CHARACTERIZATION OF NIGERIAN STRAINS OF NEWCASTLE DISEASE VIRUS

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D E D I C A T I O N

This thesis is dedicated to my lovely children

Olufunmilayo, Adewole and Oluwaseyi

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ABBREVIATIONS

AF	-	Allantoic fluid
BSA	-	Bovine Serum Albumin
CEF	-	Chicken Embryo Fibroblast
CPE	-	Cytoplathic Effect
CM	-	Centimetre
EDTA	-	Ethylenediaminetetra acetic acid
EID	-	Egg Infectious Dose
ELD	-	Fluorescent Antibody
GM	-	Geometric mean
HA	-	Haemagglutination
HI	-	Haemagglutination Inhibition
i/o	-	Intraocular
im	-	Intramuscular
I.U.	-	International Unit
MDT	-	Mean Death Time
MEM	-	Minimum Essential Medium
mg	-	Milligram
ml	-	Millilitre
mm	-	Millimetre
ND	-	Newcastle Disease
NDV	-	Newcastle Disease Virus
NP	-	Nucleoprotein

PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate Buffer Saline
PFU	-	Plaque Forming Unit
pi	-	Post Inoculation/Post Immunisation
rbc	-	Red Blood Cell
rpm	-	Revolution Per Minute
SDS	-	Sodium Dodecyl Sulphate
UAF	-	Uninfected Allantoic Fluid
VP	-	Virus Protein

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Natural outbreaks of Newcastle disease were investigated in different parts of Nigeria and samples for virus isolations were collected from vaccinated and unvaccinated commercial farms and indigenous chickens. Eleven NDV isolates were recovered from a total of 37 samples collected from chickens and one isolate was recovered from a parrot. All the 12 isolates were characterized as velogenic strains.

Two types of clones - small and large were isolated from the velogenic strains. Results of the pathogenecity characterization of the clones showed a significant difference in the degree of virulence between the small and large clones. A clone of low virulence UI-104-S similar to a lentogenic strain was successfully isolated from a velogenic strain. A further pathogenecity test on this clone is suggested with the aim of developing an attenuated live vaccine from it.

Eight of the isolated clones were antigenically compared with the 2 vaccine strains in use in Nigeria by the cross haemagglutination test, plaque reduction

neutralization test, polyacrylamide gel electrophoresis and cross protection test. All the *in vitro* tests showed significant antigenic differences between the clones derived from field samples and the vaccine strains. The *in vivo* cross protection test did not however reveal any significant antigenic diversity between the strains.

The susceptibility of chickens vaccinated with the three live vaccines in use in Nigeria to challenge by Nigerian velogenic strains was studied. Between 0 to 30% of birds vaccinated with the NDV-i/o vaccine died after challenge with the field virus. While no mortality was recorded in bird vaccinated with NDV i/o and boosted with NDV-Lasota, 10 out of 90 birds showed mild nervous signs. Birds vaccinated with NDV i/o Lasota and Komarov vaccines resisted challenge and showed no nervous signs. This result showed that the lentogenic vaccines in use in Nigeria are not immunogenic enough to confer the degree of immunity required to protect chickens against the prevalent virulent viscerotropic velogenic strains. Therefore it is recommended that the Vom recommendation on vaccination programme be strictly adhered to. However because of the prevalence of the extremely virulent velogenic viscerotropic strains of the virus a reappraisal of the

existing vaccines and vaccination procedures is advocated. It is therefore recommended that the use of inactivated oil emulsion vaccine be introduced in Nigeria.

Four experimental inactivated oil emulsion vaccines were prepared from two selected clones, UI-104-L and UI-104-S derived from Nigerian velogenic field strain and two vaccine strains currently in use in Nigeria. Result of immunogenecity and challenge tests showed that the oil emulsion vaccines prepared from the Nigerian strain UI-104L and the vaccine strain Komarov stimulated the highest antibody response in chickens. Birds vaccinated with these two vaccines resisted challenge at 10 and 20 weeks after challenge.

Results obtained from the study with the live and inactivated vaccines showed a similarity in their response and suggest that vaccines prepared from the moderately virulent and virulent strains are more effective than those prepared from the less virulent strains. Based on this, a comparative study of the duration of immunity of vaccinated chickens using both the live vaccines and inactivated oil emulsion vaccines is suggested for further study.

CHAPTER ONE

1.0 INTRODUCTION

Two decades ago, the poultry industry in Nigeria was mainly backyard based, even though one or two commercial houses were already operating in the country. The advent of the oil boom during the early seventies brought changes in many spheres of Nigerian life resulting in rapid urbanization and unprecedented demand for better quality nutrition among the ever increasing Nigerian population. The poultry industry responded magnificiently to this challenge, and an era of huge commercial, highly intensive ventures started. As the industry skyrocketted so also did the problems of intensivism.

Between 1976 and 1983 an average of N20 million was disbursed annually as loans for poultry production in the country (Nawathe, personal communication). Despite this, the poultry industry in Nigeria has not done well as evidenced by the high cost of eggs and other poultry products. Two major constraints face the industry in Nigeria, and these are high cost of feeds and disease. On one hand, the high cost of feeds can be attributed to

the importation of raw materials needed for feeds production, while on the other hand, the various poultry diseases - viral, bacterial and parasitic - have continued to take their toll in the poultry industry and this has limited the expansion of the industry.

One of the earlier diseases to be recognised was Newcastle disease (ND) and presently it is the single most dreaded disease of poultry industry. Ever since its first recognition in Nigeria in 1953 (Hill et al) ND has since spread widely and now constitutes the major threat to the industry in Nigeria. Between 1977 and 1978, 21 out of 82 reported disease outbreaks in birds were due to ND. Out of 49,931 birds with ND, 26,348 or 52.7 per cent died. This represent 77.6 per cent of all deaths due to reported disease outbreaks. By 1982, and despite improvement in vaccine delivery and better education, ND still accounted for 17 out of a total of 57 poultry disease outbreaks involving a total of 133,734 birds out of which mortality due to ND was 17,789. This represents mortality rate of 74.7 per cent of all birds dying from reported diseases in Nigeria in 1982 (Table 1).

The economic losses due to the disease is enormous in terms of mortality rate, reduced meat and egg production

Table 1: Poultry Disease Outbreak in Nigeria

	1977–1978				1982			
	No. of Outbreaks	No. of birds involved	Mortality (%)	Mortality Total (%)	No. of Outbreak	No. of birds involved	Mortality (%)	Mortality Total (%)
Newcastle disease	21	49931	26348 (52.7)	77.6	17	62621	17789 (28.3)	74.7
Gumboro Disease	38	62123	7202 (11.5)	21.2	21	28488	4962 (17.4)	20.8
Fowl Pox	19	5272	258 (4.8)	0.8	3	5700	37 (0.64)	0.20
Mareks Disease	4	5900	130 (2.2)	0.4	-	-	-	-
Chronic Respiratory Disease (CRD)	-	-	-	-	2	2000	18 (0.9)	0.08
Fowl Typhoid	-	-	-	-	5	9432	808 (8.6)	3.40
Coccidiosis	-	-	-	-	6	7000	128 (1.8)	0.54
Aspergillosis	-	-	-	-	1	10000	54 (5.4)	0.23
Avian Lgeconis	-	-	-	-	2	8500	17 (0.2)	0.07
Total	82	123226	33938	100	57	133743	23813	100

- = No outbreak reported.

Source: Federal Livestock Department, Kaduna.

resulting from poor feed conversion, impaired growth and listlessness (Nawathe, 1983).

ND is a contagious viral disease of chickens turkeys and various other birds. Man could also be affected (Utterback and Schwartz, 1973, Onunkwo and Momoh, 1980). The disease is caused by the Newcastle disease virus (NDV), belonging to the genus Paramyxovirus of the Paramyxoviridae. Today one or more forms of ND are present in all poultry producing countries of the world. Depending on the strain causing an outbreak, mortality rate among young and adult birds can be as high as 50-100 per cent. (Kranelveld, 1926, Doyle, 1927; Sahai, 1937; Cornel, 1940; Kashula, 1961; Reid, 1961). Many clinical forms of the disease have been recognised. Clinical presentation of the disease could be a very mild disease with transient respiratory signs, or a fatal disease with respiratory signs.

In some cases, severe disease may be characterised by neurologic signs, total paralysis and death. In other severe forms, the disease may be characterised by haemorrhages and necrosis of the gastrointestinal tract and a lack of neurologic signs (Beard and Brugh, 1975). Tho respiratory and nervous forms are common in Europe and North America, while the enteric form is common in the

tropics. In recent years, there had been severe epizootics starting in South-East Asia and spreading to South and Central America, Europe and South Africa, killing 100% of unvaccinated and 20% of vaccinated birds. The new strain of ND virus responsible for these epizootics have been designated as viscerotropic virulent Newcastle virus (VVNDV) because of its high pathogenecity and the production of lesions in the form of necrosis of the reticulo-endothelia system and visceral organs (Cherille et al, 1972). In Nigeria, the disease is essentially acute and peracute type, exhibiting enteric form resembling Asiatic type (Kaschula, 1961, Majiyagbe and Nawathe, 1981).

There is no treatment once the disease has set in. The major measure of control of the disease in Nigeria today is by vaccination, using the three types of vaccine produced in the National Veterinary Research Institute, Vom.

The increased recurrence of ND in vaccinated poultry is attributed to:-

1. Poor management and debilitation through latent intercurrent diseases

2. Possibility of immunological difference and difference in pathogenecity and degree of virulence between the vaccin strains and the existing wild (but unknown) strains, and
3. Improper use of available vaccines (Nawathe, 1983).

As the ND vaccines used in Nigeria are produced from virus seeds isolated from Holland and Israel, it is speculated that this may account in part for their failure. It is possible that the available vaccines are no longer adequate to protect the birds from subsequent challenge by the velogenic strains available in the country.

There is therefore an urgent need to determine strain distribution of NDV in Nigeria and compare their strain and antigenic variations with the vaccine strains in use, and if significantly different from the available vaccine strains, to produce a vaccine from a local Nigerian strain.

1.1 Objective of the Present Study

The aims and objectives of the study are:-

(a) To survey for and isolate Newcastle disease virus strains from different parts of Nigeria.

(b) To characterise antigenically and compare the isolated strains with the vaccine strains currently in use in Nigeria; and

(c) To prepare and test a vaccine from a local Nigerian strain found to be significantly different from the available vaccine strain in terms of virulence and pathogenecity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of the Disease

The agent of ND was first described by Doyle in 1926 (Doyle, 1927) following the isolation of a filtrable agent from diseased birds. The epizootic spread over the northern English coast around Newcastle-Upon-Tyne (from which the common name of the disease was derived). In England (Doyle, 1927),,Java (Kraneveld 1926) and Korea (Konno et al, 1929), where the disease was first recognised, it first occurred in and around seaports apparently as a result of commercial movement by sea. Only much later in these countries did it appear further inland away from the seaport. Accounts of the various facts of the history of ND including its recognition in other areas and possible mode of spread have been summarised and reviewed by Brandly et al, 1946; Beaudette, 1951; Hanson, 1964; and Lancaster, 1966.

2.2 Origin of the Virus

Three hypotheses exist for the origin of Newcastle disease virus (NDV) and indeed any other virus (Hanson, 1972).

1. That the virus originated at some point in time and place as a result of a major mutation or series of mutations from a related virus.
2. That the virus existed in its present form in other species, probably a vertebrate animal, and only contact with domestic chickens introduced the virus into the poultry populations, and
3. That the virus has always been present as an unrecognised and presumably mild virus disease of chicken and that changes in the nature of host population gave rise to viral mutants of greater virulence which in its turn gave rise to the present overt and destructive disease (Hanson, 1972).

While the third hypothesis seemed to be most tenable, it is however premature to decide which of the hypothesis best accounts for the origin of the virus.

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2.3 Incidence and Distribution

ND was first recognised in three widely separated countries Java (Kraneveld, 1926) England (Doyle 1927) and Korea (Konno et al 1929). The disease was later reported in India (Edwards, 1928) and the Phillipines (Farinas, 1930). Within 10 years after the first report of ND, the disease had been reported in Australia, (Johnstone, 1931), Japan (Nakama et al, 1933) and East Africa (Hudson, 1937). By the beginning of the world war II, ND had appeared in several countries of the Middle East (Komarov, 1940) and almost all the countries of Europe (Ecker', 1957). The disease was first recognised in the United States in 1944 (Minard and Jungherr, 1944) and Canada in 1948 (Walker, 1948). Today one or more forms of ND are present in all poultry producing nations of the world.

In Nigeria, a tentative diagnosis of the disease was made in April 1951 in two outbreaks in some poultry farms in Eastern Nigeria (Kirby, 1951). However, it was not until 1952 that outbreaks of ND were first reported and confirmed from Ibadan and other parts of Western Nigeria (Hill et al. 1953). Presently ND constitutes one

of the most dreaded fatal poultry diseases of economic importance in Nigeria (Uzoukwu, 1967; Fatunmbi and Adene, 1979). Between 1981 and 1985 confirmed outbreaks of ND ranged from 8 in Borno State to 74 in Lagos State (Annual Report, Nat. Veterinary Research Institute, Vom, 1981-1985)

2.4 Aetioloov of the Disease

The causative agent of ND is a filtrable virus. It is a paramyxovirus, and has an estimated particle size of 80-120mu (Burnet and Fenny, 1934). Like other members of the paramyxoviruses, it has an ether sensitive envelope with projections and spikes which contain the antigenic components responsible for the production of the haemagglutin inhibiting (HI) and virus neutralizing antibodies (NA) (Rott, 1964).

