeks for any abnormal reactions.

The 3 live ND vaccines as controls were vaccinated according to "notes on vaccines by National Veterinary Research Institute." The NDV intraocular or NDVB1 (200 doses) was reconstituted in 10ml PBS and administered to one day old chickens through the eyes at 0.05ml per chicken using dropper pipette. Three weeks later, the NDVL (200 doses) was reconstituted in 2 litres of PBS and given to the chickens to drink at 10ml per bird. After 8 weeks, the NDVK (200 doses) was reconstituted in 40ml sterile PBS and given to the chickens at 0.2ml per bird intramuscularly.

The tag-number of each chicken and the date of vaccination were recorded.

3.2.9. Titration of antibodies -haemagglutination inhibition test

(a) Serum samples collection

Blood (0.5ml) was removed by wing vein puncture from each chicken every 2 weeks for 16 weeks into bleeding tubes. The blood was allowed to clot at room temperature for 30 minutes after which it was left at 4°C over-night. The samples were spun at 5000g for 5 minutes after which the sera were collected with pasteur pipettes and placed into fresh tubes. The sera were then heat inactivated at 56°C for 30 minutes.

(b) Haaemagglutination test

The positive ND antigen was titrated by HA test as described in 3.2.2. From its titre 4 haemagglutination units (4HAU) of the antigen was calculated.

(c) Haemagglutination inhibition test

The modified form of the beta microtest technique of haemagglutination inhibition (HI) (Beard and Wikes, 1978) was carried out as follows:

1. With a dropper pipette 0.025ml of PBS was placed into each well of a microtitre plate.
2. To the first well of each row of the plate, 0.025ml of each serum was placed.
3. Dilutions were made with a microdiluter

down the row to obtain dilutions ranging from 1:2 to 1:8192.

4. Each serum dilution was given a tube number beginning with 1:2 dilution. Thus 1:2 dilution was well number 1, 1:4 tube number 2 up to 1:8192 with well number 13.
5. Separate rows for positive and negtive serum controls were set up.
6. To each well 0.025 ml of the 4HAU antigen was added and the test was left at room temperature for 20 minutes for antigen-antibody reaction to take place.
7. To each well 1% washed RBC was then added and the plate left for 45 minutes at room temperature. The Hi titre of the serum sample was the reciprocal of the highest well dilution of the serum in which there was complete haemagglutination inhibition.

(d) Antibody geometric mean titre

The results of the HI titres were expressed in geometric mean titres (GMT). Expression of serological results in arithmatic terms (e.g. reciprocal of end points like 32, 64, 128 etc.) can be deceptive and do not follow linear scale especially when large number of sera are tested (Brugh, Jr, 1978). The antibody GMT was determined using the conversion table of base-two logarithmic mean titres to geometric mean titres (Brugh,

Jr, 1978). To obtain the GMT, the HI titres were first converted to mean titres (MT). The MT was then located in the conversion table under the 1:20 column. The final value was then divided by 10 to obtain the GMT of base-two logarithmic value.

3.2.10(a) Safety and Stability t st

One day old susceptible chickens (20 in number) and 23 weeks old susceptible chickens were vaccinated with IOEV as described in 3.2.8 but with increased dose of 0.5ml per bird. the birds were kept under strick observation for 14 days for disease symptoms, swellings or any abnormal lesions at the site of inoculation. For stability test, samples of the vaccine were kept at 21-25^{o}C, 4^{o}C and 37^{o}C, and observed for one year for emulsion stability.

3.2.10(b) Potency testing

The experiment was divided into 8 schedules.

Schedule 1: Twenty-one day old chickens were vaccinated with the 3 current live vaccines as described in 3.2.8.

Schedule 2: Twenty, one day old susceptible chickens were first given live NDVB1 intraocularly. Three weeks later, they were given the IOEV at 0.4 ml as described in 3.2.8.

Schedule 3: was similar to Schedule 2 but instead of NDVB1 they were given NDVL at 12 days old (to avoid high mortality of day-1 with NDVL).

The birds were then boosted with IOEV at 3 weeks post vaccination with NDVL.

Schedule 4: Twenty, one day old chickens were vaccinated with IOEV at 0.4ml per bird without further boosting.

Schedule 5: was similar to Schedule 4 except that there was boosting with IOEV after 3 weeks.

Schedule 6: was also similar to Schedule 4 except that the boosting with IOEV was done after 6 weeks. Schedule 7 was similar to Schedule 4 except that the boosting was done with live NDVK after 6 weeks.

Schedule 8: was susceptible unvaccinated control birds.

All birds were bled before inoculation, after which they were bled bi-weekly. the antibody titres were monitored by the haemagglutination-inhibition (HI) test for at least 16 weeks as described in 3.2.9.

Challenge trials

The vaccinated chickens of 3.2.10(b) were divided into 5 groups containing 4 birds from each Schedule. Each group was then challenged with 10^5 EID_{50}/0.1ml of challenge virus as shown below.

Herts 33/50	group I
NDVP	group II
UI104	group III
K1090	group IV
OJP	group V.

All inoculations were done intramuscularly. Controls consisted of four susceptible, 16 weeks old birds per unit inoculated with each of the 5 strains at $10^5 EID_{50}$ per 0.1ml. All birds were observed for 4 weeks for disease symptoms.

3.2.11. Vaccination of larger number of chickens using Schedules2 reqimen

(a) Antibody response

A fresh batch of inactivated oil emulsion vaccine (IOEV) was prepared as described in 3.2.7. Its purity was ascertained after culturing in BA, MCA and CMM for 48 hours at 37°C. Samples of the vaccine were also observed at different temperatures, 37°C, 21-25°C, and 4°C as described in 3.2.10(a) for stability of the emulsion. Two hundred chickens were vaccinated with IOEV as shown below:

(i) All chickens were vaccinated first with live NDVB1 vaccine as described in 3.2.8.

(ii) The birds were bled before the inoculation and after 3 weeks for HI test.

(iii) After 3 weeks 100 of these birds were given IOEV subcutaneously at 0.4ml per bird while the other 100 were given IOEV subcutaneously at 0.2ml per bird.

Another set of 100 birds serving as controls were vaccinated with the 3 live vaccines as described in 3.2.8. Twenty chickens were left unvaccinated as negative controls.

All the chickens were reared as in 3.2.6. They were bled every two weeks for 22 weeks and the sera collected were tested for HI antibody.

(b) Challenge trials

The 5 velogenic viruses Herts 33/50, NDVP, UI104, K1090 and OJP used in 3.2.10(c) were used as challenge strains. Birds vaccinated with 0.4ml IOEV were divided into 5 groups so that each group received Herts 33/50, K1090, UI104, NDVP and OJP respectively. Similarly, birds vaccinated with 0.2ml IOEV and those vaccinated with live vaccines were each divided into 5 groups so that each group received the Herts 33/50, K1090, UI104, NDVP and OJP respectively.

The protocol for the challenge trials is as shown on table 3.3.

Table 3.3: Challenge trials of Schedule 2 regimen.

	Group Number	Number of test birds in the group	Number of unvaccinated birds in the group	Challenge virus strain used
IOEV (.4m) birds	I	20	5	Herts 33/50
	II	20	5	K1090
	III	20	5	UI104
	IV	20	5	NDVP
	V	20	5	OJP
IOEV (.2ml) birds	I	20	5	Herts 33/50
	II	20	5	K1090
	III	20	5	UI104
	IV	20	5	NDVP
	V	20	5	OJP
Normal live vaccine birds	I	20	5	Herts 33/50
	II	20	5	K1090
	III	20	5	UI104
	IV	20	5	KDVP
	V	20	5	OJP

3.2.12. Maternal antibody experiment

The hens of 3.2.11 started laying as from 18-20 weeks. At 20 weeks and just befort they were challenged, 50 eggs were collected from the hens vaccinated with IOEV and 30 eggs from the hens vaccinated with live vaccines. The eggs were marked differently for easy identification. They were then incubated at 37°C for 21-22 days for hatching. Twenty-five live chickens were obtained from the first 50 eggs and 16 from the 30 eggs. The two sets of chickens were bled after 2 weeks and their sera tested by HI test to find the level of maternal HI antibodies present in them.

3.2.13. Substitution of Arlacel A with 'Egusi' oil

Most of the raw materials for the preparation of inactivated oil emulsion vaccine (IOEV) can be obtained locally. Arlacel A (Manide mono-oleate) had to be imported and is expensive. Egusi or melon seed (Citrullus lunatus) is a common Nigerian household seed. It contains higher linoleic compounds (34.1%) than Groundnut oil (19.7%) or palm oil (8.8%). It also has high saponification value (Oyenuga, 1968). An attempt was therefore made to use 'Egusi' oil as an emulsifier instead of Arlacel A.

(a) Laboratory preparation of Egusi oil

Five hundred grams of Egusi seed was washed in tap water, rinsed in deionized water and then dried at 37°C. The 500g seed was ground to fine paste in a blender. The paste was transferred

into a clean mortar and pestle and 2 table spoonful of deionized water was added. The paste was then pounded for 10 minutes. The paste and the mortar was incubated at 37°C for 40 minutes for gradual release of the oil after which the oil was collected into a universal bottle. the process of pounding and incubation of paste was continued until most of the oil was released. About 300 ml 'Egusi' oil collected from 500g seed was centrifuged at 5000g for 20 minutes to pellet any coarse particles present. It was then filter sterilised and its sterility ascertained by culturing on BA, MCA and CMM as described in 3.2.4.

(b) Preparation of inactivated oil emulsion vaccine using 'Egusi' oil emulsifier

Virus culture and inactivation was done as described in 3.2.4 and 3.2.5 respectively. Two batches of inactivated oil emulsion vaccine (IOEV) were prepared as described in 3.2.7 except the following:

(i) The adjuvant of the first batch contained 10% 'Egusi' oil emulsifier.

(ii) The adjuvant of the second batch contained 10% Arlacel A emulsifier.

The two batches were kept at 37°C, 21-25°C, and 4°C to test for stability of the emulsion.

(c) Potency testing of 'Egusi' oil IOEV

Seven hundred, one day old chickens were bled and the pre-vaccination sera checked for HI antibody. The chickens were then vaccinated with NDVB1

intraocularly as described in 3.2.8. After 3 weeks the chickens were divided into two groups of 350 chickens each. One group was vaccinated with IOEV containing 'Egusi' oil emulsifier subcutaneously at 0.4ml per chickens. The second group was vaccinated with IOEV containing Arlacel A emulsifier at 0.4ml per chicken. Each chicken was bled every forthnightly for 16 weeks and the sera titrated by HI.

The birds were challenged after 16 weeks with the 5 velogenic viruses; each bird received 10^5 EID_{50} per 0.1ml as shown on table 3.4. All birds were observed for one week for symptoms of Newcastle disease. A postmortem was performed on all dead chickens.

Table 3.4: Challenge trials with IOEV containing 'Egusi' oil emulsifier.

Challenge viruses used		Number of [1]Egusi oil-IOEV vaccinates	Number of [2]Arlacel A-IOEV vaccinates	Number of vaccinated controls
Herts 33/50	I	30	30	10
K1090	II	30	30	10
UI 104	III	30	30	10
NDVP	IV	30	30	10
OJP	V	30	30	10

[1]Egusi oil-IOEV = Inactivated oil emulsion vaccine containing egusi oil emulsifier.

[2]Arlacel A-IOEV = Inactivated oil emulsion vaccine containing Arlacel A emulsifier.

3.2.14. Field trials with inactivated oil emulsion vaccine

Fresh, sterile batch of IOEV (batch 3) was prepared as described in section 3.3. Field trials were carried out on 5 small scale poultry farmers in vom area to test the efficacy of the vaccine. The layout of the field trials is as shown in the table 3.5. All birds were vaccinated with the IOEV subcutaneously at 0.4ml dose per bird. All birds except the broilers were observed for 5 months. The broilers were observed only for 5 weeks.

Wapmuk and Imogie birds were bled before IOEV vaccination, 3 months and 5 months post IOEV vaccination. Chakven broiler birds were bled before IOEV vaccination and 5 weeks post IOEV vaccination. Gbodi and Musa birds were bled before IOEV vaccination and 5 months post vaccination.

There were no challenge inoculations in the field trials.

Table 3.5: Protocol of field trial.

Name of Owner	Type and age of birds	Number of birds used	Previous ND vaccination History	Purpose of applying IOEV
Wapmuk	Layers 4 weeks	250	Received live B1 at 1-day old	To examine immunogenicity of IOEV before and during laying
Imogie	Layers 4 weeks	70	-do-	-do-
Chakven	Broilers 3 weeks	50	Received live B1 at 1-day old	To examined immunogenicity of IOEV in broiler chicks
Gbodi	Layers, over 20 weeks	250	Received B1 at 1-day old and Lasota before laying	To examine the effect of IOEV in laying birds
Musa	Layers, over 20 weeks	50	-do-	-do-

CHAPTER 4

RESULTS

4.1. Cultivation and titration of viruses

Of the eight virus isolates cultured in embryonated chicken eggs, the lentogenic strains had the lowest embryo mortality while the velogenic strains had the highest. The Newcastle disease virus B1 (NDVB1) and the Newcastle disease Lasota (NDVL) produced 0% and 8% embryo mortality respectively, the Newcastle disease virus Komarov (NDVK), 16% and all the velogenic strains, 100% embryo mortality at the end of their incubation periods (table 4.1).

The results of the haemagglutination (HA) titres of the seven viruses were shown in table 4.1. The NDVL had the highest HA titre of 1280 followed by NDVK and NDVB1 with 640 and 320 respectively. All the velogenic strains had HA titres less than 320.

The UI 104 gave the highest EID_{50} (10^{18} per 0.1ml) while the NDVL and the NDVK gave $10^{16.5}$ per 0.1ml and $10^{16.1}$ per 0.1ml respectively. The OJP had comparatively the least EID_{50} of $10^{7.0}$ per 0.1ml (table 4.1).

4.1.2. Antigen stability

The antigens (haemagglutinin) of the viruses exposed to $4^{o}C$ and room temperature (Section 3.2.3) showed that NDVL, NDVK and NDVB1 were stable at $4^{o}C$ temperature, while at room temperature their HA titres

Table 4.1: Haemagglutination and embryo infective dose fifty percent titres of vaccine and wild strains of Newcastle disease virus.

Virus strains	Incubation period in hours	embryo mortality (%)	Haemagglutination titre	EID_{50} per 0.1ml	Pathogenicity
NDVK	48	16	640	$10^{16.1}$	Mesogenic
NDVL	72	8	1280	$10^{16.5}$	Lentogenic
NDV B1	96	0	320	$10^{15.3}$	Lentogenic
NDVP	72	100	160	$10^{13.0}$	Velogenic
UI 104	48	100	160	$10^{18.0}$	Velogenic
K1090	48	100	80	$10^{14.0}$	Velogenic
OJP	48	100	80	$10^{7.0}$	Velogenic
Hwera 33/50	48	100	160	$10^{12.8}$	Velogenic

dropped only one $\log_2$. The velogenic strains (NDVP, UI 104, K1090, OJP) on the other hand were very unstable particularly at room temperature. Their HA titres dropped significantly after the room temperature (table 4.2).

4.2. Sterility of the vaccine candidate viruses

Allantoic fluids containing NDVL and NDVK, cultured onto Blood agar (BA), McConkey agar (MCA) and cooked meat medium (CMM) showed no growth at the end of 5 days incubation at $37^{o}C$.

The allantoic fluids which were also treated with specific anti ND serum before inoculating into fresh embryonated eggs and chick fibroblast monolayer cells showed no virus growth in the eggs and in the cells. The allantoic fluids were also free of mycoplasma.

4.2.1. Optimal concentration of formalin for inactivation of viruses

The effects of various concentrations of formalin on the haemagglutinin (antigen) and inactivation of the vaccine candidate viruses are shown on table 4.3. Formalin concentrations of 10^{-1} and 10^{-2} had a detrimental effect on the haemagglutinin of the viruses (reduced HA titre) while concentrations of 10^{-3} and 10^{-4} had no effect on the haemagglutinin of the viruses (no reduced HA titres). Similarly, concentrations of 10^{-1} to 10^{-3} inhibited virus growth (complete inactiva-

Table 4:2: The haemagglutinin stability of the viruses at 4°C and 22 - 25°C for 14 days.

Virus	[1]HA titres before stability test	HA titre at 4°C after 14 days	HA titre at room temperature after 14 days
NDVK	640	640	320
NDVL	1280	1280	640
NDV B1	320	320	160
NDVP	160	80	20
UI 104	160	80	20
K1090	80	80	20
OJP	80	40	20
Herts 33/50	160	80	40

Table 4.3: The effect of formalin concentration on haemagglutinin and growth of NDVL and NDVK

Formalin concentration	[1]HA titre of [2]NDVL before formalin treatment	HA titre of NDVL after formalin treatment	Growth of NDVL in eggs after formalin treatment	HA titre of [3]NDVK before formalin treatment	HA titre of NDVK after formalin treatment	Growth of NDVK after formalin treatment
10^{-1}	1280	320	-Ve[4]	640	80	-Ve
10^{-2}	1280	640	-Ve	640	160	-Ve
10^{-3}	1280	1280	-Ve	640	640	-Ve
10^{-4}	1280	1280	+Ve[5]	640	640	+Ve
Untreated Allantoic fluid control	1280	1280	+Ve	640	640	+Ve

[1]HA = Haemagglutination
[2]NDVL = Newcastle disease virus Lasota
[3]NDVK = Newcastle disease virus Komarov
-Ve[4] = No virus growth
+Ve[5] = Virus growth

tion) in eggs while 10^{-4} concentration did not.

When the above concentrations were inoculated into one day old chickens subcutaneously dilutions of 10^{-1} and 10^{-2} were toxic while 10^{-3} and 10^{-4} were on the other hand non-toxic (table 4.4).

4.3. Selection of oil adjuvant for the preparation of inactivated oil emulsion vaccine

The haemagglutination inhibition titres expressed as geometric mean titres (GMT) of the 3 oil adjuvants are shown on figure 1. Each of the oil adjuvants induced a gradual rise in antibody levels but liquid paraffin induced the highest antibody level of GMT, 194. Groundnut oil was next with GMT of 128, and palm oil was least with a GMT of 97.

Comparing the stability of their emulsions at $4^{o}C$ for 8 weeks, the emulsion of the liquid paraffin was more stable than those of the other two oils. The emulsion of palm oil separated within 48 hours, that of groundnut oil separated within a week while that of liquid paraffin never separated even at the end of one year.

4.4. Reculturing of the virus strains for preparation of emulsion

The embryo infective dose fifty (EID_{50}) of the two strains after reculturing in embryonated eggs were:

Table 4:4: Toxicity of formalin concentrations in one day old chickens.

Formalin concentration	Number of birds inoculated per concentration	Number of birds showing ^{2}toxic effect	Percentage mortality
10^{-1}	10	3	30
10^{-2}	10	1	10
10^{-3}	10	NT[1]	NT
10^{-4}	10	NT	NT
Uninoculated control birds	10	NT	NT

NT[1] = No toxic effect.

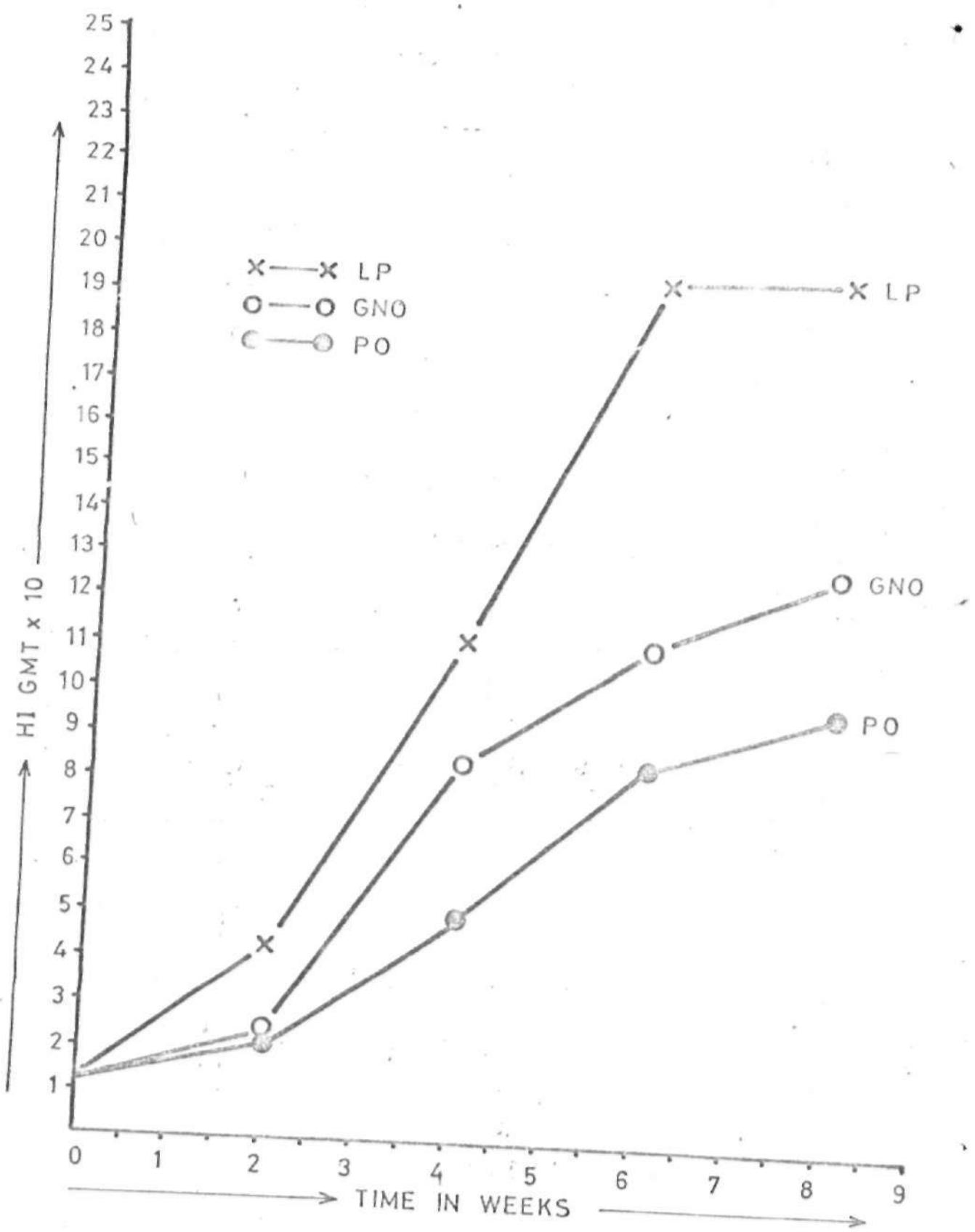
^{2}toxic effect = Sick or dead bird.

Fig. 1: The comparison of the antibody GMT of the 3 oil adjuvants containing Lasota antigen.