Newcastle disease virus possesses a number of biological and physical properties by which it may be distinguished from other myxoviruses and by which strains of NDV may be distinguished from each other. Foremost among the biological properties is the ability to adsorb to the surface of red blood cells and induce their aggregation (haemagglutination). The haemgglutination properties of NDV was first described by Burnet (1942).

The inactivating effects of chemicals on NDV is dependent on the suspending medium; formalin, beta-propiolactone (Mack and Chotisen, 1955) and phenol have been used to destroy the infectivity without severely damaging the immunogenecity of the virus. The virus may be inactivated in one minute at 100°C and in 30 minutes at 60°C. At 37°C, NDV activity declines in a matter of days. The virus is destroyed by ultraviolet light as well as several chemical disinfectants. NDV is stable within a pH range of 2 to 10 (Moses et al, 1947).

2.5 Pathogenesis and Epizootology

2.5.1 Natural and Experimental Hosts

Domestic chickens and turkeys are most susceptible to NDV. However, the virus has been isolated from starling (Gillespie, et al, 1950); dove (Hanson and Sinha, 1952) and sparrow (Gustafson and Moses, 1953). NDV has been isolated from migratory water fowls such as swan, geese and ducks (Asplin, 1947; Palmer, 1969; Majiyagbe and Nawathe, 1981). The disease may be mild or acute in this group of birds depending on the immune status of the birds and the strains of NDV. Experimental

infection of these birds via the natural routes however often results in transitory infection. Such birds become carriers shedding the virus in either the nasal secretions or in faeces and later develop antibodies following convalescence. There is no substantial evidence of seasonal differences in the prevalence of ND. The virus is known to be present in all seasons in both temperate and tropical regions.

2.5.2 Transmission and Carriers

The primary mode of transmission of NDV within a flock is by aerosol. Within 2 days after exposure and a day before showing clinical signs of disease, the infected birds begin to release virus in the air through either the nasal secretion or faeces. Within a short time the population of infective virus builds up considerably in the air (Sinha *et al*. 1954). The virus remains in the air through the turbulent activity of the birds. Larger particles settle at night while the smaller particles remain air-borne for days.

Dissemination between flocks over long distances has been frequently related to (a) movement of carriers

(Jungherr and Terrel, 1946). (b) introduction of diseased tissues such as offals into feeds (Dobson and Simmins, 1951) and (c) contamination of feed and water (Gustafson and Moses, 1953). The disease may be transmitted mechanically by man through clothing and foot gears and artifacts such as crates , feed sacks and trucks (Jungherr and Markham, 1962). It is not clear whether the virus can be passed from the eggs to the hatchling however evidence abounds that infection and death of embryo from eggs laid by diseased birds may occur during the first 4-5 days of incubation.

Other domesticated fowls may serve to transport and perpetuate the disease. Turkey which usually suffers less severely than chickens have been suspected of being the initial foci of epizootics in certain regions. In Nigeria, the local free roaming chickens have been shown to be the constant source from which the more virulent velogenic strains continue to challenge the more susceptible exotic birds (Nawathe et al, 1975, Adu et al, In Press). In other instances, incompletely inactivated vaccine, (Placidi, 1956; Spalatin and Hanson, 1968) or active virus (Hanson et al, 1967) contaminating a vaccine may be the source of infection in flock.

It has been shown by Rogoff et al (1977), that flies were able to transmit NDV for as long as 6-10 days post-exposure to the virus.

2.5.3 Clinical Signs, Morbidity and Mortality.

The incubation period of ND following exposure has been reported to vary from 2 to 15 days. The incubation time as well as severity of the disease vary with the age of the birds (Hanson, 1972). An outbreak of ND may be so acute and severe as to kill all or nearly all the birds in a flock within 3 to 4 days. On the other hand, the disease may be so mild that symptoms are scarcely noticeable.

The clinical signs, symptoms, morbidity and mortality of ND vary depending on the virulence of the strains causing the disease, environmental conditions and state of the flock (David West, 1972, Hanson, 1975). Four forms of the disease are therefore recognised:

(a) Doyle's form which is characterised by sudden onset of the disease in which birds are found dead without having shown any signs (Iyer, 1943). Often the birds may appear listless with laboured

respiration. A profuse watery greenish diarrhoea may be noticed, weakness becomes more apparent, and death, preceeded by prostration, occurs in 5-7 days.

(b) The Beach form - The Beach form is characterised by sudden onset and rapid spread of the disease with respiratory distress, coughing and gasping more marked than in the Doyle's form (Beach, 1943). Birds are off feed and egg production may drop or stop completely. Diarrhoea may not be ooserved, but nervous signs characterised by paralysis of the legs and wing and torticollis appear within a day or two after the onset of the disease. In this form mortality can be as high as 50% among adult birds and 90 per cent in young birds (Bins et al, 1949).

(c) The Beaudette form is an acute respiratory disease with coughing but rarely gasping. Egg production may fall or cease completely, and occasionally birds do not return to normal production (Platt, 1948).

(d) The Hitchner form of ND is the mildest of all the forms and is characterised by rales. This form is often inapparent in adult birds (Bankowski, 1961) and death losses are uusually negligible, although in young birds, mortality may be as high as 40 per

cent, particularly when it is complicated with other infections (Asplin, 1952). Nervous signs are not usually associated with this form.

2.6 Pathology

The pathological changes found in ND vary greatly from bird to bird, flock to flock and from one geographical region to another. Among the factors that create these differences are the virus strains involved, the route of exposure, the severity of exposure, the age and breed of birds and the environment. Readily recognizable changes are seldom found in inapparent forms and are almost invariably found in birds that died of the severe forms of the disease. Truly pathognomonic lesions have not been recognised for ND. If gross lesions are to be useful in diagnosis, it is important to remember that all the changes found in ND also occur in other diseases and that the overall patterns encountered are more significant than are individual changes.

Informations on the pathology of ND have been documented by Jungherr and Minnard, 1944; Jungherr et al, 1946; Jungherr, 1964.

The Doyle's form of the disease is characterised by dark red or purplish red haemorrhagic lesions in the intestinal wall presenting themselves as small red areas having definite but somewhat irregular borders. The diphtheroid necrotic inflammation of the intestinal mucosa simulates the "button" ulcers of hog cholera and is considered to be thoroughly suggestive of Newcastle disease. Haemorrhages are sometimes seen in the enteric form.

In other forms of ND, the primary lesions are found in the respiratory tract. A serous or catarrhal exudate is present in the nasal passages, larynx and trachea. Haemorrhages may occasionally be found in the trachea. Lungs are usually normal, although the lower anterior portion may sometimes be pneumonic (Beard and Hanson, 1984).

Few, if any, gross lesions are observed in older chickens that die from velogenic viscerotropic Newcastle disease (VVND) with a history of vaccination. However, haemorrhagic and necrotic ovaries and ruptured yolks in the peritoneal cavity are frequently seen in non-immune hens. If chickens have low levels of immunity from an earlier vaccination, they may survive infection with VVND virus for 2-3 weeks before developing nervous signs that

clincally appear indistinguishable from those resulting from Beach's neutropic virus strain (Beard and Hanson, 1984).

Histologically, hypaeraemia, oedema, haemorrhages and other blood vessel changes are found in various organs. Other vessel changes consists of hydropic degenerations of the media, hyalinization of capillaries and arterioles, development of hyaline thrombosis in the small vessels and finally, necrosis of the endothelia cells of the vessels. Details of histological changes in the trachea, lung, liver and intestines have been well documented (Jungherr, et al. 1946, Decock, 1954).

Encephalitis is not present in the inapparent forms of the disease, and may not be present in some birds affected with more severe enteric forms; however, abnormality of the central nervous system (CNS) seems to be the most histologically significant features of all the forms wherever they are present. It is generally believed that glia foci, neurotial degeneration, peri-vascular lymphatic infiltration and hypertrophy of endothelia cells are found in CNS of affected chickens.

Lesions are fairly distributed throughout the CNS except the cerebrum (Beard and Hanson, 1984).

2.7 Immunity

All NDV strains are capable of provoking antibody response in chickens, rabbit and other species into which they are introduced. The ND virion contains several antigens. The antigens that induce the virus neutralizing antibody and the haemagglutinating antibody are associated with the envelope of the virus. The nucloprotein (NP) or soluble antigen is associated with the nucleoprotein portion of the virus and is distinguishable from the HA antigen and the virion associated antigen.

The virus neutralizing antibody effectively blocks the ability of the virus to totally infect chickens, chicken embryos and cell cultures. Such birds may develop subclinical infection and shed the virus for a short period of time. Resistance to reinfection is usually associated with the presence of moderate to high titre of neutralizing antibodies (Levine et al, 1950). Antibodies appear in the serum of chickens 6-10 days and reach peak titre 3-4 weeks after introduction of infection.

Minor antigenic differences exist among NDV strains (Upton et al, 1953). Strains can be distinguished on the basis of the gel diffusion test, and the virus neutralization tests. These differences are both qualitative and quantitative in nature (Schloer, 1964).

2.8 Diagnosis

The unequivocal method of diagnosing ND is the isolation and identification of the causative agent, and subsequent reproduction of the disease in susceptible chickens. The virus can be easily isolated from the trachea and cloaca of live birds and the lungs, brain, spleen, liver, kidney and bone marrow of deceased birds.

Various systems and tests have been established for the diagnosis of ND. The embryonated chicken eggs is preferred for isolation. Fertile eggs from healthy non-immune hens which have been incubated for 9-11 days at $37^{o}C$ are injected with suspensions of suspected tissue into the allantoic chamber. Antibiotic agents usually a mixture of penicillin and streptomycin are added to the inoculum to suppress any bacterial agent which may be present.

Newcastle disease virus can be readily propagated in primary cells of avian origin and in some mammalian primary cells (Tepacio, 1934; Chanock, 1955). However, chicken

embryo fibroblast or chicken embryo kidney cells grown as monolayers are more commonly used for isolation of the virus (Bankowski, 1964).

The Haemagglutination (HA) and Haemagglutination-Inhibition tests (HI) have provided a simple and useful diagnostic method.

The fluorescent antibody procedure allows more rapid detection of NDV infection than HI or regular egg inoculation (Maestrone and Caffin, 1961). The procedure has been used in the studies of pathogenesis of the disease (Beard and Easterday, 1967). However, the widespread use of the viable vaccines has made the demonstration of NDV antigen by the FA test in diagnostic applications of limited significance. Mcferran et al, (1978) have reported the use of electron microscopy in identifying NDV in avian clinical specimens.

Other diagnostic tests include ELISA (Synder, et al, 1983; Miers, et al, 1983) and peptide mapping of the NDV proteins (Naggi et al, 1985).

2.9 Prevention and Control

The simplest and most logical measure against ND is to prevent contact of the virus with the susceptible birds. Another is by vaccination which gives the bird a degree of protection against infection. The latter is most

significant and generally accepted means of control, however a combination of sanitary management (to reduce chance of exposure) and a vaccination programme is required to combat a highly contagious disease such as ND wherever it has become widely established in a community, region, or country. Both measures must be systematically carried out on an area basis if control is to be reasonably effective.

2.9.1 Vaccination

Control of ND by the use of vaccines will only be fully successful when vaccination itself is fully effective. Programmes of vaccination must be such that an effective immune response is achieved as early in life as possible and factors affecting vaccinations must be more clearly understood and controlled.

Patterns of immunization developed in different areas depend on the nature and virulence of the local field strains. In tropical and mediterranean countries, the field virus has tended to be more virulent, and a system involving primary vaccination with a mesogenic form of the virus has been found to be more effective.

Two types of vaccines - live attenuated vaccines and inactivated vaccines are generally in use. Live NDV

vaccines are usually prepared by growing in embryonating eggs modified or "weakened" strains of the virus (Brandly et al, 1946; Hitchner, 1964; Lancaster, 1964), or naturally occurring strains of lentogenic and mesogenic origins (Hitchner and Johnson, 1948; Winterfield et al, 1957). Avian cell cultures have also served as a source of ND vaccine (Bankowski and Boynton, 1948, Bankowski 1950).

Inactivated or killed NDV vaccines are prepared by growing suitable antigenic strains of the virus in embryonating eggs, harvesting the dead or dying embryos and tissues (Hanson, et al, 1951; Hofstad, et al, 1963) and inactivating the virus usually by chemical agents such as formaldelhyde (Brandly et al, 1946) or beta-propiolactone (Mack and Chotisen, 1955). Adjuvants are usually added to inactivated vaccines to prolong and increase the immunising effects (Traub, 1944).

While both live and inactivated vaccines will stimulate a substantial degree of immunity in a large proportion of healthy fowl (Levine and Falscricant, 1952), both vaccines have some advantages and disadvantages over each other. Live vaccines are easy to apply and are usually required in smaller doses while inactivated vaccines are more labour intensive in terms of application, and require usually higher titre of the virus to stimulate immune response. The titre evoked by living vaccines are usually

higher than those by inactivated vaccines, yet, only the later may be relied upon to prevent some undesirable effects of vaccination in laying flocks or in stocks suffering from other diseases (Gross, 1961). Inactivated vaccines are more likely to withstand less favourable environmental conditions than the live vaccines.

Many workers have recently reported oil emulsion vaccines to be effective in inducing long lasting high titres of haemagglutination inhibition (HI) antibodies in chicks that had low level of maternal antibodies (Box, 1965; Cessi and Narcelli, 1974). Other reports show the effectiveness of oil emulsion vaccines when administered either alone or in combination with live vaccines (Phillips, 1973; Partadiredja, et al, 1979).

Since the first reported case of ND in Nigeria, the National Veterinary Services, had adopted several prophylactic measures, the most significant of which is vaccination. NDV vaccine was introduced into Nigeria ever before the disease was recognised (Hill, et al, 1953). Following outbreak of the disease in the neighbouring Congo, the Komarov vaccine (Komarov and Goldsmit, 1946), was imported from South Africa to protect the local flock. For quite a long time, the Komarov vaccine was the only one in use. Difficulties encountered with the imported

Komarov vaccine from South Africa as a result of poor transportation network within the country eventually led to the beginning of local production of the vaccine in Vom in 1953 (Crowther, 1953).

In an attempt to find a mild vaccine, devoid of the disadvantages of hot strain vaccine, the intraocular vaccine strain obtained from Israel was introduced into Nigeria (Thorne, et al, 1960). This was followed in 1964 by the production of the Lasota vaccine, (Owolodun 1968) the seed virus of which was obtained from Holland. Through continued research work, a comprehensive policy of immunization was established using the three vaccines now produced at the National Veterinary Research Institute, Vom.

CHAPTER THREE

3.0 CLASSIFICATION OF NIGERIAN ISOLATES OF NEWCASTLE DISEASE VIRUS

3.1 Introduction

NDV isolates are classified on the basis of virulence, biological and physical properties into three strains - the lentogenic, mesogenic and velogenic strains (Hanson and Brandly, 1955). However, individual member of each strain shows genetic heterogeneity and exhibits differences in ability to induce diseases and respond to laboratory test (Cranoff, 1964; Hanson and Daniel, 1968).

The lentogenic strain is the avirulent form and is the strain used for producing vaccines for young chickens. The mesogenic strain is a milder strain of moderate virulence and is the strain used for vaccine production for older chickens, while the velogenic strain is the strain with the highest virulence and is often used as challenge virus. The three strains exhibit differences in their pathogenecity for adult chickens. While both the lentogenic and mesogenic strains will cause a drop in egg production with rapid and complete recovery, the

velogenic strain will kill the chicken in 4-14 days.

In this present study, isolations from ND outbreaks in vaccinated and unvaccinated poultry flocks in Nigeria were studied, using the different techniques as described in "Methods of Examining Poultry Biologics and for identifying and quantifying Avian Pathogens" (National Academy of Sciences, Washington D.C., 1971).

3.2 Materials and Methods

3.2.1 Sample Collection

Natural outbreaks of ND were investigated in different parts of Nigeria and samples for virus isolation were collected from vaccinated and unvaccinated flocks in commercial farms and backyard poultry farms. Exotic and indigenous breeds of chickens were involved in these outbreaks. Samples which included spleen, trachea, liver, intestine and brain were collected separately in universal bottles and preserved in ice until transported to the laboratory.

3.2.2 Embryonated Eggs

Nine to ten day-old embryonated eggs used for virus isolation were supplied from the flocks of the National Veterinary Research Institute, Vom. The flocks were certified free of pathogenic organisms and negative for NDV antibodies.

3.2.3 Virus Isolation

Twenty percent organ suspensions in phosphate buffered saline (PBS) were made and centrifuged at 3000 rpm for 15 minutes at $4^{o}C$. The suspensions were supplemented with 250 I.U. of penicillin and 250mg of streptomycin per ml. One-tenth ml each of a 10^{-1} dilution of the suspension was inoculated into the allantoic sac of the embryonated eggs, using 10 eggs per specimen.

Inoculated eggs were incubated at $37^{o}C$ and candled twice daily for 15 days. Dead embryonated eggs were separated and chilled over-night before ready for harvest. Allantoic fluids (AF) were harvested by first disinfecting the eggs with 10% iodine tincture. Using a sterile pair of scissors and forceps, the eggs were cut open and the AF were gently sucked out with a sterile 20ml syringe. Allantoic fluids were clarified by centrifugation at 3000 rpm for 15 minutes at $4^{o}C$ and subpassaged undiluted

at a volume of 0.1ml into a new set of 9-10 day old embryonated eggs. Three to four eggs passages were done before virus identification. The viruses were identified by the haemagglutination (HA) and Haemagglutination Inhibition (HI) Tests.

3.2.4 I Haemagglutination (HA) and Haemagglutination Inhibition (HI) Tests

The haemagglutination test was done in 96 - well Linbro U-bottom microtitre plates (Linbro Scientific, Inc) using 50 microlitre droppers and diluters. Serial two fold dilutions of the viruses were made in 0.85% normal saline starting from 1:5 dilution. Fifty micro litres of 0.5 per cent red blood cells (rbc) were added to each well and the plates were allowed to stand at room temperature for 30-60 minutes before readings were taken. The HA titres were recorded as the dilution of the viruses showing 50 percent haemagglutination (Allan and Gough, 1974, Allan et al, 1978). This was used to calculate the 4 haemagglutination unit used in the HI test.

The beta method i.e. constant antigen varying serum dilution method was used in the HI test. Serial two fold dilutions of a known NDV positive serum was made in 0.85% normal saline starting from 1:5. Each well contained 50

microlitres of the serum. An equal amount of the virus diluted to contain 4HA units was added to all the wells, and mixed by shaking. Fifty microlitres of 0.5% rbc were added to each well and the plate was incubated at room temperature for 30-60 minutes. A control test, using a known NDV negative serum was done simultaneously and both results were read at the same time.

3.2.5 Virus Stock Preparation and Titration

Stock viruses were prepared in 9-10 day old embryonated eggs. Each of thirty 9-10 day old embryonated eggs was inoculated with 0.1 ml of a 10^{-5} dilution of NDV isolate at the 4th passage and incubated at 37°C. Eggs were candled twice daily. Dead and moribund eggs were chilled overnight at 4°C until ready for harvest. Eggs were harvested as described above and the AF were collected in centrifuge tubes. The AF were clarified at 3000 rpm for 15 minutes after which the clarified AF was supplemented with 250 I.U. of penicillin and 250mg of streptomycin per ml.

The AF from each isolate was titrated in 10 day old embryonated eggs. Ten fold serial dilutions of 10^{-5} to 10^{-15} of the AF was made in PBS and 0.1ml of each dilution inoculated into five embryonated eggs. The eggs were

incubated at 37°C and candled twice daily. The titre of each isolate was calculated by the method of Reed and Muench (1938).

3.2.6 Characterization of NDV Isolates

Strain characterization of isolates was done as described in "Methods of examining poultry Biologics and for identifying and quantifying Avian Pathogens. Controls were the vaccine strains - NDV i/o, NDV-Lasota and NDV-Komarov and one challenge strain - NDV-Herts 33/56. The following characteristics were tested for: Mean Death Time (MDT), Minimal Lethal Dose (MLD), Intracerebral Pathogenecity Index (ICPI) for day old chicks, Heat stability of haemagglutinatinin; Rate of elution, Haemagglutination of mammalian erythrocytes and plaque formation in cell culture monolayers. The procedures of the tests are given below.

3.2.6.1 Determination of Mean Death Time (MDT)

Ten fold serial dilutions of the AF were made in PBS and 0.1ml of the dilutions from 10^{-1} to 10^{-10} were each inoculated into five 9-10 day old embryonated eggs at 8 a.m. The eggs were incubated at 37°C.

At 4.00 p.m., fresh serial dilutions of the AF were made and inoculated as described for the 8 a.m. innoculation into five 9-10 day old embryonated eggs and similarly incubated at 37°C. By inoculating five embryos per dilution at 8 a.m. and repeating the titration with the same dilutions at 4 p.m., observations of the death time were made at 8 hourly intervals within the usual work day thus avoiding the error of unequal observation periods. Both sets of inoculated eggs were candled twice daily at intervals of 8 hours (8 a.m. and 4 p.m.) beginning 24 hours and terminating 128 hours post inoculation. Embryo dying at 24 hours were counted. Dead eggs were recorded against the hour post inoculation. The Mean Death Time was calculated using the formula.

$$MDT = \frac{(\text{No. of dead eggs at A hours}) \times (\text{A hours}) + (\text{No. of dead eggs at B hours}) \times (\text{B hours}) + \frac{(\text{No. of dead eggs at N hours}) \times (\text{N hours}) \text{ etc}}{\text{Total number of dead eggs.}}}{}$$

Where

A, B and N are the respective hours post inoculation when dead eggs were observed.

3.2.6.2 Minimal Lethal Dose (MLD)

The highest dilution at which all the inoculated embryos died was regarded as the minimal lethal dose.

3.2.6.3 Intracerebral Pathogenecity Index (ICPI) for day old chicks

0.05ml of a 10^{-1} dilution of the AF was inoculated intracerebrally into each of ten 1-day old chicks. Chicks were observed daily for 8 days. At each observation, birds were observed for state of health and scored as follows: 2 for dead, 1 for birds sick or showing signs of ND and 0 for normal birds. All deaths were considered specific since NDV can kill intracerebrally in less than 24 hours. Birds that died in previous observations were always recorded as dead at each subsequent observation. The ICPI was calculated as the total sum of the values divided by the numbers of observations.

3.2.6.4 Heat Stability of Haemagglutinin

One ml each of undiluted virus preparations was placed in a lyophilizing ampoule and flame-sealed. Ampoules were submerged in a water bath of 56°C. One ampoule was removed at a time interval of 0, 15, 30, 60 and 120 minutes and

tested for haemagglutinin by the standard methods described above. Samples removed at 0 minute were used as control. Absence of haemagglutination at a dilution less than 1:5 was considered negative, and not stable at the given time interval.

3.2.6.5 Absorption of Haemagglutinin by Brain Cells

Brain cell suspension was prepared from the heads of 12 day old chicks. Whole brains from 25 chicks were washed in PBS to remove the membranes and debris. The brains were mercerated in a 10 ml syringe and resuspended in 10 ml of PBS. The suspension was centrifuged at about 3000 rpm for 15 minutes at 4^{o}C. A 1:10 dilution of the supernatant in PBS was used for the absorption experiment. To 0.5 ml of undiluted AF was added 2 ml of the supernatant in a tube. Absorption was allowed to take place at 4^{o}C for 15 minutes in an orbital shaker, and the tubes were centrifuged again at 3000 rpm at 4^{o}C for 15 minutes. HA test was done on both the absorbed and unabsorbed virus preparation. The difference between the HA titre of the unabsorbed and the absorbed virus represents the amount of haemagglutinatinin absorbed.

3.2.6.6. Haemagglutination of Mammalian Erythrocyte

0.5 percent rbc from whole bovine and equine blood cells in normal saline were used in HA tests as described above. The plates were incubated at room temperature and the HA titre of each virus was read. Haemagglutination is considered negative if the HA titre is less than 1:5.

3.2.6.7 Rate of Elution

A standard haemagglutination test was performed on the virus at 4°C and the HA titres were determined after the chicken erythrocytes had settled. Incubation was continued at 4°C for 24 hours when the titre was read again. The erythrocytes were then resuspended and the titres were read again after 2 hours at 4°C and again left for 24 hours before the final titre was read. All procedures were carried out at 4°C for reproducible results. The rate of elution was determined by the length of time agglutination persisted.

3.2.6.8 Plague Formation in Cell Culture Monolayers

Chicken embryo fibroblast (CEF) cells were prepared from 9-10 day old embryonated eggs. The eggs were disinfected with 10% iodine tincture, and cut open with sterile scissors. The embryo was carefully removed and placed in a beaker containing Hank's medium.

The extremities and the heads were removed and the remaining washed in Hank's medium to remove cell debris. The washed embryos were marcerated by passing through a 10 ml syringe, and further washed in Hank's medium. 20ml of 0.25% trypsin was added to the cell in an Erlenmenyer flask containing a magnetic stirring rod. Trypnization was done for 1 hour at 37°C on a magnetic stirrer. Cells were passed through a sterile muslin and centrifuged at 3000 rpm for 30 minutes at 4°C. The supernatant was discarded and the cell precipitate was diluted in medium E. 199 containing 10 percent calf serum and supplemented with 250 I.U. penicillin, 250 mg steptomycin and 250mg of fungizone. Petri dishes (60 mm) were seeded with 5 ml of cell suspension containing 250,000 cells per ml, and incubated at 37°C in an atmosphere containing 5% CO_2 for 24-48 hours. Confluent CEF were inoculated with 0.1 ml of virus dilutions containing between 10 to 10,000 units

of virus per ml of the isolates and 5 ml of E. 199 containing equal amount of twice concentrated E. 199 and supplemented with 5% calf serum and 1.6 percent Noble Agar were added. Plates were incubated at 37°C for 3 days in 5 percent CO_2 after which another 5 ml of a medium containing equal amount of 2X E.199 supplemented with 2% calf serum, 1.6% Noble Agar and Neutral Red at a final concentration of 0.01 percent were added. Plaque type and morphology was read 2-4 hours after.