LP = Liquid paraffin adjuvant

GNO = Groundnut oil adjuvant

PO = Palm oil adjuvant.


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TIME IN WEEKS

(i) NDVL = $10^{16.47}$ per 0.1ml.

(ii) NDVK = $10^{16.21}$ per 0.1ml.

This compared very favourably with their previous EID_{50} of $10^{16.5}$ and $10^{16.1}$ per 0.1ml respectively.

Their HA titres also did not change after reculturing in embryonated eggs. They were:

(i) NDVL = 1280.

(ii) NDVK = 320.

4.4.1. Viability of the antigen after after formalin inactivation

After treatment of the viruses with 1:1000 concentration of formalin for 24 hours at room temperature, they did not grow in embryonated egg. Their HA titres also did not drop (NDVL = 1280, KDVK = 640) after the formalin treatment.

4.5. Safety and stability of the vaccine

The two sets of birds (1-day and 3 weeks old) vaccinated with the IOEV showed no disease symptoms during the 14 days observation. Particular attention was paid to the site at which each bird was inoculated. There was neither swelling nor any abnormal lesions at the site of inoculation during the 14 days of observation.

The results of the temperature stability test is shown on table 4.5. The IOEV was unstable after three months at 37°C. It also lost almost all its HA titre at the end of one year at this temperature.

Table 4.5: The effect of temperature on the stability of [4]IOEV

Temperature	[5]HA titre before exposure	Months 1	2	3	4	5	6	7	8	9	10	11	12	HA titre after exposure	Remarks
37°C	640	+	+	+	+	±[1]	-	-	-	-	-	-	-	10	Unstable
21-25°C	640	+[3]	+	+	+	+	+	+	+	+	+	±[2]	±	320	Stable
4°C	640	+	+	+	+	+	+	+	+	+	+	+	+	320	Stable

[1] ± = Complete separation of the emulsion

[2] ± = Partial separation of the emulsion

[3] + = No separation of the emulsion

[4] IOEV = Inactivated oil emulsion vaccine

[5] HA = Haemagglutination

At room temperature and at $4^{o}C$, the emulsion was quite stable and the HA titre dropped only one dilution step.

4.6. Potency of the Vaccine

Antibody geometric mean titres (GMT) in chickens vaccinated with IOEV are shown in fig. 2. Its Statistical analysis (table 4.6) showed that Schedules 2,3 and 6 induced significantly higher antibody response compared to Schedule 1. Of the three, Schedule 2 (Primed with NDVB1 at one day old than IOEV at 3 weeks) induced the best antibody response when compared to Schedule 1 (Standard live vaccine).

Mortality of birds during the 16 weeks of serum monitoring are shown on table 4.7. There was no death in Schedule 1,2,3 and 8. Two birds died in unit 4 leaving 18, while in Schedule 5 and 6 four birds each died leaving 16 birds. Schedule 7, three birds died leaving 17 birds. All the deaths which took place within 24 hours after each vaccination were therefore regarded as non-specific deaths due to stress.

4.6.1. Challenge with wild viruses
16 weeks after vaccination

All the susceptible controls of unit 8 died within 4-6 days, inducing no protection against the 5 virulent strains. They showed typical Newcastle disease (ND) symptoms like respiratory, gastrointestinal and central nervous system involvements before their death. All deaths among vaccinated chickens showed only mild

Fig. 2: The comparison of the antibody GMT induced by the different schedules of IOEV.

Schedule 1 = The 3 live vaccines.

Schedule 2 = Primed with NDVB1 at one day old than IOEV at 3 weeks old.

Schedule 3 = Primed with NDVL at 12 days old then IOEV at 3 weeks old.

Schedule 4 = IOEV given to one day old chicken. No further boosting.

Schedule 5 = IOEV given to one day old chicken, boosted at 3 weeks with more IOEV.

Schedule 6 = IOEV given to one day old chicken, boosted with more IOEV at 6 weeks.

Schedule 7 = IOEV given to one day old chicken, boosted with live NDVK at 6 weeks.

Schedule 8 = Susceptible unvaccinated chickens

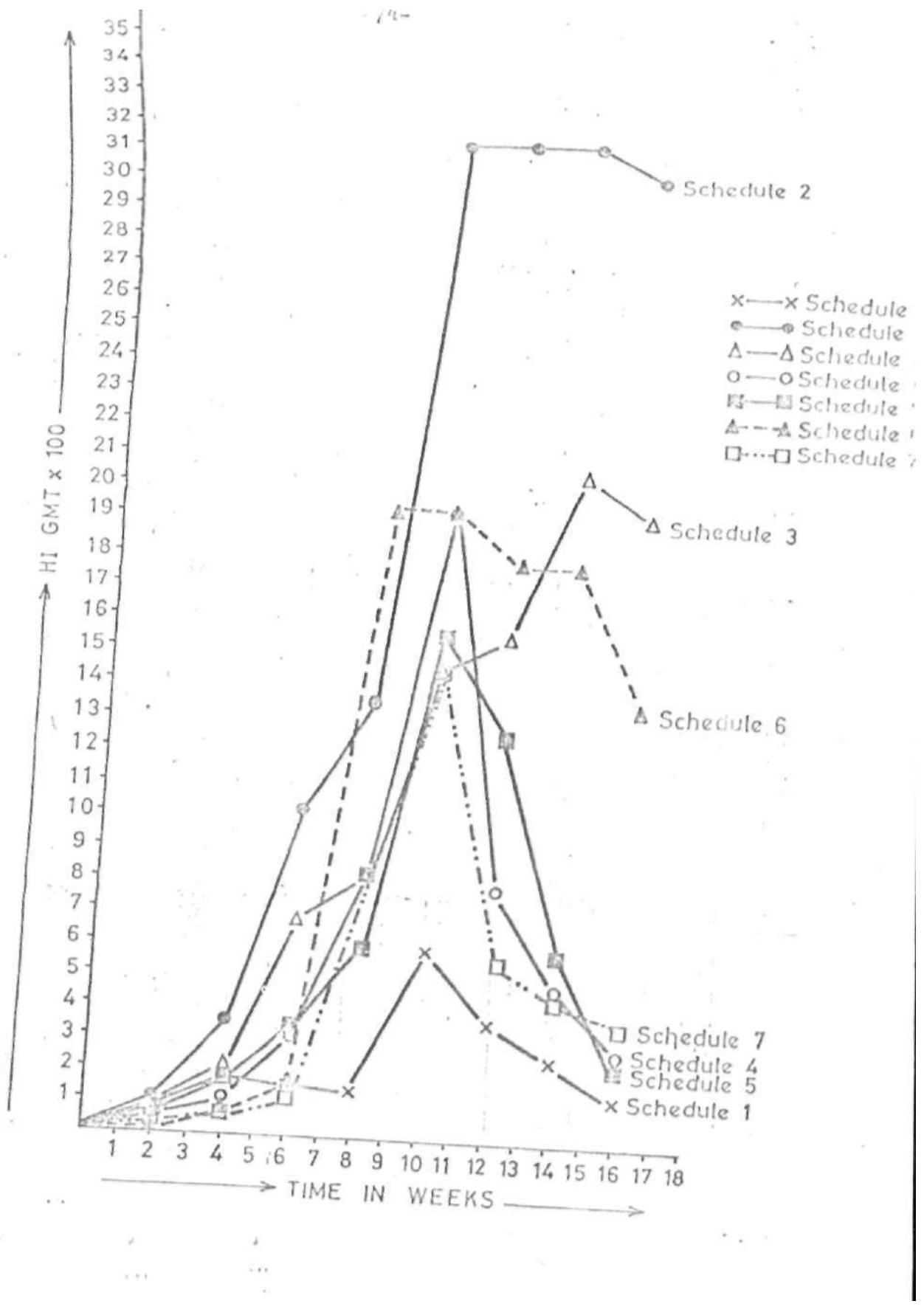

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TIME IN WEEKS

Table 4.6: Statistical analysis of antibody response by different schedules of IOEV compared to the live vaccines

Student's T test (two-tailed)					
Schedule	Mean	T	P	dt	Statistical significance with Schedule 1
1	214.222	Standard	Standard	16	-
2	1674.011	3.163681	6.019891E-03	16	Significantly different
3	976.500	2.833201	1.199025E-02	16	Significantly different
4	532.933	1.547232	.413567	16	Not significantly different
5	530.766	1.669655	.1144325	16	Not significantly different
6	1002.789	2.58427	1.996827E-02	16	Significantly different
7	437.633	1.322098	.2047262	16	Not significantly different

T = T test value
P = Probability
df = Number of observations

Model of computer = Olwetti personnel Computer M24 with Programme epistat.

Table 4.7: Mortality of chickens vaccinated with different schedule of [3]IOEV.

[2] Schedule	No of birds per unit	Week																Live/dead ratio	% Live	Percentage mortality
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th			
1	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20/20	100	0
2	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20/20	100	0
3	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20/20	100	0
4	20	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18/20	90	10
5	20	18	18	18	16	16	16	16	16	16	16	16	16	16	16	16	16	16/20	80	20
6	20	19	19	18	18	18	17	17	16	16	16	16	16	16	16	16	16	16/20	80	20
7	20	19	19	19	19	19	19	18	17	17	17	17	17	17	17	17	17	17/20	85	15
8	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20/20	100	10

[1] IOEV = Inactivated oil emulsion vaccine

Schedule 1 = The 3 live vaccines

Schedule 2 = Primed with NDVB1 at one day old then vaccinated with IOEV at 3 weeks old

Schedule 3 = Primed with NDVL at 12 days old then vaccinated at 3 weeks with IOEV

Schedule 4 = IOEV given at one day old. No further boosting

Schedule 5 = IOEV given to one day old chickens, boosted at 3 weeks with more IOEV

Schedule 6 = IOEV given to one day old chickens, boosted with more IOEV at 6 weeks

Schedule 7 = IOEV given to one day old chickens, boosted with live NDVK at 6 weeks

Schedule 8 = Susceptible unvaccinated chickens.

symptoms. Post-mortem results indicated tht control birds showed haemorrhagic lesions of the intestine and the nervous system typical of ND.

The protection induced by each Schedule against the 5 virulent strains are shown on table 4.8. Schedules 2,3 and 6 induced 100%, 95%, and 94% respectively. Although Schedule 4 did not induce significantly higher antibody response, its protective immunity was very higher. This could be due to cell mediated immunity.

4.7. Vaccination of larger number of birds with IOEV (batch 2) using Schedule 2 regimen

The stability of IOEV batch 2 at 37°C, room temperature and 4°C was similar to batch 1 (table 4.5). The haemagglutination-inhibition geometric mean titre (GMT) induced by IOEV (0.4ml and 0.2ml) and the 3 live vaccines over a period of 22 weeks are shown on fig. 3. The IOEV 0.4ml and 0.2ml gave significantly higher antibody response than the live vaccines (table 4.9). However, IOEV 0.4ml gave significantly higher antibody response than IOEV 0.2ml (table 4.9).

4.7.1. Challenge trial with wild viruses

The results of the challenge with wild viruses after IOEV and live vaccine vaccination are shown on table 4.10. The protection induced by IOEV (0.4ml dose) against the 5 virulent strains was 96%, the IOEV (0.2ml dose) was 89% and the 3 live vaccines was 78%.

Table 4.8: The percentage protection induced by different schedule of [1]IOEV after 5 challenge with wild viruses.