3.3 Results

3.3.1 Virus Isolation

A total of 38 samples (37 from chickens and 1 from a parrot) were collected between 1980 and 1983). Eleven NDV isolates were recovered from the chicken samples while the only parrot specimen yielded another NDV isolate. Three isolates were recovered from Oyo State while two isolates each were recovered from Anambra, Plateau and Imo States. Sokoto, Kano and Kaduna States recorded one isolate each. Details are shown in Table 2.

Table 2. Newcastle Disease Virus Strains isolated in different parts of Nigeria.

Year of Isolation	Location of Isolation (State)	Isolate	Organ	Source	Passage level in embryonated
1980	Sokoto (Sokoto State)	SLS - 1075	Lung	Backyard poultry	4
1980	Kano (Kano State)	K - 1090	Lung	Indigenous local chicken	4
1980	Bukuru (Plateau State)	NDV - P	Pool Organ	Parrot	4
1982	Ibadan (Oyo State)	UI - 104	Brain	Commercial poultry	4
1982	Kaduna (Kaduna State)	K - 1084	Spleen/ Liver	Commercial poultry	4
1983	Ibadan (Oyo State)	NY - 1	Intestine	Commercial poultry	4
1983	Ode-Omu (Oyo State)	OM - 1	Spleen/ Lung	Vaccinated Commercial poultry	4
1983	Vom (Plateau State)	KB	Intestine/ Lung	Backyard poultry (Broilers)	4
1983	Umudike (Imo State)	UM - 651	Lung	Indigenous local chicken	4
1983	Umudike (Imo State)	UM - 151	Lung	Vaccinated Commercial poultry	4
1983	Orji River (Anambra State)	OJR - 360	Liver	Vaccinated poultry	4
1983	Orji River (Anambra State)	OJR - 753	Lung	Commercial poultry	4

3.3.2 Virus Identification

The 12 virus isolates were identified as NDV strains by the demonstration of haemagglutinin which in HI test was inhibited by a known specific NDV antiserum. The HA titre ranged between $Log2^{7.3}$ and $Log\ 2^{9.3}$ HA_{50}, and were all inhibited by the NDV specific antiserum at a dilution of 1:320.

3.3.3 Characterization of NDV Isolates

Results of strain typing of the field isolates are summarised in Table 3.

3.3.3.1 Mean Death Time (MDT)

The MDT of all the field isolates ranged from 45 to 56 hours which is similar to the MDT of NDV-Herts. The MDT of the intraocular, Lasota and Komarov strains are 120, 120, and 68 respectively.

3.3.3.2 Minimal Lethal Dose (MLD)

All the isolates had an MLD between 10^{-9} and 10^{-10}, which is similar to the MLD of NDV-Herts. The intra-ocular, Lasota and Komarov strains had MLD of 10^{-0}, 10^{-0} and 10^{-7} respectively.

3.3.3.3 Intracerebral Pathogenecity Index (ICPI)

The ICPI of the field isolates ranged from 1.3 to 1.9. The ICPI for the intraocular, Lasota, Komarov and Herts were 0.00, 0.00, 0.53 and 1.5 respectively.

3.3.3.4 Heat Stability of Haemagglutinin

All the isolates were stable at 56°C for 120 minutes except K-1090 and OM-1 which were stable for 15 and 10 minutes respectively. NY-1 was not stable at any of the given time. The intraocular and the Lasota vaccine strains were not heat stable but the Komarov and the Herts strains were stable for 120 minutes at 56°C.

3.3.3.5 Absorption of Haemagglutinin

The percentages of the HA of the field isolates absorbed by the brain cells were between 87.5 and 99.5 per cent. The HA of both the intraocular and Lasota was not absorbed by the brain cells, while 85.5 and 99.3 per cent of the HA of NDV-Komarov and NDV-Herts were absorbed respectively.

3.3.3.6 Haemagolutination of Mammalian Erythrocytes

Six of the isolates (UI-104, SLS-1075, K-1084, UM-151, OJR 753 and NY-1) failed to agglutinate both the bovine and equine erythrocytes while three (OM-1, K-1090 and OJR 360) agglutinated both erythrocytes. KB and UM 651 agglutinated either of the mammalian erythrocytes.

Among the vaccine strains, the intraocular and the Lasota agglutinated erythrocytes from both mammals but Komarov did not agglutinate any of the erythrocytes. Both erythrocytes were agglutinated by the Herts strain.

3.3.37 Rate of Elution

Those strains that did not agglutinate the chicken rbc in all dilutions 24 hours before and after resuspension were considered fast eluters, while those strains where agglutination persisted 24 hours before and after resuspension were considered slow eluters.

All the isolates except K-1084 were slow eluters. The intraocular and Lasota strains were fast eluters while Komarov and Herts were slow eluters.

3.3.3.8 Plaque Formation in Cell Culture

All the 12 isolates formed small and large plaques in CEF. The plaques were either red or clear types and were round or oval with diameters ranging from 0.7 to 4.5mm. Both the intraocular and Lasota did not form any form of plaque under the condition of the experiment. Komarov formed only small plaques while the Herts strain formed plaques similar to the field strains. Figure 1 shows the different plaque forms of some of the strains.

3.4 Discussion

In nature ND virus exhibits great variation resulting in mild, moderate and severe forms of ND depending upon their virulence and ability to spread. Based on their pathogenecity, virus strains are grouped into velogenic, mesogenic and lentogenic pathotypes.

The velogenic or highly pathogenic strains have an MDT of less than 60 hours, an ICPI of more than 1.5, are heat stable for at least 15 minutes. They give 50-100 percent absorption of HA and are unable to agglutinate

A.

B.

C.

Fig. 1. Plaque formation by the different strains of Newcastle disease virus:

(A) one type of plaque of NDV-Komarov

(B) Small and large plaque of the field isolate

(C) No plaque by NDV-Lasota.

Table III. Strain characterization of Nigerian Isolates of Newcastle Diseases virus

Isolates	Mean Death Time	Minimum Lethal Dose	Heat Stability at 56°C (Min)	HA of Mammalian erythrocytes A		Intracerebral pathogenecity index	% absorption of HA by brain cells	Type of B plaques	Rate of elution	Type
				Bovine	Enquire					
UI-104	51.3	9	120	-	-	1.7	87.5	SLC	Slow	Velogenic
SLS-1075	54.9	10	120	-	-	1.6	99.2	SLCR	Slow	"
K-1090	46.7	9	15	±	+	1.3	93.7	SCLC	Slow	"
NY-1	49.0	9	0	-	-	1.3	99.0	SCLC	Slow	"
OM-1	56.6	10	10	+	+	1.3	87.5	SLC	Slow	"
K-1084	48.4	10	120	-	-	1.5	87.5	SLC	Fast	"
KB	52.3	10	120	+	-	1.9	99.5	SLCR	Slow	"
UM-151	44	10	120	-	-	1.8	99.2	SLCR	Slow	"
UM-651	48.9	9	60	+	-	1.5	88.6	SLC	Slow	"
OJR-360	45.5	10	120	+	+	1.3	87.5	SLC	Slow	"
OJR-753	50.8	10	120	-	-	1.7	98.4	SLC	Slow	"
Komarov	68.4	7	120	-	-	0.53	85.5	SC	Slow	Mesogenic
Lasota	120	0	0	+	+	0.00	0.00	NP	Fast	Lentogenic
Intraocular	120	0	0	+	+	0.00	0.00	NP	Fast	"
Herts	52.5	10	120	+	+	1.5	99.3	SLCR	Slow	Velogenic

Legends: A = No. haemagglutination; ± = doubtful haemagglutination; L = Large plaques; C = Clear plaques; R = Red plaques, and NP = No plaques.

mammalian erythrocytes but form two types of plaques in CEF.

The mesogenic or moderately pathogenic strain have an MDT of 70 to 100 hours, an ICPI of between 1.0-1.5 and variable heat stability, absorption by brain cells and the agglutination of mammalian erythrocytes. This group contains isolates that show greatest variation and there is no sharp line of demarcation between this and the lentogenic group. They however, differ from the lentogenic strain in that they form one type of plaques.

The lentogenic or very slightly pathogenic strains have an MDT of more than 90 hours, an ICPI less than 0.5. Most of them are heat labile and lack HA absorption by brain cells, but are able to agglutinate mammalian erythrocytes. The lentogenic strain does not form any form of plaque in CEF in the absence of magnesium ions. There seemed to be a degree of overlapping among the three strains in the properties enumerated above. However, a number of workers (Schloer and Hanson, 1968; Schloer and Hanson, 1971; Lomiczi, 1975), have shown the relationship between plaque size and virulence and have concluded that plaque forming capacity can to a certain degree be regarded as conclusive in differentiating the strains.

On the basis of this, all the 12 Nigerian isolates can be classified as velogenic strains.

Two of the isolates OM-1 and UM-151 were recovered from vaccinated commercial farms. Result obtained from the characterization of these two isolates indicated that the viruses were not the vaccine strain, but a challenging velogenic strain. Although the birds were said to have been vaccinated, the immune status of the birds were not known at the time of the outbreak. It is quite possible that the HI titre of the birds had dropped to the non-protective level. A titre of between 1.20-1.40 may not protect against a highly virulent strain (Beard and Brush, 1975; Allan et al, 1978).

Although Nawathe et al (1975) had earlier classified 13 Nigerian isolates into velogenic strain, it was still necessary to assess NDV strain distribution in the country following reported vaccine failures from the field.

The viruses isolated by Nawathe et al (1975) were from the Northern and Eastern States of the country. The virus strains isolated in this study were recovered in different locations all over the country. The findings in both studies confirmed the earlier findings (Ann. Rep. Fed. Dept. Vet. Res. 1955-1956) that isolations of

new field strains of the virus have shown no divergence from the usual extremely virulent strains.

CHAPTER FOUR

4.0 PLAQUE PURIFICATION OF NIGERIAN STRAINS OF NEWCASTLE DISEASE VIRUS

4.1 Introduction

Newcastle disease virus strains, with few exceptions are heterogeneous population of plaque mutants. In a series of viral genetic studies using plaque markers, Granoff (1959, 1961, 1962 and 1964) described the plaque types occurring in strains of Newcastle disease virus. During the course of these studies, it was found that considerable variation in a number of virus properties existed not only between related strains of NDV but within virus populations of individual strains as well. It was also discovered that heterogeneity of plaque population is particularly characteristic of all velogenic strains.

Plaque morphology has been used as a distinguishing feature of NDV strains. The velogenic strains develop both large and small plaques while the mesogenic strains develop only small plaques. On the other hand, the lentogenic strains do not develop plaques (Hanson, 1967,

Daniel et al, 1968 and Schloer and Hanson, 1971), although recent developments have shown that plaque assays can now be performed on avirulent strains (Nagai et al, 1976).

Furthermore, Daniel et al (1968) and Schloer and Hanson (1968) have shown that the virulence of the strains bears a direct relationship to the plaque size; Large plaques being associated with high virulence and small plaques with reduced virulence. Previous reports have also shown that with mutants of ND virus, slight antigenic differences do occur (Schloer, 1974). Estupian (1971) and Schloer (1974) isolated stable clones which differ in biological characteristics, virulence, cell affinity and antigenecity from mixed plaque populations of NDV. This chapter describes the plaque purification and pathogenecity of isolated plaques from the Nigerian strains of Newcastle disease virus.

4.2 Materials and Methods

4.2.1 Viruses

Four of the field strains - NDV-104, NDV-P, NDV-SLS, NDV-KB and two vaccine strains NDV-K and NDV-L were used.

Isolated history of the viruses had already been presented in Chapter 3 section 3.3.1.

4.2.2 Chicken Embryo Fibroblast (CEF)

Chicken embryo fibroblast cells were prepared as described above in Chapter 3 section 3.2.6.8.

4.2.3 Plaque Assay and Isolation of Clones

The procedure for obtaining the plaques had been described in Chapter 3. Small (0.75-2.5mm) and large (2.6-4.5mm) well separated plaques were individually picked as described by Granoff (1964). Briefly, a sterile cotton wool plugged pipette, fitted with a rubber bulb, was gently flamed and cooled. The pipette tip was dipped into the centre of the plaque holding the pipette at right angle to the petri dish. This was sucked gently and carefully into the pipette such that the agar plug sucked out was close to the tip. The content of the pipette was immediately expelled into 0.1ml of the suspending E.199 medium in a bijou bottle and mixed thoroughly with the aid of the pipette. This was then inoculated into another confluent monolayer in a 60mm petri dish. Cells were again incubated at 37^{o}C in an atmosphere of 5% CO_2. After 75% of the

monolayer has been destroyed, cells were frozen at -20°C, thawed and centrifuged at 3000 rpm for 15 minutes. The supernatant was stored at -20°C and designated small (S) or large (L) first clone passage depending upon the size of the plaque. 0.1ml of each clone was inoculated into another set of confluent monolayer and overlayed as described above. Plaques were checked for characteristic morphology and then picked as described above.

Two types of plaques were normally observed. These were the clear and the red plaques, however only the clear plaques were picked. This is because the clear plaques have been found to be more stable and their rate of mutation, if any, lower than the red plaques (Estupian and Hanson, 1971). The procedure was continued until uniform and stable clones were derived from the parent strains.

4.2.4 Clone Stock Preparation

After stable clones have been obtained, stock viruses were prepared from the isolated clones by inoculating 0.1ml each of the clone preparation into thirty 9-10 day old embryonated eggs. Eggs were candled twice daily and

dead eggs were separated and chilled before harves. Eggs dying 24 hours post inoculation were discarded. Allantoic fluid were harvested as already described and stored at -70°C until used. All the AF were designated S or L clone derivatives of the parent strain. Clone stocks were titrated in embryonated eggs and in 24 well plates to obtain the ELD_{50} and plaque forming unit (PFU) respectively.

4.2.5 Pathogenecity Characterization of Clones

Virulence of clones were determined by the mean death time, intracerebral pathgenecity index for day old chicks and effect of clones in CEF cells. Details of the first two tests have been given in Chapter 3 sections 3.2.6.1 and 3.2.6.3.

4.2.5.1 Cythopatic Effect of Isolated Clones on CEF

Viral clones were diluted in cold PBS to contain approximately 50 PFU per 0,1ml, and 0.1ml each was inoculated into monolayer CEF cultures in 60mm petri dishes, using five replicates per clone.

Virus absorption was allowed to take place at 37°C for 1 hour. The monolayer was then washed with PBS pH 7.4 to remove any unabsorbed virus. The monolayers were covered with 5ml of medium E.199 supplemented with 2% calf serum, and observed daily for cytopathic effect.

4.3 Result

4.3.1 Isolation of Clones

Monolayers were examined for plaque size and morphology on day three p.i. Two types of plaques were detectable among the field strains at the first passage in CEF - large plaques with mean diameter ranging between 2.6mm-3.5mm and 3.0mm-4.5mm, and small plaques with mean diameter ranging from 0.50mm-2.5mm to 0.80mm-2.5mm. The mesogenic vaccine strain, NDV-Komarov formed only small plaques, while NDV-Lasota under the condition of this experiment formed no plaque. At the first passage in CEF the ratio of large to small plaques ranged between 3:1 and 5:1 (Table 4). At subsequent passages, when large plaques were inoculated, the ratio of large to small plaque increased until at the 4th or 5th passage no small plaques were observed. Similarly, when small

Table 4: Plaque morphology of Nigerian Strains of NDV Isolates at first passage in Chicken Embryo Fibroblast.

Inoculum	Type of Plaque		Size of Plaque (mm)		Ratio of L to S Plaques	
	L	S	L	S	L	S
UI-104	+	+	2.6 - 4.6	0.75 - 2.5	4	1
NDV-P	+	+	2.6 - 4.0	0.50 - 2.5	3	1
NDV-KB	+	+	3.0 - 4.5	0.75 - 2.0	5	1
SLS	+	+	2.6 - 3.5	0.80 - 2.5	3	1
NDV-Komarov	-	+	-	0.5 - 2.0	-	-
NDV-Lasota	-	-	-	-	-	-

Source of inoculum = 4th egg passage of parent stock in embryonated egg.

L = Large plaque

S = Small plaque

\+ = Presence of plaque

\- = Absence of plaque

plaques increased until no large plaques were observed. Stable clones which did not revert from large to small and vice-versa were observed during five subsequent passages.

4.3.2 Mean Death Time and Intracerebral Pathogenecity Index

All the L-clones had MDTs similar to their parent strains while the S-clones had MDTs of either mesogenic or lentogenic strains. Four of the S-clones had MDTs between 60.5 and 67.0 hours which fall within the MDT of a mesogenic strain, while one UI-104-S had an MDT of 105 hours which is similar to the MDT of a lentogenic strain. The MDTs for the velogenic and lentogenic strains are 60 and 120 hours respectively while the MDT for mesogenic is 70-100. No significant difference was observed in the ICPI of the L clones and the parent strains however, significant differences were detected between the ICPI of the S-clones and the parent strains. The ICPI of the parent strains ranged from 1.6 to 1.9, while the ICPI of the S-clones ranged between 0.35 and 1.0. The velogenic strains have an ICPI above 1.5, mesogenic strain between 1.0-1.5 while the ICPI for lentogenic strain fall between 0.1-0.5. Results of both tests are shown in Table 5.

Table 5. Mean Death Time (MDT) and Intracerebral Pathogenecity Index of Nigerian Newcastle Disease Virus Clones.

Viruses	Mean Death Time (Hours)			Intracerebral Pathogenecity Index		
	Parent Strain	Clones		Parent Strain	Clones	
		L	S		L	S
UI-104	51.3	53.7	105	1.7	1.5	0.35
NDV-P	46.3	53.1	67.0	1.6	1.6	0.75
KB	52.3	53.1	60.5	1.9	1.7	0.9
SLS	54.9	60.0	62.2	1.6	1.3	1.0
NDV-K	68.0	-	66.9	0.53	-	0.49
NDV-Lasota	120	-	-	0.00	-	-

L = Large clones

S = Small clones

- = Not tested

4.3.3 Cytopathic Effect of Clones on CEF

All the clones show cytopathic effect on CEF. Complete cytopathic effect with L-clones occurred in 48 hours while with the S-clones it took 96 hours and above The mesogenic Komarov strain destroyed the cells in 96 hours while the lentogenic Lasota strain did not show any cytopathic effect on the CEF monolayer.

4.4 Discussion

Many wild strains of NDV isolated from different geographical areas exhibit heterogeneity in plaque morphology (Granoff, 1964; Schloer, et al. 1968 and Schloer and Hanson, 1968). Similar results were obtained in the present study for NDV strains. Plaque heterogeneity was more commonly shown with the velogenic strains. Only one type of plaque was observed with the mesogenic Komarov strain. The lentogenic Lasota did not develop Plaque under the condition of this experiment.

Plaque size diversity is thought to be a reflection of genetic differences within a strain (Schloer and Hanson,

1968). In the present study all of the velogenic NDV strains examined were genetically mixed populations of small and large clones. This is in agreement with the earlier findings of Schloer and Hanson (1968). According to Granoff (1955) and Schloer (1968) the presence of large plaques within a viral population is related to virulence of strains of NDV even when these plaques were a minor component of the population. While the presence of the small plaque mutants did not appreciably reduce virulence, the absence of large plaques either in clones or strains was associated with reduction in virulence for the chick embryo. This was clearly demonstrated in the present study where L-clones had MDTs and ICPIs similar to the parent strains whereas the S-clones were less virulent. In the same vein there was a significant difference between the behaviour of the L and S clones in CEF monolayers. Complete cytopathic effect by the L clones was detected in the CEF monolayers 24 hours earlier than the S clones. According to Kato et al (1972), there is an inherent difference between the intracellular multiplication of the two clones. Small size of the S-clones, were not due to inhibitors, but are probably due to the rate

at which the virus was synthesized in CEF cells and released from infected cells. This appeared to be one of the factors responsible for the difference in plaque size of the L and S clones.

No reversion from small to large plaques was observed in the present study, although there had been reports of isolation of large plaque revertants from a clone of small plaques (Granoff, 1961; Granoff, 1964 and Thiry, 1964).

In this study, a clone, UI-104-S of low virulence was successfully isolated from a Nigerian velogenic strain. This is the first time a clone of low virulence is being isolated from a Nigerian velogenic strain. It is speculated that attenuated strains of NDV must have arisen in a situation where small plaque mutants with extremely low rate of reversion to large plaque size arose and persisted. If this speculation is correct the isolation of a stable clone of a low virulence from a velogenic wild Nigerian NDV strain is a first step in developing an attenuated live vaccine from locally isolated NDV strain. It is on record that no isolation of natural mesogenic or lentogenic strain had been reported in Nigeria.

CHAPTER FIVE

5.0 ANTIGENIC AND STRAIN VARIATIONS OF NIGERIAN NEWCASTLE DISEASE VIRUS CLONES

5.1 Introduction

Antigenic differences in Newcastle disease virus have been reported by several workers (Waterson et al 1967; Schloer and Hanson 1971; Alexander and Allan, 1973; Gomez-Lillo et al 1974; Schloer et al 1975). The magnitude of the differences are similar to those found among strains of the type 1 polio virus (McBride, 1959; Osaki et al, 1965). Consequently the strains are therefore placed in a single serotype. Antigenic differences in NDV apparently involved the two envelope antigens, the neuraminidase (NA) and the haemagglutinin (HA). However, the study of antigenic diversity of NDV isolates is complicated by the presence in most field isolates as well as most laboratory cultures of plaques with differing character (Granoff, 1964, Estupian and Hanson 1971). Several methods including reciprocal cross neutralization test, the kinetic neutralization test and the haemagglutination inhibition test have been used in

the antigenic characterization of different isolates of ND, and in detecting the antigenic relationship between different strains and clones of NDV (Bankowski and Kinjo, 1965; Schloer, 1974; and Schloer and Hanson, 1975). The reciprocal plaque reduction test has also been used by Gomez-Lillo et al (1974) to evaluate between the viscerotropic velogenic strains and some vaccine strains. Lancaster et al, working at the Central Veterinary Laboratory, Weybridge (1978) used both the plaque reduction and the HI methods and the cross challenge test to determine the serological differences between 14 isolates of NDV. Nagy and Lomniczi (1984) employed the one dimensional peptide mapping to serologically differentiate the different strains, of NDV. All of the workers came to the conclusion that antigenic variations exist among NDV isolates, and that the antigenic variation found among mutants is similar to that found within a serotype.

The often reported outbreaks of ND in vaccinated flocks in Nigeria may be due to the presence of antigenic differences between the vaccine strains in use and the indigenous wild strains existing in the country. Limited information is available about the antigenic relationship

between the vaccine strains and the prevalent wild velogenic strains of the virus existing in Nigeria (Nawathe et al, 1975; Adu et al, 1984).

To provide more information concerning the strain variation of Nigerian isolates of NDV, 8 clones derived from the velogenic strains and two vaccine strains were compared serologically and antigenically using the plaque reduction, the haemagglutination inhibition, the cross protection tests and the polyacrylamide gel electrophoresis methods.

5.2 Materials and Methods

5.2.1 Reagent For Protein Determination

(i) Reagent A: 2% Na_2CO_3 in 0.1N NaOH. It was prepared by first dissolving 2gm of Na_2CO_3 in 10ml of 0.1N NaOH and subsequently adding 90ml of 0.1N NaOH.

(ii) Reagent B: 2% Na-K-Tartarate. The 2% Na-K-Tartarate was made by dissolving 2 gms of this reagent in 100ml of distilled water.

(iii) Reagent C: 1% $CuSO_4.5H_2O$. This was made by dissolving 1gm of copper sulphate in 100ml of distilled water.

(iv) Reagent D: Reagent D was made by mixing the above three reagents in the following proportions: 50ml of reagent A, 0.5ml of reagent B and 0.5ml of reagent C. Reagent B was added first to avoid cloudiness.

(v) Reagent E: This is the Folin-Ciocalteu reagent which consists of phosphomolybdie-tunstic complex (LiSO4) and bromine water. The reagent is commercially available as a 2N solution and is diluted to 1N with distilled water just before protein assay.

5.2.2 Reagents for Polyacrylamide gel Electrophoresis

5.2.2.1 Running gel and Stacking gel

The running and stacking gels are prepared as detailed below.

		Running gel	Stacking gel
(i)	30% W/V Acrylamide, 0.8% bis-acrylamide	8ml	1.3ml
(ii)	1.5m Tris-HCl, 8m MEDTA 0.4% sodium dodecyl Sulphate (SDS) $CH_3(CH_2)_{11}OSO_3Na$)	5ml	2ml
(iii)	N N. ^{1}N. ^{1}N. ^{1}N. 1 tetramethyle-thylene-diamine (TEMED)	0.01	0.005
(iv)	Distilled water (H_2O)	6.9	4.60
(v)	10% Ammonium persulphate $(NH_4)_2S_2O8$)	0.1	0.16
(vi)	Final concentration	12%	5%

The Ammonium persulphate which acts as the catatyst to promote gelation of the acrylamide and bis-acrylamide is added just before use.

5.2.2.2 Electrode Buffer

The electrode buffer is made from the following reagents:

Tris Base ($C_4H_{11}NO_3$)	24gm
Glycine ($N_2NCH_2CO_2H$)	115.2gm
Sodium dodecyl sulphate ($CH_3(CH_2)_{11}OSO_3Na$(SDS)	4.0gm
Ethylenediamine tetracetic acid (EDTA)	2.7gm

5.2.2.3 Sample Buffer

This is prepared as a 3X concentration using the following reagents.