Challenge viruses used		Alive/dead							
		2 Units of IOEV							
		1	2	3	4	5	6	7	8
Herts 33/50	1	3:1	4:0	3:1	4:0	3:0	3:0	3:1	0: 4
NDVP	II	3:1	4:0	3:1	4:0	3:0	3:0	3:0	0: 4
UI 104	III	4:0	3:1	4:0	3:0	2:1	3:0	2:1	0: 4
K1090	IV	3:1	4:0	4:0	3:0	4:0	3:0	3:0	0: 4
OJP	V	2:2	4:0	4:0	4:0	3:0	3:1	4:0	0: 4
Alive/Dead ratio		15:5	19:1	18:2	18:0	15:1	15:1	15:2	0:20
Percentage protection		75	95	90	100	94	94	88	0

[1]IOEV = Inactivated oil emulsion vaccine
Schedule 1 = 3 live vaccine
Schedule 2 = Primed with NDVB1 at one day old then vaccinated withIOEV at 3 weeks old
Schedule 3 = Prince with NDVL at 12 days old then vaccinated at 3 weeks with IOEV
Schedule 4 = IOEV given at one day old. No further boosting
Schedule 5 = IOEV given to one day old chickens boosted with more IOEV at 3 weeks
Schedule 6 = IOEV given to one day old chickens boosted with more IOEV at 6 weeks
Schedule 7 = IOEV given to one day old chickens boosted with live NDVK at 6 weeks
Schedule 8 = Susceptible unvaccinated chickens.

Fig. 3: The comparison of the HI antibody of IOEV at different doses with those of the 3 live vaccines.

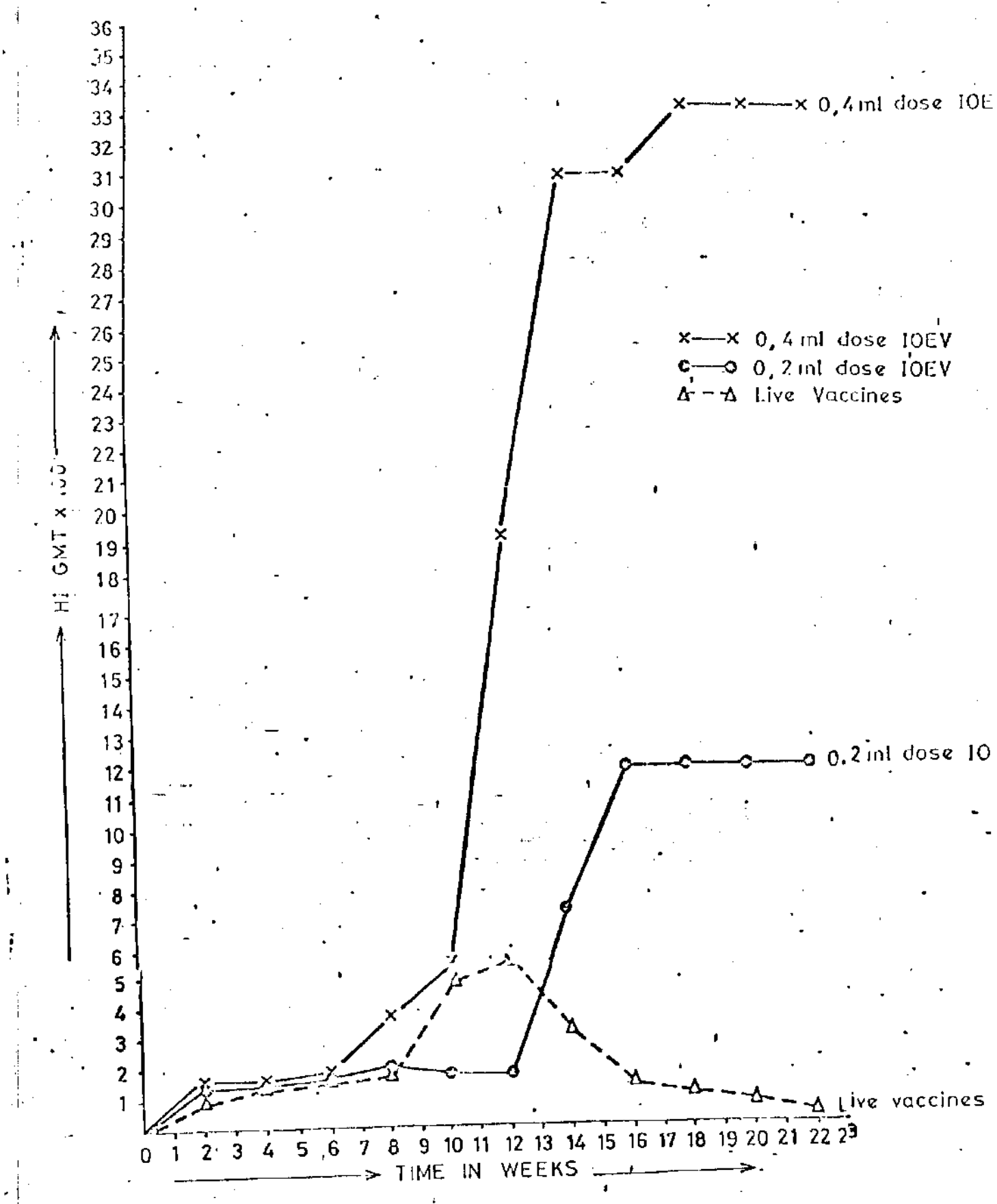

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Live Vaccines
0.2 ml dose IO
Live vaccines

Table 4.9: Statistical analysis of the antibody response by different doses of IOEV

		Student's T test (two tailed)				
	Vaccine dose	Mean	T	P	df	Statistical significance
I	IOEV 0.4ml	1626.267	3.283102	3.395438E-03	22	Significantly different
	IOEV 0.2ml	534.416	2.238989	3.559423E-02	22	Significantly different from Standard
	Live vaccine	191.383	Standard	Standard	22	-
II	IOEV 0.4ml	1626.270)	8.9187	5.34093E-04	22	Significantly different
	IOEV 0.2ml	534.416)				from each other

IOEV = Inactivated oil emulsion vaccine
T = T test Value
P = Probability
df = Number of observations

I = Comparison of IOEV 0.4 and 0.2ml with live vaccine

II = Comparison of IOEV 0.4ml and 0.2ml with each other

Computer = Owetti personel computer M24 with programme epistat.

Table 4.10: The percent protection induced by 2 different doses of [1]IOEV

Challenge viruses	No of birds	IOEV (0.4ml) survivor/ death ratio	IOEV (0.2ml) survivor/ death ratio	Live vaccine (usual dose) survivor/ death ratio	Susceptible controls survivor/ death ratio
Herts 33/50 I	20	19:1	18:2	16:4	0:15
NDVP II	20	19:1	18:2	16:4	0:15
UI 104 III	20	19:1	19:1	15:5	0:15
K1090 IV	20	20:0	16:4	15:5	0:15
OJP V	20	19:1	18:2	16:4	0:15
Percentage protection		96	89	78	0

[1]IOEV = Inactivated oil emulsion vaccine.

4.8. Maternal antibody experiment results

The comparison of the maternal antibody level of the progeny of the IOEV vaccinated parents, progeny of live vaccinated parents and NDVB1 primed chickens at 2 weeks Schedule 2) are shown on table 4:11. The GMT of the progeny of IOEV vaccinated parents was 147.0 and that of the progeny of the live vaccinated parents was 29.9. The GMT of the NDVB1 primed chickens at 2 weeks (the last titre before the administration of IOEV at 3 weeks) was 111.4.

4.9. Stability of inactivated oil emulsion vaccine containing 'Egusi' oil emulsifier

The results of the stability of the emulsion containing Egusi oil emulsifier at different temperatures are shown on table 4:12. The IOEV containing 'Egusi' oil emulsifier was more stable at 37^{o}C than that of Arlacel A. It separated after 5 months while that of Arlacel A separated at 4 months. Both emulsions did not separate at room temperature and at 4^{o}C even after 8 months.

4.10. Potency of inactivated oil emulsion vaccine containing 'Egusi' oil emulsifier

The results of the serological response and statistical analysis 16 weeks post vaccination are shown on fig. 4 and table 4:13 respectively. The IOEV containing Arlacel A and IOEV containing Egusi oil emulcifier induce significantly higher antibody response than the live vaccines containing no emulcifier (table

Table 4.11: Maternal antibodies of progeny of IOEV vaccinated and live vaccines vaccinated parents.

Chickens	Number of birds	Antibody level (GMT)
Two weeks old progeny of IOEV vaccinated parents	30	147.0
Two weeks old progeny of live vaccinated parents	16	29.9
Two weeks old NDVE1 - primed chickens before IOEV administration at 3 weeks (from table 6, unit 2)	20	111.4

Table 4.12: Effect of temperature on the stability of IOEV containing 'Egusi' oil emulsifier

Temperature	[4]HA titre before exposure	Time in months								HA titre after exposure	Emulsifier used in [5]IOEV
		1	2	3	4	5	6	7	8		
	640	1+	+	[2]±	3-	-	-	-	-	10	Arlacel A
37°C	640	+	+	+	+	-	-	-	-	10	'Egusi' oil
Room temperature	640	+	+	+	+	+	+	+	+	320	Arlacel A
	640	+	+	+	+	+	+	+	+	320	'Egusi' oil
4°C	640	+	+	+	+	+	+	+	+	320	Arlacel A
	640	+	+	+	+	+	+	+	+	320	'Egusi' oil

[1]+ = No separation of emulsion
[2]+ = Partial separation of emulsion
3- = Complete separation of emulsion
[4]HA = Haemagglutination
[5]IOEV = Inactivated oil emulsion vaccine

Fig. 4: Antibody response to IOEV containing 'Egusi' oil emulsifier.