0.5M NaP, pH 7.6	1.2ml
Glycerol ($HOCH_2CH(CH)(H_2OH)$	6.0gm
SDS ($CH_3(CH_2)_{11}OSO_3Na$)	1.5gm
Dithiothreitol ($HSCH_2CH(CH)CH(CH)CH_2SH$)	30gm
Bromophenol group (BrC_6H_4OH)	10
Distilled water (H_2O) To make up to	20ml

5.2.3 Viruses

Eight clones from the parent velogenic field strains and one clone each from the mesogenic and lentogenic vaccine strains were used. The methods for isolating the clones from their parent strains had been described in Chapter Four Section 4.2.3.

5.2.4 Infection of Embryonated Eggs

Each virus clone was diluted to contain between 50-100 PFU per 0.1 ml and inoculated into 9-10 day old embryonated eggs using 60 eggs per clone. The inoculated eggs were incubated at 37°C and were candled twice daily. Dead and moribund eggs were chilled overnight at 4°C before harvesting, all embryos dying before 24 hours were discarded. Allantoic fluids (AF) were harvested and clarified at a speed of 3000 rpm for 15 minutes at 4°C.

5.2.5 Concentration and Purification of Viruses

The AF were concentrated by centrifugation at 40,000 rpm for 1 hour in an MSE 65 cold centrifuge type rotor 43114-120. The virus pellet was resuspended in one-fortieth the original volume in Tris buffer.

Purification of the virus was carried out by layering 1ml of the concentrated virus on a 10-50% Sucrose cushion and centrifuging at a speed of 30,000 rpm in a swing out type rotor 43127-115 rotor. Virus fractions were identified in a sphegrophotometer at an optical density of 450nm. Virus fractions were then centrifuged at 50,000 rpm for 1 hour and the pellet suspended in 1ml Tris buffer. Allantoic fluid harvested from uninoculated 9-10 day old embryonated eggs were similarly concentrated and purified and used as control.

3.2.6 Protein Determination of Purified Clones

The protein determination of the purified clones was done according to the method of Lowry (1950).

A set of 16 pairs of tubes were used in the determination of protein. The first five pairs of tubes were used for the standard, while the remaining 11 pairs were for the virus samples. The protocol (including the volumes of standard reagents and test samples used for determining the Protein concentration) is shown in Table 6. Tubes were allowed to stand for 30 minutes, and the optical density (OD) read at 750nm. The mean OD was calculated from each pair of tubes. The mean OD of the blank was substracted from the mean OD of the BSA to obtain the OD

for each sample. The result was plotted against the microgram of protein. The OD values of samples were plotted against protein concentration and calculated from the formula.

$$\text{Gradient of graph} = \frac{\text{protein concentration (ug)}}{\text{OD.}} \quad \text{(Fig. 2)}$$

5.2.7 Antisera

Antisera were prepared in adult white rabbits obtained from the Small Animal Division of the National Veterinary Research Institute, Vom. Each rabbit received two injections of 0.5ml of the purified virus at 10 days apart. Two weeks after the second injection the animals were sacrificed and bled to death through the jugular vein. The clotted blood were incubated at 37°C for 3 hours and subsequently kept overnight at 4°C. Serum was separated by centrifugation at a speed of 3000 rpm for 15 minutes and stored at -20°C until used. All sera were inactivated at 56°C for 30 minutes before use in the HI and plaque reduction neutralisation tests.

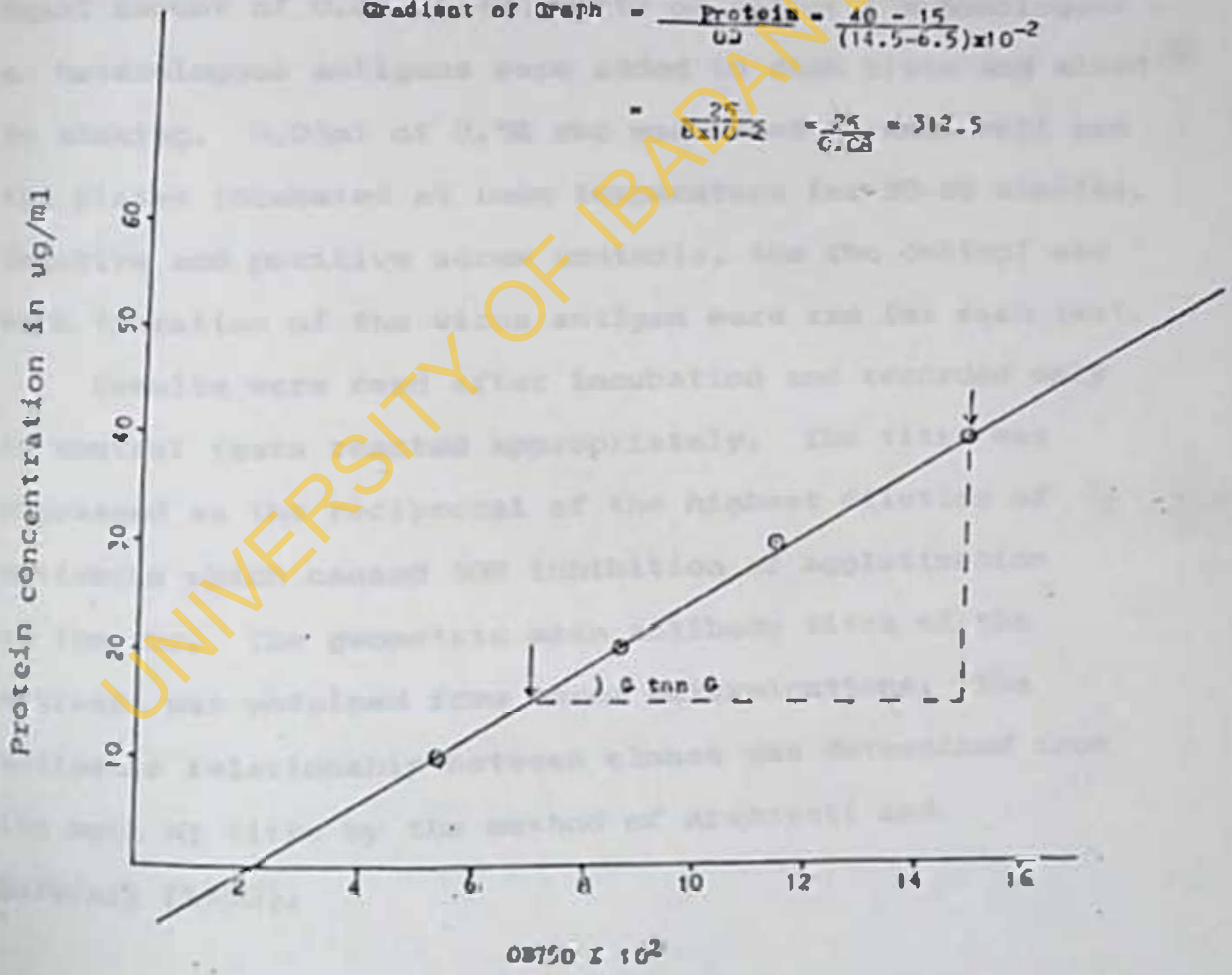
TABLE 6: Protocol Determination of protein concentration in virus clones (Lowry, 1950)

Items	Tube 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	Contents															
	STANDARD					104-S	SLS-	PL	PG	KEL	KES	104L	SLS-L	HDV-L	VD -K	UAC
Volume of Bovine Serum Albumin (ul)	0	10	20	30	40	0	0	0	0	0	0	0	0	0	0	0
Volume of virus clones preparation (ul)	0	0	0	0	0	30	30	30	30	30	30	30	30	30	30	30
Volume of water (ml)	.60	.59	.58	.57	.56	.57	.57	.57	.57	.57	.57	.57	.57	.57	.57	.57
Volume of Reagent D (ml)	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Volume of Reagent E (ml)	.3	.3	.3	.3	.3	.3	.3	.3	.3	.3	.3	.3	.3	.3	.3	.3
OD1 (750 nm)	.068	.120	.153	.180	.216	.073	.083	.103	.220	.137	.159	.119	.220	.181	.185	.057
OD2 (750 nm)	.066	.122	.153	.184	.218	.081	.089	.114	.246	.154	.181	.137	.183	.192	.157	.062
Mean OD	.067	.121	.151	.182	.217	.077	.086	.109	.233	.147	.170	.128	.202	.187	.171	.059
Mean OD – Blank	0.00	.054	.086	.115	.150	.032	.041	.064	.188	.102	.125	.061	.135	.120	.104	.013
Protein Concentration (mg/ml)						0.34	0.44	1.11	2.02	1.1	1.34	0.96	2.11	1.90	1.63	0.15

Fig. 2 - Determination of protein concentration of virus clone.

Gradient of Graph = $\frac{\text{Protein}}{OD} = \frac{40 - 15}{(14.5-6.5)\times10^{-2}}$

$= \frac{25}{8\times10^{-2}} = \frac{25}{0.08} = 312.5$



5.2.8 Cross Haemagglutination Inhibition Assay

The beta (constant antigen varying serum dilution) method was used. Each of two fold serial dilutions of sera was reacted with 4HA units of virus antigen (Allan and Gough, 1974; Allan et al, 1978; Brugh et al, 1978). Each serum was diluted in 0.85% normal saline and tested in triplicates in a 96-well U-botton Linbro microtitre plates. Equal amount of 0.05 ml 4HA units of either the homologous or heterologous antigens were added to each plate and mixed by shaking. 0.05ml of 0.5% rbc was added to each well and the plates incubated at room temperature for 30-60 minutes. Negative and positive serum controls, the rbc control and back titration of the virus antigen were run for each test.

Results were read after incubation and recorded only if control tests reacted appropriately. The titre was expressed as the reciprocal of the highest dilution of antiserum which caused 50% inhibition of agglutination of the rbc. The geometric mean antibody titre of the antisera was obtained from three determinations. The antigenic relationship between clones was determined from the mean HI titre by the method of Archtetti and Horsfall (1950).

5.2.9 Plaque Reduction Neutralization Test

Plaque reduction neutralization test was carried out in Linbro tissue culture plates. Cells prepared from 9-10 day old embryonated eggs as described above were suspended in MEM medium supplemented with 5% calf serum, 250 I.U. penicillin, 250mg of streptomycin and 100mg fungizone. Cells were diluted in MEM medium to contain 75 x 10^3 cells per ml. Each well was seeded with 1ml of medium and incubated at 37°C in an atmosphere of 5% CO_2 and used for the PRNT 24-48 hours when they were already confluent.

For the neutralization test, the beta method was used. Two fold serial dilution of homologous or heterologous sera were made in MEM medium without calf serum. Virus preparations were diluted to contain approximately 100 PFU per 0.1ml. 0.5ml of the virus preparation was added to an equal volume of each of 2 fold serial dilution of either the homologous or heterologous sera. The serum virus mixtures were incubated at 37°C for 1 hour. Three wells were inoculated with 0.2ml each of the serum-virus mixture and incubated at 37°C for another 1 hour. Cell were overlayed with 1ml medium containing equal amount of 2 x MEM and 1.6% Ionagar as earlier desribed above. Virus control

was run for each replicate. Both control and experimental plates were incubated at 37°C for 72 hours after which a second overlay containing neutral red at a final concentration of 1:100,000 was added. Plaque reduction neutralization antibody titre of the serum was determined as the highest dilution of serum which reduces the average number of plaques in the control well by 80%.

5.3.0 Antigenic Relationship Between Clones

The antigenic relationships among the clones were determined from the result of the cross haemagglutination and the plaque reduction neutralization tests. The degree of relatedness was computed from the titre ratios using the formula of Archetti and Horsfall (1950) which states that $r = \sqrt{r_1 \times r_2}$. The ratio r reflects the extent of the antigenic differences between 2 clones when both clones and both antisera were used in reciprocal cross-serological reaction.

The ratio r_1 was found by dividing the heterologous titre of virus 2 with the homologous titre of virus 1. The ratio r_2 was found by dividing the heterologous titre

of virus 1 by the homologous titre of virus 2. Where there is no antigenic difference between two clones, the value of r is 1. Because the ratio r is the geometric mean of the two ratios obtained with heterologous viruses and homologous viruses and homologous sera, it yields a value which gives equal weight to differences found in either direction. The ratio is expressed in % in order to simplify the data.

5.3.1 Polyacrylamide gel Electrophoresis of NDV Clones

5.3.2 Preparation of Slab gel

The running gel was degassed using a suction pump for 5 minutes and poured into the gel assembly to a level of about 3cm below the maximal filling level. It was allowed to polymerise for 1-2 hours. The top of the polymerised gel was rinsed with water for 1-2 minutes and allowed to drain. Five ml of the stacking gel was carefully layered with a pasteur pippette, and the assembly comb was inserted to obtain slots of the desired depth.

5.3.3 **Preparation of Samples**

The concentrated and purified clones containing the determined amount of proteins were prepared before loading into the slab gel. The samples were calibrated in such a way to contain equal amount of protein using the sample buffer and water. The samples were allowed to sit at room temperature for 20 minutes.