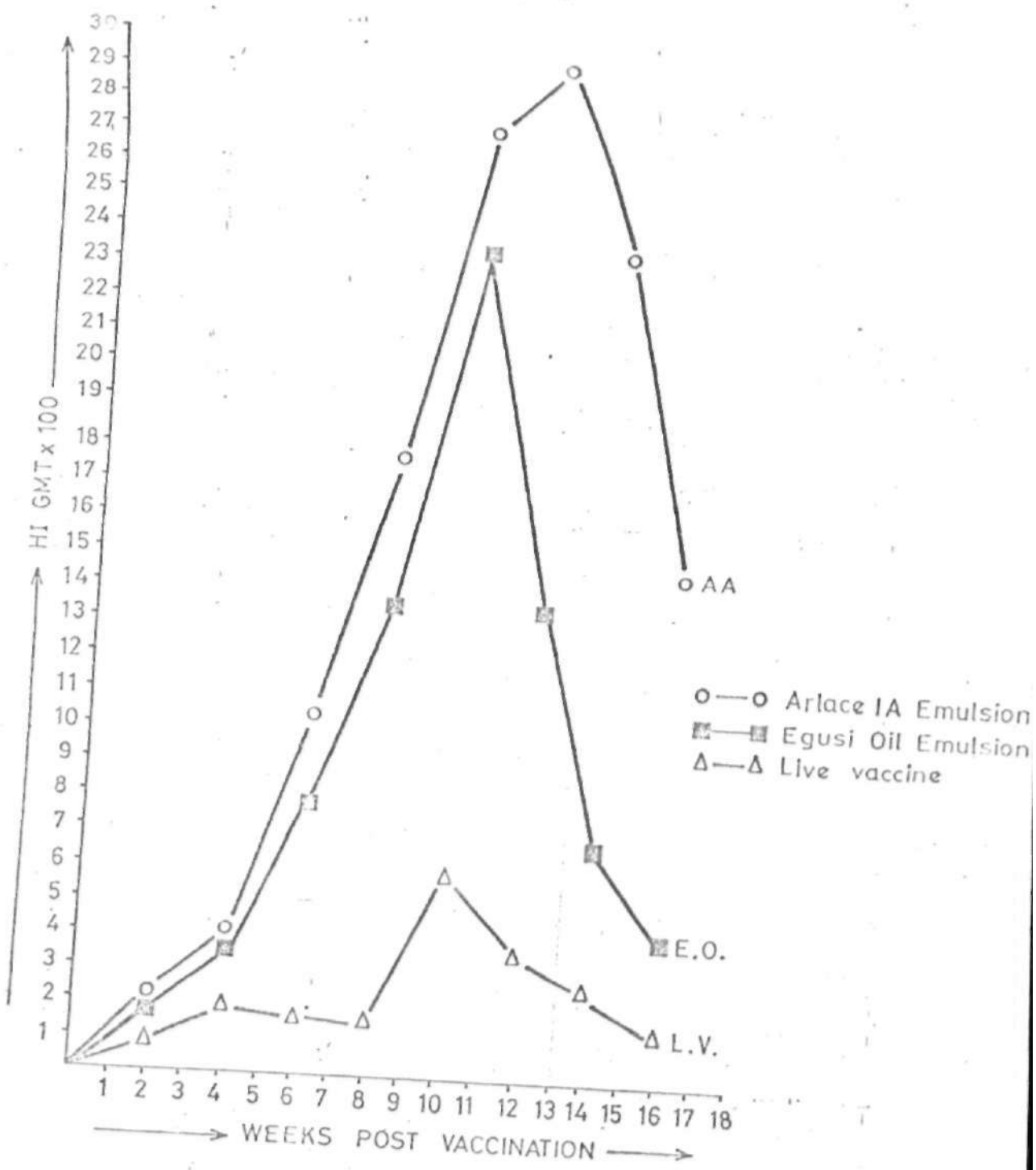

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▪—▪ Egusi Oil Emulsion
Δ—Δ Live vaccine
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WEEKS POST VACCINATION

Table 4.13: Statistical analysis of antibody response to IOEV containing 'Egusi' oil emulcifier

	Student's T test (two-taited)					
	Emulcifier	Mean	T	P	df	Statistical difference
I	A A Emulcifier	1443.111	3.351395	4.055262E-03	16	Significantly different from Standard
	EO Emulcifier	860.888	2.525707	2.247942E-02	16	Significantly different from standard
	No Emulcifier Livewvaccines	214.222	Standard	Standard	16	-
II	AA Emulcifier	1443.111	1.323715	.2042002	16	Not significantly different from each other
	EO Emulcifier	860.888				

AA = Arlacel A
EO = "Egusi" oil
T = T test value
P = Probability
sf = Number of observations

I = Comparison of IOEV containing AA Emulcifier, and EO emulcifier with Live vaccines
II = Comparison of IOEV containing AA and EO emulcifiers with each other
= Olwetti personel computer M24 with Computer programme epistat.

titre). However the antibody titre induced by IOEV containing Arlacel A is not significantly higher than IOEV containing Egusi oil (table 4.13II). The protection induced by IOEV containing both emulcifiers against the 5 virulent challenge viruses was similar (table 4.14).

4.11. Field trials with [illegible] oil emulsion vaccine

The results of field trials with the inactivated oil emulsion vaccine (IOEV) [illegible] on table 4.15.

The first category (Mapmoor/Imegie) which examined the immunogenic and side effects of IOEV on layers before and at the beginning of the laying period showed an increase in haemagglutination inhibition geometric mean titre (GMT) from 111.4 to 2828.2 before laying and to 3104.2 at the beginning of laying period. There was no adverse reaction of IOEV on the birds after administration. There was no increase in mortality (ND) reported in the flock 10 weeks after vaccination.

The second category (Obudifia) which examined the side effects of IOEV (especially the rate of egg production) in birds already laying showed no symptoms of stress or reduction in the rate of egg production. The antibody titre in the birds also rose from 512.0 to 2048.0 before the IOEV administration. There was no reported case of mortality in the flock before their disposal a month later.

Table 4.14: Challenge trials after vaccinating with IOEV containing 'Egusi' oil emulsifier 16 weeks after vaccination.

Challenge strain		[1]Egusi-IOEV Live/death ratio	[2]Arlacel A-IOEV Live/death ratio	Unvaccinated control Live/death ratio
Herts 33/50	I	30:0	29:1	0:10
K1090	II	29.1	30:1	0:10
UI 104	III	30:1	30:0	0:10
NDVP	IV	29:1	30:0	0:10
OJP	V	30:0	30:0	0:10
Total live/ death ratio		148:2	149:1	0:50
Percentage protection		98.7	99.3	0

[1]Egusi-IOEV = Inactivate oil emulsion vaccine containing 'Egusi' oil emulsifier.

[2]Arlacel A-IOEV = Inactivate oil emulsion vaccine containing Arlacel A emulsifier.

Table 4.15: Field trials with [1]IOEV in 3 categories of birds

Area of observation in field trial	Bird Owners	Number of birds	Pre-IOEV [2]GMT	Post-IOEV GMT	Antibody GMT before laying	Antibody GMT at beginning of laying	Effect of IOEV on rate of laying	Effect of IOEV on broilers
Immunogenicity of IOEV in layers before and at beginning of laying	Wapmuk/ Imogie	320	111.4	[6]ND	2896.3	3104.2	[5]NA	NA
Effects of IOEV in births already laying	Gbodi/ Musa	300	512.0	2896.3	NA	NA	[3]NER	NA
Immunogenicity of and side effects of IOEV in broiler chickens	Chakven	50	104.0	776.0	NA	NA	NA	[4]NSE

[1]IOEV = Inactivated oil emulsion vaccine
[2]GMT = Geometric mean titre
[3]NER = No egg reduction
[4]NSE = No side effects
[5]NA = Not applicable
[6]ND = Not done

The third category which were broiler chicken showed increase in antibody GMT from 104.0 to 776.0 after the administration of the IOEV. There was no adverse reaction due to IOEV in the chickens after administration, neither was there any report of ND incidence until their disposal at the age of 8-10 weeks.

CHAPTER 5

DISCUSSION

In order to attain a successful viral vaccine production, a cheap means of propagating the virus which would ensure large yield of antigenmust be sought. In this study embryonated chicken eggs were used. Apart from this medium being more economical and easier to handle, ND viruses have high affinity for its allanto-amniotic membrane cells. Pure mature virus particles are shed into the allantoic fluid, at the end of the replication cycle which can be harvested as source of antigen (Beard and Hanson, 1984). Embryonated chicken egg was therefore selected as the virus propagation medium for the production of inactivated oil emulsion vaccine (IOEV).

The selection of virus strain for any vaccine development is of strategic importance. With live vaccines development emphasis is usually laid on a well attenuated strain that would not easily revert to virulence during virus replication in the body. In this study high stable haemagglutination (HA) titres with minimum embryo mortality and good infectivity in embryonated chicken eggs were the major characteristics used to select strains for the production of the IOEV. Of the eight available local isolates cultured the four velogenic strains had low HA titres because

they killed their hosts (embryo) before attaining a reasonably high HA titre (table 4.1). Their haemagglutinin antigen were also unstable when exposed to room temperature and 4^{o}C (table 4.2). They were selected for use as challenge strains. The ND virus Lasota (lentogenic) and the ND virus Komarov (mesogenic) on the other hand gave the highest HA titres with minimum embryo mortality and high infectivity in embryonated eggs (table 4.1). Their HA titres did not drop significantly at room temperature and at 4^{o}C. The two virus strains were therefore chosen for the production of the inactivated oil emulsion vaccine (IOEV).

Inactivation is another major area in the development and production of IOEV. In this project a lentogenic and a mesogenic strain were incorporated together to form a plyvalent IOEV. Although formalin inactivation was carried out for human polio vaccine, this technique has not been successful for Newcastle disease IOEV (Hitchner, 1984). In fact previous workers preferred beta propiolactone to formalin for fear that the formalin would damage the haemagglutinin antigen. This study has demonstrated that this assertion is not completely true. A low concentration of formalin (1:1000) effectively inactivated the ND viruses without any loss in haemagglutinin titre. It also did not produce any toxic effect in one day old chickens (table 4.3; table 4.4). The IOEV produced from the formalin inactivation was given to chickens 3 weeks and one day

old without any ill effects. This could not have been possible with live mesogenic vaccines like the ND Komarov. Although beta propiolatone could have taken only 2 hours to inactivate instead of 24 hours taken by formalin, beta propiolactone is rare in Nigeria and carcinogenic (Allan et al, 1979). This technique was therefore more appropriate and was recommended for use in developing countries, like Nigeria, where facilities for handling dangerous drugs in the laboratories are not available.

The wide acceptance of IOEV in recent years depends largely on the adjuvant effect of its oil component. Of the three oils tested in this project (palm oil, groundnut oil and liquid paraffin), liquid paraffin had the highest adjuvant effect (fig. 1) and produced the most stable emulsion and was therefore selected for use in the production of IOEV. This finding agreed with those of several workers who had compared the adjuvant effect of liquid paraffin with other oils (Cernik, 1970), Brugh et al, 1983; Garba et al, 1987).

That this new IOEV is a combination of a lentogenic and a mesogenic strain is a new enovation. Previous workers of IOEV have used only lentogenic strain, especially the ND Lasota (Stone et al, 1983; Brugh et al, 1983; Ernawati and Ibrahim, 1984). This IOEV is intended to reduce the burden and the expenses on Nigerian poultry farmers, who administer live ND Lasota vaccine several times (2 to 3 times) on their

parent stock and just before they lay they are again given the live ND Komarov vaccine. With this protocol, a farmer with 1000 young layers spends at least ₦700 to ₦1,000 on the ND Lasota and ND Komarov vaccines alone before the birds start to lay (all the ND vaccines now cost at least 10k a dose). With the IOEV the layers are vaccinated only once before they lay and at estimated price of 40k per dose it will cost only ₦400.

The efficacy of the IOEV can be clearly seen when its potency is compared with that of the current live vaccines. One of the IOEV Schedules (Schedule 2) produced about 5 times more antibody geometric mean titres (GMT) over the current live vaccines. When they were challenged with 5 virulent isolates from different parts of the country, IOEV induced protection ranging from 88-100% while the current live vaccines induced only 78% protection (table 4.8; fig. 2). This findings confirms the results of other workers (Thomson et al, 1969; Aitken and Survashe, 1974; Stone et al, 1981; Garba et al, 1987) that killed oil emulsion vaccinates consistently induced the highest antibody titre and protection. The highest average protection induced by IOEV in this study was 98% while that of the live vaccines was 76.5%

A closer look at Schedule 4 which consisted of one day old chickens vaccinated with IOEV without further boosting showed that it has low antibody response,

but its protection was 100%. The protection could be from cell mediated immunity. Schedule 5 which were boosted at different times between 3 weeks had rather diminished antibody response and protection. Although the reasons for this is not clear, it is obvious that the IOEV is capable of inducing protective immunity in chickens from one day to at least 16 weeks without any need for a booster. However, the direct inoculation of one day old chicken, was tedious to perform and led to non-specific deaths of chickens that were still too fragile to handle (table 4.7). Schedule 2 which consisted of birds primed at one day old with NDVB1 before IOEV vaccination at 3 weeks appears to have provided a good solution to the problem. This Schedule gave the highest serological response and it was next to Schedule 4 in terms of protection against challenge with 5 virulent isolates (table 4.8, fig. 2). It is therefore recommended that one day old chickens whose parents received only live vaccines be primed with NDVB1 to protect them for 3 weeks before they are vaccinated with IOEV. At this age the birds are easier to handle.

It is particularly noteworthy that the two week old progeny of the IOEV vaccinates had antibody GMT of 147.0, while those of the live vaccine vaccinates was only 29.9 and the NDVB1-primed chickens at 2 weeks old (the last observed GMT before IOEV administration) was 111.4 (table 4.11). Since the last observed GMT

of the primed chickens before IOEV administration is less than the GMT of the progeny of the IOEV-parents at 2 weeks old, it becomes obvious that the progeny from vaccinated IOEV-parents need not be primed with NDVB1. Maternal antibody from their IOEV vaccinated parents is enough to keep them protected for 3 weeks. It is therefore recommended that chickens of IOEV vaccinated parents need not be primed with NDVB1 before vaccinated with IOEV at 3 weeks. This will further reduce the expenses of the poultry farmer.

The dosages of killed emulsion vaccines are usually larger than those of the live vaccines. This is understable because killed vaccines need large antigenic mass. Stone et al. (1981) and Brugh et al. (1983) used 0.5ml dose containing at least 1×10^{10} EID_{50} of the virus. Since the EID_{50} of the virus strains used were at least 1×10^{16} per 0.1ml (table 4.1) and since the IOEV was 1:1 antigen/oil ratio (section 3.2.7 chapter 3) the EID_{50} content of the IOEV was 0.5×10^{16} or 5×10^{15} per 0.1ml. The 0.4ml dose therefore contained 2×10^{16} EID_{50} of the virus. The protective immunity induced by different regimen of IOEV using this dose has already been discussed. A repeat of schedule of vaccine using regimen 2 experiment with fresh batch of IOEV and 0.4ml gave a reproducible result (Fig. 3; table 4.10. The 0.2ml (containing 1×10^{16} EID_{50} of the virus) induced less serological response and protected chickens less than 0.4ml dose.

It however induced higher serological response and gave better protection than the current live vaccines (table 4.9; fig. 3; table 4.10). Therefore the 0.4ml dose is recommended for chickens at 3 weeks old. However, if it is desirable to give IOEV to one day old chickens directly, the 0.2ml dose should be used.

The results of the safety and stability tests of the IOEV is of particular interest. That it is safe and without side reactions in all the ages of chickens tested makes it superior to the live vaccines. Live ND Lasota vaccine causes post-vaccination respiratory symptoms (Allan et al, 1978) while ND Komarov vaccine is never recommended for chickens under 8 weeks due to its adverse side reactions (David-West, 1972, Allan et al, 1978). The length of time an IOEV can stay unseparated irreversibly from its oil adjuvant at a particular temperature without appreciable loss of HA titre determines its shelf-life at that temperature. That the IOEV remained at least one year at room temperature and at least 4 months at 37^{o}C without irreversible separation and significant loss of HA titre (table 4.5), like Nigeria, than the live vaccines. It can be stored without the expensive cold chain facilities required for live vaccines. All live ND vaccines maintain potency up to 1 year if only they are properly stored at -20^{o}C. In fact most reports of loss of potency of ND vaccines in Nigeria have been due to virus inactivation by the tropical heat in the field.

The field trials with IOEV conducted in 3 categories of chickens revealed more advantages of this vaccine over the current live vaccines. Its high immunogenicity and freedom from side reactions was not only confirmed in layer chickens but also in broiler chickens (table 4.15). This observation is in line with that of Partadiredja et al. (1979) that killed oil ND vaccine induced rapid and high titre antibody response in broiler breeder chickens that lasted for a long time. More interestingly was the fact that the IOEV given to chickens already laying did not affect their rate of egg production. This is in contrast with the report of David-West (1972) which stated that vaccination of laying hens with live ND vaccine Komarov produced marked drop in egg production for a period of up to six weeks.

The source of raw materials for the production of the IOEV is relevant. It is important to note that the IOEV was prepared without the usual antigen concentration. Therefore the sophisticated ultracentrifuge equipment needed for antigen concentration was not required. It has been demonstrated that the allantoic fluid used contained sufficient antigen to produce highly immunogenic IOEV without further antigen concentration. This has confirmed the observation of Stone et al. (1983) who showed that highly potent ND killed oil emulsion vaccine could be produced using unconcentrated virus-laden allantoic fluid as antigen.

In fact they further demonstrated that increasing antigen content beyond certain limits might lead to diminishing serological returns in that titres did not continue to increase proportionaly to the antigen content of the vaccine. Other sophisticated equipments and materials like freeze-drying machines, capping machines, vials, rubber caps and enamel caps which are essential imported items for the production of live ND vaccines are not required for the production of IOEV. The 50ml screw capped bottles into which the IOEV was dispensed are now manufactured at Kaduna, in Kaduna State of Nigeria. The formalin, embryonated eggs and liquid paraffin which are essential components of IOEV are locally available.

The initial problem of the IOEV with regards to the raw materials was the Arlacel A, an important emulsifier-component of the vaccine. Arlacel A (Manide mono-oleate) is not available in Nigeria and therefore very expensive to import. While sourcing for a local substitute, it was discovered that 'Egusi' oil contained Oleic acid similar to Arlacel A (Oyenuga, 1968). This oil was used in place of Arlacel A as an emulsifier for the production of IOEV. The 'Egusi'-IOEV was equally stable at room temperature and at $4^{o}C$. It was even more stable at $37^{o}C$ than the emulsions of Arlacel A (table 4.12). 'Egusi'-IOEV was less viscous and hence easier to inject subcutaneously into chickens than those of Arlacel A. The serological response and challenge trials with 'Egusi'-IOEV were similar

to those of Arlacel A (fig. 4; table 4.13 and table 4.14). This study has therefore demonstrated that 'Egusi' oil can be used as an emulsifier instead of Arlacel A for the production of the IOEV.

In summary, the following objectives have been achieved in this project:

1. Inactivation

   Betapropiolactone a common inactivating agent for ND viruses is carcinogenic and can only be handled by skilled personnel. It is also rare in Nigeria and expensive to import. Formalin at a concentration of 10^{-3} has proved to be a good substitute for β-propiolactone by inactivating the virus without any detrimental effect on the haemagglutinin antigen of the viruses. It is non-carcinogenic, easy to handle, and commonly available.

2. Bivalent nature of the IOEV

   The ND virus Lasota (lentogenic) and the ND virus Komarov (mesogenic) have been successfully combined into one vaccine. Previous workers of IOEV have used only one strain. As a result of this achievement the IOEV can be used in chickens not only at 3 weeks old but even at one day old. The live ND Komarov vaccine cannot be used in chickens less than 8 weeks old because of its virulent nature.

3. Substitute for Mannide mono-oleate (Arlacel A)

Arlacel A, the common emulsifier used in the production of IOEV is rare in Nigeria and it is expensive to import. A litre of Arlacel A costs about ₦7000.00. Egusi oil has proved to be a good substitute for Arlacel A. One litre of this oil can easily be prepared in the laboratory at a cost of less than ₦10.00.

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4. Cost of production

The IOEV is relatively cheaper to produce. About 90% of the raw materials needed for its production was obtained locally. The cost of raw materials for production is estimated to be 40k per dose. However, it is relatively more expensive to apply the vaccine into chickens considering the cost of labour. The current live vaccines are relatively more expensive to produce. Most of the vital raw materials like vials, caps, rubber stoppers and freeze-drying machines are still being imported. Maintenance of the freeze-drying machines needs foreign experts. The cost of raw material for the production of the current live vaccines is more than 80k per dose. The production of IOEV with local raw materials is in perfect agreement with the government's

local sourcing of raw materials and self reliance programme.

5. Avoidance of Multiple vaccination

The IOEV is retained in the body for a much longer time than the live vaccines, hence the immunity induced by IOEV lasts much longer. Repeated vaccination (multiple vaccination) is not necessary. With IOEV layer chickens are vaccinated only once before they lay and it is estimated that a farmer with 1000 chicken would spend ₦400 to ₦600 for IOEV including labour of application. With live vaccines, immunity wains very fast hence the need for multiple vaccination. With multiple vaccination a farmer with 1000 chickens now spends at least ₦800 to ₦1,000 on live ND Lasota and ND Komarov vaccines alone before the birds start to lay.

6. Better Potency and efficacy

The IOEV induces 5 times antibody response of the 3 live vaccines and 98% protective immunity as against the 76.5% protection of the live vaccines. Outbreaks of ND even among vaccinates of live vaccines have been reported whereas there was no incidence of ND among vaccinates of IOEV throughout the period of the field trials.

The IOEV is therefore not only more potent but also more efficacious than the current live vaccines.

REFERENCES

[illegible], W.W. (1964). Cell surface phenomena of Newcastle disease virus. In Newcastle Disease Virus An Evolving Pathogen. ed. by R.P. Hanson, Univ. Wisconson Press, Madision, U.S.A.

Adu, F.D., O. Oyejide, and B.O. Ikede (1985). Characterization of Nigerian Strain of Newcastle disease virus. Avian Dis. 29: 829-831.

[illegible]ken, I.D. and B.D. Survashe (1974). Observation of the serological and dermal responses of turkeys to a single subcutaneous inoculation of inactivated Newcastle disease vaccine in aircraft oil adjuvant. Avian Pathol. 3: 211-222.

Alexander, D.J. and W.H. Allan (19[illegible]). [illegible] disease. The nature of the virus [illegible] Bull. int. Epiz., 79 (1-2): 15-26.

Alexander, D.J. (1980). Avian [illegible]. Vet. Bull. 50: 737.

[illegible], D.J. (1982). Avian paramyxoviruses other than Newcastle disease virus. World's [illegible]. 38: 97-104.

[illegible] and R.E. [illegible] (1974). A rapid haemagglutination inhibition test for Newcastle disease. [illegible] comparison of [illegible]. 147-153.

Allan, W.H., J.E. Lancaster and B. [illegible] (1978). [illegible] tle [illegible] e Vaccine. FAO Animal Production and Health Paper. Food and Agricultural Organization of the United Nations, Rome.

[illegible]ws, C.H., F.B. Bang and F.M. [illegible] (1965). A short description of the myxovirus group (including related viruses) Virology 25: 176-184.

[illegible] and F.D. [illegible]). Interaction of virus [illegible] erythrocytes. J. [illegible] 15: [illegible].

[illegible] Interaction of [illegible] cells, a [illegible] the st[illegible]. 247-261.

[illegible]splin, F.D. [illegible] disease [illegible] and Obse[illegible] resist[illegible] Vet. Rec. 64: [illegible].

Bankowski, R.A. (1964). Cytopathogenicity of Newcastle disease virus. In Newcastle Disease Virus An Evolving Pathogen. ed. By R.P. Hanson, Univ. Wisconson Press, Madison, U.S.A.

Beach, J.R. (1944). The neutralization in vitro of avian pneumoencephalitis virus by Newcastle disease immune serum. Science, 100: 351.

Beard, C.W. and W.J. Wilkes (1973). A simple and rapid microtest procedure for determining Newcastle haemagglutination inhibition (HI) antibody titres. Proc. 77th Annual Meeting, U.S. Anim. Health Assoc. P. 596-600.

Beard, C.W. and R.P. Hanson (1984). Newcastle disease. In Disease of Poultry, ed. by M.S. Hofstad, Iowa State Univ. Press, Ames, Iowa, U.S.A.

Beaudette, F.R. (1943). A review of the literature on Newcastle disease. Proc. 47th Annual Meeting, U.S. Livestock Sanit. Assoc. Chicago, p. 122-177.

Beaudette, F.R., J.A. Bivins and B.R. Miller (1949). Newcastle disease immunization with live virus. Cornell Vet. 39: 302-334.