An SDS molecular weight marker (Sigma Chemicals Company, Missouri, USA) containing 13.5mg of a lyophilized mixture of the following 6 proteins with their approximate molecular weight was used as standard while an uninfected allantoic fluid which was concentrated and purified in the same way as the clones was used as control.

Protein	Mol. Weight
Lysozyme (Egg white)	14,300
H-Lactoglobulin (Bovine MVK)	18,400
Typsinogen	24,000
Pepsin	34,700
Albumin, Egg (Oralbumin)	45,000
Albumin, (Bovine plasma)	66,000

0.5 ug of Gamma globulin (MW 220,000) was incorporated into the above solution to provide high molecular weight marker. The procedure for the preparation of samples before loading into the gel is presented in Table 7..

5.3.4 Loading and Running of Samples

Between 20-40 ul of each sample and the standard was applied into each well of the gel with a Hamilton syringe. The gel slab was then clamped into the electrophoresis chamber and both chambers were filled with the electrode buffer. The gel was run at a constant current and voltage of 30mA and 50 volts at $4^{o}C$ for 16 hrs using the continuous buffer system of Laemmli (1970).

5.3.5 Staining

After the run, the gel was fixed for 30-60 minutes in 10% Trichloro-acetic acid (TCA) and 50% methanol. The gel was further rinsed and stained for 1-2 hours with 0.25% Coomassi blue, 50% methanol and 7.5% acetic acid. It was destained in 20% methanol and 7.5% acetic acid.

Table 7. Preparation of Samples before loading into gel slab

Sample	ug Protein	Vol. ul	Water (ul)	Sample Buffer (ul)
104-L	300	312	88	100
104-S	"	206.9	-	50
SLS-L	"	141	59	50
SLS-S	"	379.7	20.3	100
KB-L	"	178.6	21.4	50
P-L	"	ND	ND	ND
P-S	"	148.5	51.5	50
X	"	183	17	50
L	"	159	41	50
UAF	"	441	-	59

UAF - Uninfected alantoic fluid.

5.3.6 Cross Protection Test in Susceptible Chickens

The L-clones and the two vaccine strains were used for this test. The virus preparations were inactivated with Beta propiolactone (Sigma Chemical Company) at a final concentration of 1:1000. Each of 140 three week old NDV-susceptible chickens was inoculated with one of the inactivated virus preparation in the thigh muscle in 0.5ml amounts. A group of 140 unvaccinated susceptible chickens served as controls.

Twenty-one days post-immunisation birds from each of the vaccinated and control group were challenged intra-nasally with either the homologous or heterologous virus. Ten fold serial dilutions from 10^{-1} to 10^{-7} of each challenge virus was prepared and 0.2ml of each virus dilution was inoculated into 5 vaccinated and 5 unvaccinated control chickens. The challenge birds were observed daily for 15 days. End point titres were calculated by the method of Reed and Muench (1939), and the protective indices were calculated by substracting the titre in the vaccinated chickens from the titre in the control vaccinated chickens.

5.4.0 Results

5.4.1 Protein Determination

The concentration of protein in the virus preparation ranged from 0.34-2.11 for AF of virus infected embryonated eggs and 0.15 for the uninfected allantoic fluid (AUF) (Table 6).

5.4.2 Cross Haemagglutination Inhibition Test

Result of HI test showed a wide variation in the inhibitory capacity and titre between the heterologous and the homologous sera. HI titre for homologous strains ranged from 40 to 290.7. The heterologous HI titre differ from strain to strain. For example, the heterologous titre between UI-104L and the other strain ranged between 80 to 290 while the heterologous titre between NDV-Lasota and the other strain ranged between 5 and 20. Three of the clones had higher heterologous titre than their homologous titres. Clone 104-S had a heterologous HI titre of 80 with clone 104-L from the same strain and heterologous titre of 100.7 with clone KBL. Clone SLS-L with a homologous titre of 100.7 had a heterologous titre of 126.9 and 290.7

with 104-L and PS respectively. Clone PL had a heterologous titre of 100.7 with SLS-S and PS. NDV-Lasota had an HI titre almost four-fold less than its homologous titre with all the field strains, except SLS-S and 104-L. Heterologous titre ranged as low as 5 with the other field strains. In the same way, NDV-Komarov had HI titre comparatively low to its homologous titre. Heterologous titres with the other field strains ranged between 20 and 40. Results of the haemagglutination inhibition test are shown in Table 8.

5.4.3 Antigenic Relationship Between Clones

The antigenic relationship between strains were calculated from the HI titre ratio (Table 9) using the method of Archetti and Horsfall. The HI test did not reveal any definite antigenic patterns among the clones. However the degree of relatedness in order of departure from the homologous titre is less evident among the field clones than the two vaccine clones. NDV-Lasota maintained a significantly low antigenic relationship with all the field strains except SLS-S and 104-L where the r-values are 52 and 63 respectively. In the same manner, NDV-Komarov clone only exhibited close antigenic relationship

TABLE 8 Result of Haemagglutination-Inhibition Test of homologous and heterologous clone

Serum	Virucco									
	104L	104S	SISL	SLSS	KBL	KBS	PL	PS	K	L
104L	290.7	290.7	201.5	201.5	290.7	290.7	290.7	290.7	80.0	290.7
104S	80	50.9	50.9	50.9	100.7	50.9	50.9	50.9	50.9	50.9
SLS-L	126.9	290.7	100.7	50.9	74.2	74.2	290.7	40	80	80
SLS-S	50.9	6.2	50.9	50.9	50.9	50.9	6.2	50.9	20.0	80
KBL	40	6.2	50.9	50.9	50.9	50.9	20	80	50.9	50.9
KBS	80	40	15.8	80	50.3	50.3	40	40	40	20
PL	80	80	80	100.7	80	80	40	100.7	40	100.7
PS	40	20	40	20	20	10	40	40	40	25
NDV-K	40	40	20	40	40	40	20	40	104	40
NDV-L	20	6.2	25	20	5	6.2	5	5	25	80

(a) - Geometric mean HI titres of three determinations.

TABLE 9 Titre ratio (a) among clones calculated from HI titres.

Serum	Viruses									
	104L	104S	SLSL	SLSS	KBL	KBS	PL	PS	K	L
104L	1	1	0.7	0.7	1	1	1	1	0.27	1
104S	1.6	1	1	1	2.0	1	1	1	1	1
SLS-L	1.26	2.88	1	0.50	1.45	1.45	2.88	0.39	0.79	0.79
SLS-S	1	0.12	1	1	0.78	0.12	1	0.39	0.39	1.57
KBL	0.78	0.12	1	1	1	0.39	1.57	1	1	1
KBS	1.57	0.78	0.31	1.57	1	1	0.78	0.78	0.78	0.39
PL	1	1	1	1.25	1	1	0.50	1.25	0.50	1.25
PS	1	0.5	1	0.5	0.5	0.25	1	1	1	0.62
K	0.38	0.38	0.19	0.38	0.38	0.38	0.19	0.38	1	0.38
L	0.25	0.07	0.31	0.06	0.07	0.07	0.06	0.06	0.31	1

(a) Value obtained by dividing the heterologous titre of virus[1] with the homologous titre of virus[2].

with 104-S, KBL and KBS with r values of 62, 62, and 54 respectively.

The field strains exhibited closer antigenic relationship among themselves than with the two vaccine strains. For example clone 104-L showed a considerable antigenic relatedness with all the clones except the two vaccine strains where the r values were 10 and 52 respectively. In general clones derived from the same strain demonstrated close antigenic similarity between themselves than with clones from other strains.

The degree of relatedness between the clones is presented in Table 10.

5.4.4 Plaque Reduction Neutralization Test

Result of PRNT showed a wide variation in the neutralising capacity and titre of the heterologous and homologous sera. Homologous titre among the strains ranged from 340 to 900. Heterologous titres among strains varied with individual strains. The heterologous titre between 104-L and the other field strains ranged from 300 to 860 while the heterologous titre between the vaccine strain NDV-Komarov

TABLE: 10 Antigenic relationship between NDV clones expressed as an r-value and calculated from HI titres.

Antisera	Clones									
	104 L	104 S	SLS L	SLS S	KHL L	KBS S	PL L	PS S	K	L
104-L	100	126	94	84	88	125	100	100	10	52
104-S		100	170	35	49	88	100	71	62	26
SLS-L			100	71	120	45	170	62	39	49
SLS-S				100	100	111	39	71	38	63
KB-L					100	100	62	87	62	24
KB-S						100	88	44	54	16
P-L							100	71	30	27
P-S								100	24	19
K									100	34
L										100

ranged between 340 and 570 (Table 11). Two clones 104-S and KBS had higher heterologous titres of 605 and 505 respectively with clone KBL.

NDV-Lasota was not used in the PRNT because it did not form plaque under the condition of this experiment.

5.4.5 Antigenic Relationship Between Clones

The antigenic relationship between the clones based on PRNT was calculated as described above using the Archetti formula (Table 12). The PRNT revealed a wider antigenic variation among the clones. NDV-Komarov exhibited a closer antigenic relationship with the field strain with r value ranging from 32 to 77 (Table 13).

The degree of antigenic divergence between homologous and heterologous titres vary with the different strains but less pronounced in clones derived from the same strain than clones originating from other strain.

TABLE 11. Result of Plague Reduction Neutralization Test

Serum	Clones								
	104 L	104 S	SLS L	SLS S	KBL	KBS	PL	PS	K
104-L	860	860	340	380	340	300	500	480	400
104-S	500	500	300	500	605	380	480	400	500
SLS-L	380	310	680	600	280	320	480	480	450
SLS-S	240	530	840	900	380	400	600	500	580
KBL	500	550	600	380	600	300	320	410	480
KBS	480	400	480	300	505	340	400	380	200
PL	405	400	360	500	450	600	840	840	440
PS	400	206	450	350	340	402	600	640	308
K	460	390	520	340	400	570	530	460	640

Highest serum dilution that neutralized 80% of the observed plagues

TABLE 12. Titre Ratio [a] of Plague Reduction Neutralization Test

Serum	Clones								
	104 L	104 S	SLS L	SLS S	KBL	KBS	PL	PS	K
104-L	1	1	0.39	0.44	0.39	0.34	0.58	0.55	0.46
104-S	1	1	0.6	1	1.21	0.76	0.96	0.80	1
SLS-L	0.55	0.45	1	0.88	0.41	0.47	0.70	0.70	0.66
SLS-S	0.26	0.58	0.93	1	0.42	0.44	0.66	0.55	0.64
KBL	0.83	0.92	1	0.63	1	0.50	0.53	0.68	0.8
KBS-S	1.4	1.2	1.4	0.88	1.5	1	1.2	1.1	0.58
PL	0.48	0.47	0.42	0.60	0.54	0.71	1	1	0.52
PS	0.62	0.32	0.70	0.54	0.53	0.62	0.93	1	0.46
K	0.71	0.63	0.81	0.53	0.63	0.90	0.83	0.71	1

[a] = Obtained by dividing the heterologous titre of virus 1 with the homologous titre of virus 2.

TABLE 13. Antigenic relationship between NDV-Clones expressed as r-values and calculated from the Plaque reduction neutralization test

Serum	Clones								
	104 L	104 S	SLS L	SLS S	KBL	KBS	PL	PS	K
104-L	100	100	46	34	57	69	52	58	32
104-S		100	51	76	105	95	67	51	77
SLS-L			100	90	64	81	54	70	73
SLS-S			100	51	62	62	54	58	
KBL				100	87	53	60	70	
KBS					100	92	83	68	
PL						100	96	65	
PS							100	57	
K								100	

5.4.6 Result of Polacrylamide gel Electrophoresis

All the clones had distinct polypeptide patterns. No polypeptide band was however found in the uninfected allantoic fluid. Although position of virus protein 1 (VPI) was found to be common to all the clones, all the other clones had specific bands that slightly distinguished them from each other. Clones KOBL and KOBS shared identical peptide bands and therefore exhibited the greatest similarity among the different clones originating from the same strain. The positions of VP2 and VP3 were shared by clones KOBL, KOBS, NDV-Komarov and 104-L, while positions VP5, VP6 and VP7 were shared by NDV-Komarov and 104-L. The position of VP9 was shared by NDV-Lasota, KOBL and KOBS and SLS-L while the position of VP10 was shared by NDV-Lasota, NDV-Komarov and SLS-L. The greatest disimilarity was exhibited by clones 104-S and NDV-Lasota (Fig. 3). The diagramatic representation of the polypeptide patterns of the clones is shown in Fig. 3a.

Fig. 3 – Polypeptide patterns of Newcastle disease clones in polyacrylamide gel.

Fig. 3 – Polypeptide patterns of Newcastle disease clones in polyacrylamide gel.