Becht, H., U. Hammerling and R. Rott (1971). Undisturbed release of influenza virus in the presence of univalent antineuraminidase antibodies Virology, 46: 337-343.

Blair, C.D. and W.S. Robinson (1968). Replication of Sendai virus I. Comparison of the viral RNA and virus specific RNA synthesis with Newcastle disease virus. Virology, 35: 537-549.

Box, P.G. and L.G.S. Furminger (1975). Newcastle disease antibody levels in chickens after vaccination with oil emulsion adjuvant killed vaccine. Vet. Rec. 96: 108-111.

Brandly, C.A., H.E. Moses, E.L. Jungherr and E.E. Jones (1946). Epizootiology of Newcastle disease in poultry. Amer. J. Vet. Res. 9(24): 243.

Bratt, M.A. and W.S. Robinson (1967). Ribonucleic acid synthesis in cells infected with Newcastle disease virus. J. molec. Biol. 23: 1-21.

Bratt, M.A. and L.A. Clavell (1972). Haemolytic interaction of Newcastle disease virus and chicken erythrocytes I. Quantitative comparison procedures. Appl. Microbiol. 23: 454-460.

Brown, F. (1984). Synthetic viral vaccines. Ann. Rev. Microbiol. 38: 221-235.

Brugh, M. and H.S. Siegel (1978). Inactivated Newcastle disease vaccines: influence of virus concentration on the primary response. Poult. Sci. 57: 892-896.

Brugh, M., H.D. Stone and H.W. Lupton (1983). Comparison of inactivated Newcastle disease viral vaccines containing different emulsion adjuvants. Am. J. Vet. Res. 44(1): 72-75.

Brugh, M. (1985). How maternal antibody affects early vaccination. Proc. 1984 U.S. Health Seminar, September 1984, South Eastern poultry and Egg Assoc., published in Poultry Digest, January 1984 ed. p. 24.

Burnet, F.M. (1942). The affinity of Newcastle disease virus to the influenza virus group. Aust. J. exp. Biol. Med. Sci. 20: 81-88.

Carlson, J.K. (1986). Development and application of genetically engineered viral vaccines of poultry. Avian Dis. 30(1): 24-27.

Cernik, K. (1970). The selection of adjuvants for the preparation of inactivated viral vaccines. Vet. CAS. 13(6): 179-187.

Choppin, P.W. and R.W. Compans (1975). Reproduction of paramyxoviruses. In Comprehensive Virology, Vol. 4: p. 95-178, ed. by Fraenkel - Contrat and R.R. Wigner, New York plenum press, U.S.A.

Clavell, L.A. and M.A. Bratt (1972). Haemolytic interaction of Newcastle disease virus and chicken erythrocytes II. Determining factors. Appl. Microbiol. 23: 461-470.

Cockrill, W.R. (1974). Economic loss from poultry disease - world aspect. In Poultry Disease and World Economy, ed. by R.F. Gordon and B.M. Freeman, British Poultry Science Ltd., Edinburgh, U.K.

Crowther, R.W. (1964). Newcastle disease - A serological study of immunity using two live vaccines. J. Comp. Path. 74: 151-162.

Cruickshank, J.G. (1964). In Cellular Biology on Myxovirus Infections: Ciba foundation symposium p. 5, ed. by G.E.W. Welstemhohme and J. Knight, Churchhill Ltd., London, U.K.

Daniel, M.D. and R.P. Hanson (1968). Differentiation of representative Newcastle disease virus strains by their plaque forming ability on monolayers of chick embryo fibroblast. Avian Dis. 12: 423-433.

Davenport, F.M. (1968). Seventeen years experience with mineral oil adjuvant and influenza vaccines. Ann. Allergy, 26: 288.

David-West, K.B. (1972). Newcastle disease in Nigeria - Retrospection and anticipation. Bull. Epizoot. Dis. Africa, 20: 291-295.

De Vries, P., R.S. Vanbinnendijk, P. Vandermarel, B.D. Sundquist, A.L. Vanwesel, H.D. Voorma, F.G.C.M. Uytdehaag, and A.D.M.E. Osterbaus (1988). Measles virus fusion protein presented in an immunostimulating complex (ISCOM) induces haemolysis - inhibiting and fusion inhibiting antibodies, virus specific T cells and protection in mice. J. gen. Virol. 69: 549-559.

Doyle, T.M. (1927). A hitherto unrecorded disease of fowls due to a filter passing virus. J. Comp. Path. 40: 44.

Eidson, C.S., P. Willegas and S.H. Kleven (1980). Field trials with an oil emulsion Newcastle disease vaccine in broiler breeders. Poult. Sci. 59: 702-707.

Eidson, C.S., S.G. Thayer, P. Villegas and S.H. Klevel (1982). Further studies with an inactivated oil emulsion Newcastle disease vaccine in broiler breeders. Poultry Sci. 61: 1309-1313.

Ernawati, R. and A.L. Ibrahim (1984). Newcastle disease vaccination in Malaysia: Application of oil emulsion vaccine. Vet. Res. 96: 108-111.

Fatunbi, O.O. and D.F. Adene (1979). Susceptibility of the the Nigerian local chicken to fulminating Newcastle disease outbreaks. Nig. Vet. J. 8(2): 30-32.

Fenner, F. (1976). The classification and nomenclature of viruses. Inter Virol. 6: 1-12.

Fruend, J. (1956). The mode of action of immunologic adjuvants. Adva Tuberc. Res. 7: 130-148.

Garba, S.A. and R.J. Terry (1986). Immunogenicity of oil based contagious bovine pleuropneumonia vaccine in cattle. Vaccine 4: 265-270.

Garba, S.A., R.J. Terry and D.A. Adegboye (1987). The development of killed adjuvanted vaccines against contagious pleuropneumonia: preparation and toxicity and potency tests in mice. Indian Vet. J. 64: 187-191.

Garten, W., T. Kohama and H.D. Klenk (1980). Proteolytic activation of the haemagglutinin-neuraminidase of Newcastle disease virus involves loss of a glycopeptide. J. gen. Virol. 51: 207-211.

Giambrone, J. (1985). The importance of inactivated vaccines. In Canada Poultryman, April ed. p. 38-40. Vineland laboratories intercontinental, affiliate of Immunogenetics Inc. NJ. U.S.A.

Gordon, R.F. (1971). The effects of ill health. Vet. Res. 89: 496.

Haddow, J.R. and J.A. Idnani (1946). Vaccination against Newcastle (Ranikhet) disease. Ind. J. Vet. Sci. 16: 45-53.

Halbert, S.P., Smolens and S. Mudd (1945). Reduced acute toxicity of antigens in saline-in mineral oil emulsions. J. Immunol. 51: 39.

Hanson, R.P. (1980). In Isolation and Identification of Avian Pathogens. p. 63-69, ed. by S.N. Hitchner, O.H. Domermuth, H.G. Purchase and J.E. Williams. Am. Assoc. Avian Path., College Station, Texas, U.S.A.

Hill, D.H., O.S. Davis and J.E.H. Wilde (1953). Newcastle disease in Nigeria. Brit. Vet. J., 109(9): 381-385.

Hitchner, S.B. and E.P. Johnson (1948). A virus of low virulence for immunizing fowls against Newcastle disease (Avian pneumoencephalitis) Vet. Med. 43(12): 525-530.

Hitchner, S.B. (1984). A re-evaluation of inactivated vaccines. Res. World., 6: 4-6.

Hofstad, M.S. (1953). Immunization of chickens against Newcastle disease by formalin inactivated virus. Am. j. Vet. Res. 14: 586-589.

Hofstad, M.S., H.J. Barnes, B.W. Calnek, W.M. Reid and H.W. Yoder Jr (1984). In Diseases of Poultry, p. 453-467, Iowa State Univ. Press, Ames. Iowa, U.S.A.

Huang, A.S., D. Baltimore and M.A. Bratt (1971). Ribonucleic acid polymerase in virions of Newcastle disease comparison with the Vesicular Stomatis Virus polymerase. J. Virol., 7:389-394.

Huygelene, C. and J. Peetermans (1963). Studies on the growth of a vaccine strain (Komarov) of Newcastl disease virus in bovine kidney tissue culture and preparation of vaccine. Res. Vet. Sci. 4: 294-303.

Iorio, R.M., J.B. Borgman, R.L. Glickman and M.A. Bratt (1986). Genetic variation within a neutralizing domain in the haemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. J. gen. Virol. 67: 1393-1403.

Iyer, S.G. and N.A. Dobson (1940). A successful method of immunization against Newcastle disease of fowls. Vet. Res. 52: 889-894.

Jorgensen, E.D., P.L. Collins and P.T. Lomedico (1987). Cloning and nucleotide sequence of Newcastle disease virus haemagglutinin-neuraminidase mRNA; Identification of a putative sialic acid binding sites. Virology, 156: 12-24.

Kaliannan, K.G.L. Seawright and J.B. Derbyshire (1975). Adequacy of commercial lentogenic vaccines against Canada strains of Viscerotropic Newcastle disease virus. Canad. J. Comp. Med. 39(2): 139-145.

Kaverin, N.V. and N.L. Varich (1971). Virus specific RNA formed in Newcastle disease virus - infected cells after suppression of protein synthesis by cyclohexamide. Arch. ges. Virusforsch, 38: 183-191.

Kelleher, C.J., D.A. Halvorsen, and J.A. Newman (1987). Evaluation of Newcastle disease virus spray vaccination program of market turkeys. Avian Dis; 31(3): 431-437.

Kessler, N., M. Aymard and A. Kalvet (1979). Study of a new strain of paramyxoviruses isolated from wild ducks: antigenic and biological properties. J. gen. Virol., 43: 273-282.

Kiham, L. (1949). A Newcastle disease virus (NDV) haemolysin. Proc. Soc. exp. Biol. Med. 71: 63-66.

Kingsbury, D.W. (1970). Replication and function myxovirus ribonucleic acids. Progr. Med. Virol. 12: 49-77.

Kohn, A. (1965). Polykaryocytosis induced by Newcastle disease virus in monolayer of animal cells. Virology, 26: 228-245.

Komarov, A. and L. Goldsmit (1946). Preliminary observation on the modification of a strain of Newcastle disease virus by intracerebral passage through ducklings. Vet. J. 102: 212-218.

Kraneveld, F.C. (1926). A poultry disease in the Dutch East Indies. Med. Ind. Bl. Diergeneesk, 38: 448-451.

Lancaster, J.E. (1966). Newcastle disease, a review, 1926-1964: Monograph No. 3 Canada Department of Agriculture Publications, Canada.

Lancaster, J.E. (1974). Viscerotropic, Velogenic Newcastle disease in Canada. Proc. XV, World's Poultry Congr. p. 420-421. New Orleans, Aug. 11-16, 1974.

Lancaster, J.E. (1981). The control of Newcastle disease. World's Poultry Sci. J. 37: 84-95.

Levine, P.P. (1964). World dissemination of Newcastle disease. In Newcastle Disease Virus ed. by R.P. Hanson, Univ. Wisconson Press. Madison, U.S.A.

Lwoff, A., R. Horne and P. Tournier (1962). A system of viruses. Cold Spr. Habour Symp. quant. Biol. 27: 51-55.

Mackett, M., G.C. Smith and B. Moss (1984). General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J. Virol. 49: 857-864.

Majiyagbe, K.A. and D.R. Nawathe (1981). Isolation of virulent Newcastle disease virus from apparently normal ducks. Vet. Rec. 108-187.

Marcus, P.I. (1962). Dynamics of surface modification in myxovirus infected cells. Cold Spr. Habour Symp. quant. Biol. 27: 351-365.

Martone, F., D. Bonaduce, P. Pagnini, E. Biondi and P. Striglioni (1976). Immunologic research into Newcastle disease. Resistance of fowls immunized with attenuated Lasota vaccine to newly isolated strain. Clinica Veterinaria, 99(10): 521-525.

Maychew and T.M. Liow (1974). Newcastle disease in Singapore. Proc. XV World's Poultry Congr. P. 406-408, New Orleans, Aug. 11-15, 1974.

MaCinnes, L.W., A. Wild and T.G. Morrison (1987). Nucleotide sequence of the genes encoding the Newcastle disease virus haemagglutinin-neuraminidase protein and comparison of paramyxovirus haemagglutinin-neuraminidase protein sequence. Virus Res. 7: 187-202.

McKinney, R.W. and F.M. Davenport (1961). Studies on the mechanism of action of emulsified vaccines. J. Immunol., 86: 91-100.

McMillan, C., C. Fellenz, and R.P. Hanson (1986). The effect of multiplicity of infection on viral sub-populations during passage of Newcastle disease virus. Avian Dis. 30(1): 122-125.

Millar, N.S., P. Chambers and P.T. Emmerson (1988). Nucleotide sequence of the fusion and haemagglutinin-neuraminidase glyco-protein genes of Newcastle disease virus, strain Ulster: molecular basis for variation in pathogenicity between strains. J. gen. Virol. 69: 613-620.

Morein, B. and R. Bergman (1972). Effect of parainfluenza 3 neuraminidase on bovine secretions. Infect and Immunity, 6: 174-177.

Morein, B., B. Sundquist, S. Hoglund, K. Dalsgaard and A. Osterhaus (1984). Iscom a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature, London, 308: 457-459.

Morein, B. and K. Simons (1985). Subunit vaccine of envelope viruses: Virosomes, miscelles and other protein complexes. Vaccine 3 83-93.

Nagai, Y., H.D. Klenk and R. Rott (176). Proteolytic cleavage of the viral glycoprotein and its significance for virulence of Newcastle disease virus. Virology; 72: 494-508.

Nagai, Y. and H.D. Klenk (1977). Activation of precursors to both glycoproteins of Newcastle disease virus by proteolytic cleavage. Virology, 77: 125-134.

National Academy of Science (1971). Methods for examining poultry biologics and for identifying and quantifying avian pathogens. Nat. Acad. Sci. p. 66-98.

National Veterinary Research Institute Manual (1984). Routine Laboratory methods in the production of live viral vaccines. 1984 revised ed. p. 42-43.

Nawathe, D.R., K.A. Majiyagbe and S.O. Ayoola (1975). Characterization of Newcastle disease virus isolates from Nigeria. Bull. off. int. Epiz. 83 1097-1105.

Nawathe, D.R. (1986). Newcastle disease and its control. Paper given at OAU-STRC-IBAR Conference on Virology at Nairobi 2-6 June, 1986.

Nawathe, D.R. (1987). Newcastle disease and poultry farming in Nigeria. Nig. Livestock Farmer, 7(1-4): 7-10.

Nobuto, K. (1968). Newcastle disease in Japan. Bull. off. int. Epiz.

Novilla, M.N. and J.A. Navarro (1969). A preliminary study on the identification and classification of Newcastle disease virus strains obtained from field outbreaks in the Philippines, Philipp. Jour. Vet. Med. 8(12): 25.

Okoh, A.E.J. (1979). Newcastle disease in Falcons. J. Wildlife Diseases, 15(3): 479-480.

Okoye, J.O. and S.V.O. Shoyinka (1983). Newcastle disease in a vaccinated flock. Trop. Anim. Hlth, Prod. 15: 221-224.

Onunkwo, O. and M.A. Momoh (1981). Characterization of a Newcastle disease virus isolated from a Parrot. J. Wildlife Diseases, 17(3): 463-465.

OAU/STRC Inter African Bureau for Animal Resources (1982). Geographical distribution of Newcastle disease in Africa. Bull. Anim. Hlth. Prod. Afri., 1982.

Owolodun, B.Y. (1968). Newcastle disease. Comparative evaluation of serological response of birds at Vom, Nigeria, to vaccination with Intraocular (cyprus originated), Komarov and Lasota vaccines. Bull. epizoot. Dis. Afr. 16: 345-351.

Oyenuga, V.A. (1968). Nigeria's food and feeding Stuff. Their Chemistry and nutritive value. P. 72-89, Ibadan University Press, Nigeria.

Partadiredja, M., C.S. Eidson and S.H. Kleven (1979). Immunization of broiler breeder chickens against Newcastle disease with an oil emulsion vaccine. Avian Dis., 23(3): 597-606.

Peck, H.M., A.F. Woodhour and D.P. Metzgar (1964). New metabolizable immunologic adjuvant for human use. Short term animal toxicity tests. Proc. Soc. exp. Biol. Med. 116: 523-530.

Piercy, S.E., A.H. Macleod and J.D. Blaxland (1964). Duration of immunity trials with betapropiolactone inactivated Newcastle disease vaccine. Vet. Rec., 76: 1084-1089.

Piscard, W.K. (1928). Pseudo fowl pest. Vet. Bull. No. 65, Dept. Agric. Ind. and Commerce in the Dutch East Indies.

Reeve, P. and D.J. Alexander (1970). Plaque formation cell fusion and haemadsorption by Newcastle disease virus. Cytobios, 2: 53-58.

Reeve, P., D.J. Alexander, G. Pope and G. Poste (1971). Studies on the cytopathic effects of Newcastle disease virus: metabolic requirements. J. Gen. Virol., 11: 25-35.

Reeve, P. and G. Poste (1971). Studies on the cytopathogenicity of Newcastle disease virus: Relation between virulence, polykaryocytosis and plaque size. J. gen. Virol., 11: 17-24.

Reeve, P., G. Poste, D.J. Alexander and G. Pope (1972). Studies on the cytopathic effects of Newcastle disease virus: cell surface changes. J. gen. Virol. 15 219-225.

Rott, R. (1964). Newcastle Disease Virus: An Evolving Pathogen, p. 133-146, ed. by R.P. Hanson, Univ. Wisconson Press, Madison, U.S.A.

Rott, R. and C. Scholtissek (1967). In Modern Trends in Medical Virology, p. 25-48, ed. by R.B. Heath and A.P. Waterson, Butterworth, London.

Salk, J.E. (1954). Formaldehyde treatment and safety testing of experimental poliomyelitis vaccine. Amer. J. Public Health, 44: 563-570.

Samson, A.C.R., M. Nesbit, A.M. Lyon and G. Meulemans (1988). Identification and haemagglutinin-neuraminidase antibody binding sites by Western blot analysis of antibody resistant mutant and partial digest fragments of Newcastle disease virus. J. gen. Virol. 69: 473-480.

Scolnick, E.M. (1984). Clinical evaluation of health adults of a hepatitis B vaccine made by recombinant DNA. J. Am. Med. Assoc. 251: 2812-2815.

Scheid, A. and P.W. Choppin (1973). Isolation and purification of the envelope proteins of Newcastle disease virus. J. Virol. 11: 263-271.

Schloer, G.M. and R.P. Hanson (1968a). Relationship of plaque size and virulence for chickens of 14 representatives of Newcastle disease virus strains. J. Virol. 2: 40-47.

Schloer, G.M. and R.P. Hanson (1968b). Plaque morphology of Newcastle disease virus as influenced by cell type and environmental factors. Am. J. Vet. Res. 29: 883-895.

Shope, R.E. (1964). The birth of a new disease. In Newcastle Disease Virus, ed. by R.P. Hanson, Univ. Wisconson Press, Madison, U.S.A.

Smith, G.L., B.R. Murphy and B. Moss (1983). Construction and characterization of an infectious vaccinia virus recombinant that expresses the haemagglutinin gene and induces resistance to influenza virus infection in hamsters. Proc. Natl. Acad. Sci. 80: 7155-7159.

Steiner, J.W., B. Langer and D.L. Schatz (1960). The local and systemic effects of Fruend's adjuvant and its fractions. Arch. Path. 70: 424-434.

Stone, H.D., M. Brugh, G.A. Erickson and C.W. Beard (1980). Evaluation of inactivated Newcastle disease oil emulsion vaccine. Avian Dis. 24(1): 99-121.

Stone, H.D., M. Brugh and C.W. Beard (1981). Comparison of three experimental inactivated oil emulsion Newcastle disease vaccines. Avian Dis., 25(4): 1070-1076.

Stone, H.D., M. Brugh and C.W. Beard (1983). Influence of formulation on the efficacy of experimental oil emulsion Newcastle disease vaccines. Avian Dis., 27(3): 688-697.

Thayer, S.G., B.N. Nersessian, B. Rivetz and O.J. Fletcher (1987). Compaarison of serological tests for antibodies against Newcastle disease virus and Infectious bronchitis virus using Immunocomb solid-phase immunoassay, commercial Enzyme linked immunosorbent-assay and the haemagglutinin-inhibition assay. Avian Dis., 31: 459-463.

Thomson, R.O., I. Batty, A. Thomson, J.B. Kerry, H.B.G. Epps and W.H. Foster (1969). The immunogenicity of a multi-component clostridial oil emulsion vaccine in sheep. Vet. Rec. 85: 81-85.

Tumova, B., A. Stumpa, V. Janout, M. Uvizl and J. Chmela (1979a). A further member of the Yucaipa group isolated from the common wren (Troglodytes troglodytes), Acta Virol., 23: 504: 504-507.

Tumova, B., J.H. Robinson and B.C. Easterday (1979b). A hitherto unreported paramyxo virus of turkeys. Res. Vet. Sci., 27: 135-140.

Vasic, N.V. (1965). A contribution to the examination of immunogenic properties of attenuated Newcastle disease viruses grown on tissue culture. Acta Vet. Hung. 15: 57-67.

Waterson, A.P. (1962). Two kinds of myxovirus. Nature, London, 193: 1163-1164.

Waterson, A.P. and J.D. Almeida (1966). Taxonomic implications of myxovirus. Nature, London, 210: 1136-1140.

Waterson, A.P. (1968). Introduction to Animal Virology, p. 1-78, 2nd ed. Cambridge Univ. Press, U.K.

Weir, D.M. (1979). Handbook of Experimental Immunology, p. 373-387, Blackwell Scientific publication, Oxford, London.

Wells, K.F. (1975). In Newcastle Disease Virus and Spread, Monograph No. 11, Canada Department of Agriculture publication.

WHO Expert Committee (1971). Revised system of nomenclature for influenza viruses. Bull. Wld. Hlth. Org. 45: 119-124.

Wildly, P. (1971). Classification and nomenclature of viruses. Monographs in Virology, Vol. 5.

Wilson, G.S. and A.A. Miles (1955). The antibody forming apparatus and its reactions. In Topley and Wilson's Principles of Bacteriology and Immunology. Williams and Wilkins Co. Baltimore, U.s.A. 2: 1258-1296.

Wilson, D.E. (1958). The inactivation of Newcastle disease virus haemolysin by antiserum and high energy electrons. Proc. Soc. exp. Biol. Med. 99: 205-208.

Winterfield, R.W., C.L. Goldman and E.H. Seadale (1957). Vaccination of chicken with B1, F and Lasota strains of Newcastle disease virus administered through drinking water. Poult. Sci. 36(5): 1076-1088.

Yoder, H.W. and M.S. Hofstad (1964). Characterization of avian mycoplasma. Avian Dis. 8: 481-512.

Zakay-Rones, Z. and R. Levy (1973). Immunogenic response of chicks to inactivated Newcastle disease virus. Avian Dis., 17: 450-452.