Gammaglobulin (MW 22,000)

Bovine serum albumin (66,000)

Egg Albumin (45000)

Pepsin (34,000)

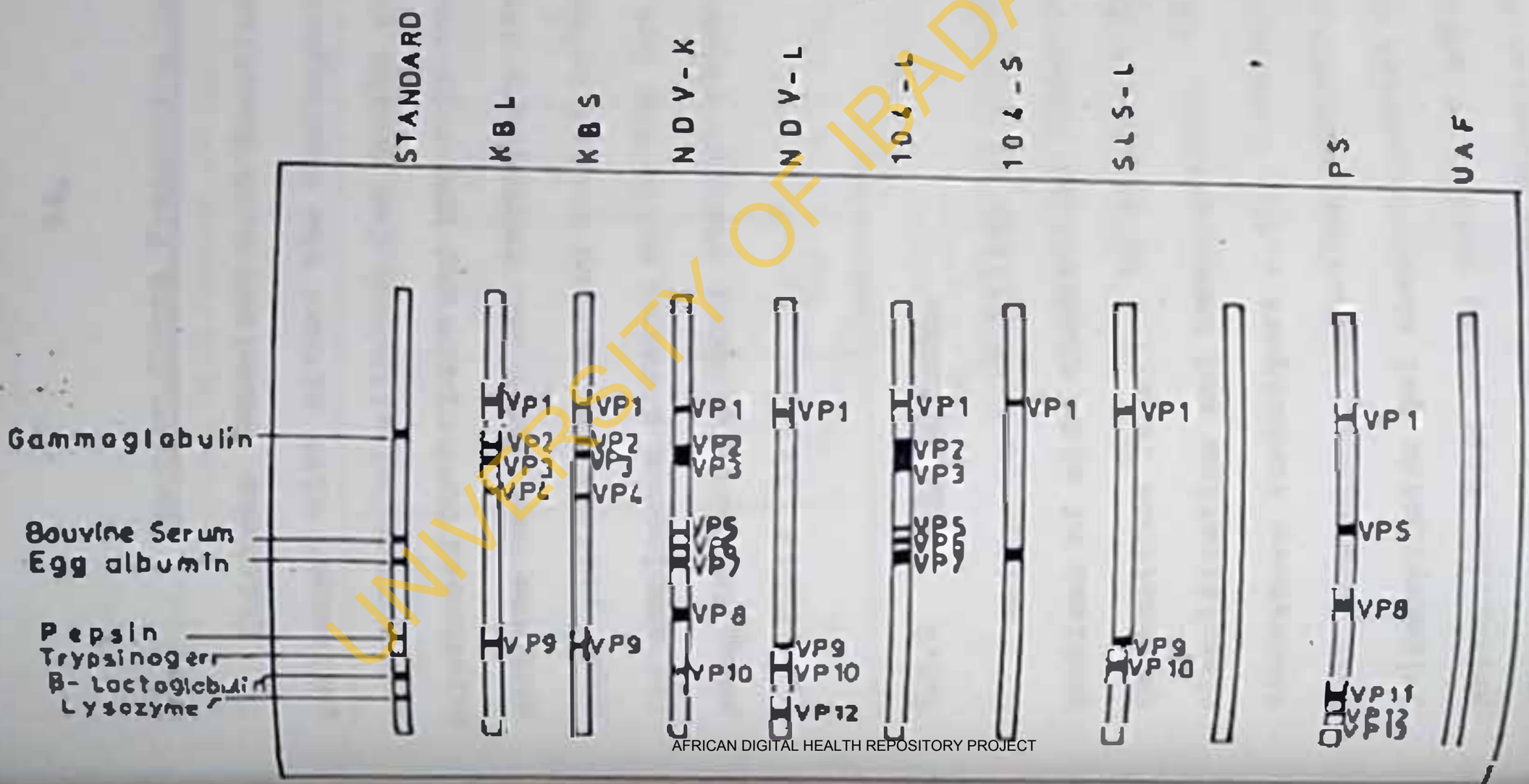
Trypsinogen (24,000)

β Lactoglobulin (18400)

Lysozyme (14,300)

92.

Fig. 3a - Diagramatic representation of fig 3 showing the positions of the different virus proteins.



5.4.7 In-vivo Cross Protestion Test

Chickens immunised with inactivated viruses and challenged with either the homologous or heterologous viruses were protected although few deaths were recorded in the lower dilution of both UI-104 and NDV-P in those birds immunised with the mesogenic and lentogenic strains. However, the Log10 protective indices ranged between 6.8 and 7.3 with the homologous strains while the Log 10 protective indices among the heterologous strains ranged between 6.6 and 7.3 (Table 14).

5.5.0 Discussion

Antigenic instability of viruses raises some serious Problems of wide theoretical interest and obvious practical implications relative to disease epidemics, identification, classification and immunization. Using four different laboratory techniques - the haemagglutination inhibition test, the plaque reduction neutralization test, the polyacrylamide gel electrophoresis and the *in vivo* cross protection test - the extent of antigenic variations among clones isolated from some Nigerian NDV virulent strains

Table 14. Cross Protection Test of Chickens immunised with inactivated viruses and challenged with homologous and heterologous Newcastle disease virus strains

Challenge viruses	Log 10 Protection Index*					
	104-L	SLSL	KBL	NDV-PL	NDV-K	NDV-L
104-L	7.2**	7.2	7.2	7.2	6.5	6.3
SLS-L	7.3	7.3	7.3	7.3	7.3	7.3
KBL	6.8	6.8	6.8	6.8	6.8	6.8
PL	7.3	7.3	7.3	7.3	7.3	7.3

* Log 10 Protection Index = Difference between Log 10 titre of the immunized group and Log 10 titre of the unimmunized group.

** Homologous Protection Index underlined.

and the two vaccine strains have been elucidated. In all tests, antigenic differences were found among these NDV clones and vaccine strains. This is in agreement with the finding of earlier workers. Upton et al (1953) and Bankowski et al (1965) observed that there is a great diversity in the antigenic components among the strains of ND virus. Schloer (1974) and Schloer et al (1975) using the kinetic neutralization tests further observed antigenic differences between mutants of ND virus.

There is a marked variation in the inhibition activities of the different sera despite the fact that the same pool of sera was used in all the tests. Results presented in this study showed that antigenic and strain variation was greatest among clones from different strains, and to a lesser extent between clones from the same strain. Of particular interest is the antigenic relationship of the two vaccine strains to the field strains. Antigenic divergence was exhibited by these two strains in both the haemagglutination inhibition test and the PRNT as expressed by the r values for both the tests, although NDV-Komarov exhibited a closer antigenic relationship in the PRNT than in the HI test.

It is quite apparent that the antigenic patterns of the highly velogenic strains and the two vaccine strains can be differentiated. The clones derived from the velogenic strains had closer antigenic relationship among themselves than between the vaccine strains of lower virulence. This antigenic divergence is probably due to the fact that antigenic variations are the functions of the external proteins which determine some of the biological functioning of the virus. MacPherson and Swain (1956) observed that even though the major antigens were shared by both the highly virulent strains of NDV, and the less virulent strains, some of the antigenic components of the virulent type are lacking in the less virulent strains of ND virus. This is clearly seen in the peptide pattern of NDV-Lasota in relation to the other field clones (Fig. 3). Such may be the immunological situation of ND in Nigeria where the field strain is the very virulent velogenic strain in contrast with the lentogenic and mesogenic vaccine strains.

One of the problems associated with an antigenic classification of ND is the avidity factor associated with many of the clones within a strain (Bratt and Gallaher, 1972). Cross reactions between some NDV strains may be due to the presence of some mutants which react more broadly with heterologous viruses (Bratt and Gallaher, 1977). In this present study 4 of the 8 clones (104-S, SLS-L, PL and KBS) had higher r values with heterologous strain than with the homologous strains. Therefore in addition to antigenic differences between strains differences in avidity are also detected. The data presented above clearly show antigenic divergence in addition to avidity.

It has been suggested that the changes and differences in the external proteins might have a definite correlation with the evolution of strains with different pathogenecity (Nagai et al 1980) and may be responsible for the difference in virulence of the different strains. A review of the previously published protein patterns of NDV reveals strain dependent differences of the viral glycoprotein (Iomniczi, 1971). A similar result was obtained in this study where the peptide bands obtained in PAGE were specific

for each strain along, apart from the high molecular weight protein which seemed to be common to all the clones. Nagai et al (1976) observed that virulent and avirulent strains exhibit specific differences in their glycoprotein which proved to be of high importance for the biological activity of the virus.

Newcastle disease virus has six major polypeptides, three of which are associated with the internal RNA genome: the neucleocapside protein (NP) the phosphoprotein (P) and the largest (L). The other three are membrane-associated; a large glycoprotein possessing both the viral haemagglutinating (HA) and neuraminidase (NA) activities, and a smaller protein (F) which is required for cell fusing activity and a third protein (M). While there is remarkable structural homology in the internal proteins, a variety of changes was revelaed for the external glycoproteins, thus, the external proteins would represent the regions of the virus which might undergo structural changes. It is these structural changes in the glycoproteins of the mutants that account for the antigenic distinctions. Although NDV may be a single serotype, sufficient antigenic differences are found to clearly distinguish among isolates and strains.

In an attempt to confirm the *in vitro* antigenic variations observed in the HI, PRNT and the PAGE, an *in vivo* cross protection test was done in three week old NDV susceptible chickens. Results of the *in vivo* cross protection test has shown that antigenic variations observed *in vitro* occur as a serological phenomenon only, as in this study the *in vitro* differences observed between the clones were not detected in the *in vivo* test. The antigenic dissimilarities expressed in all the tests between the clones may as well provide confirmatory evidence for the concept that antibodies measurable by *in vitro* techniques are not identical with those measurable by the *in vivo* procedure. Even though there were a few death among the birds vaccinated with the moderate and avirulent vaccine strains when challenged with velogenic strain of lower dilution the protective index was not significant. A protective index higher than 1 is regarded as significant. The implication of such a finding is that birds vaccinated with the less virulent strains can come down with the disease if exposed to high concentration of the velogenic strains. This appears to be the case in the field, but it is subject to confirmation.

CHAPTER SIX

6.0 STUDIES ON LIVE AND INACTIVATED VACCINES

6.1 Introduction

Results obtained so far in this study have shown significant antigenic variations between the wild field strains of the prevalent Newcastle disease virus and the vaccine strains in use in Nigeria. Significant differences have also been observed in the pathogenecity and virulence of the two strains. These factors may be responsible for the frequent post-vaccination outbreaks often reported from the field. Severe mortality attributed to ND outbreaks in vaccinated flocks has continued to occur. There has been occasions when post vaccination reactions following the use of the current vaccines have assumed alarming proportions. For example, Ugochukwu (1982) reported a post vaccination ND outbreak in a farm in the Eastern State with a mortality of 1288 or (10%) of 12,000 vaccinated birds. The first reported outbreak of ND in Nigeria (Hill, 1953) was reported in flocks vaccinated with the NDV-Komarov vaccine from South Africa. Although

it is seldom necessary to select a vaccine according to the antigenic type of the field virus occurring in any locality (Buxton, 1977), an appraisal of the existing vaccines and vaccination proceedures may become necessary if vaccination programmes that have once protected chickens against ND are no longer adequate in protecting birds from subsequent challenge by the existing viscerotropic and neurotropic velogenic strains.

From field report, it is likely that the present vaccines in use in Nigeria have not proved sufficiently immunogenic to give the birds the degree of protection against the velogenic strains of NDV responsible for most of the outbreaks. Because of this it was decided to test the efficacy of the current live vaccines in Nigeria in protecting birds against these virulent viscerotropic velogenic strains and to compare the efficacy of currently available vaccine with a vaccine prepared from a local Nigerian strain.

In this Chapter, are reported the response of vaccinated chickens to experimental challenge with the Nigerian velogenic strains, as well as the preparation and testing of inactivated oil emulsion (OE) vaccines prepared from selected clones of Nigerian velogenic strain and two vaccine strains.

6.2.0 Experiment I:

Response of chickens vaccinated with live NDV vaccines to experimental challenge by wild isolated Nigerian velogenic strains of Newcastle disease virus.

6.2.1 Materials and Methods

6.2.1.1 Vaccines

The three live vaccines - the NDV intra ocular (i/o), NDV-Lasota and NDV-Komarov were supplied by the Virology Division of the National Veterinary Research Institute, Vom. ND i/o Batch No. 135 had an EID_{50} of Log $10^{-6.5}$ NDV-Lasota Batch No. 240 had an EID_{50} of Log $10^{-8.6}$ while the Komarov vaccine Batch No. 85 had an EID_{50} of Log $10^{10.3}$.

6.2.1.2 Challenge Viruses

Nine of Nigerian velogenic strains were used. The characterization of the strains had been described in Chapter 3 Section 3.2.6.

6.2.1.3 Chickens

A total of 540 white Leghorn day old chicks, supplied by the Poultry Division of the National Veterinary Research Institute were used for this study. Birds were fed ad lib with mash ration and were housed in semi-isolation rooms with adequate heat supply.

6.2.1.4 Vaccination

The birds were divided into three groups of 180 each. Each group consisted of 90 test and 90 control chicks. At day old, the test birds in all the groups received one drop each of NDV-i/o into each of the eyes. The control birds received the same amount of sterile diluent in each of the eyes.

At day 21, the test birds in group II and group III were vaccinated with NDV-Lasota dissolved in chlorine free water after they had been deprived of water for 8 hours. The control birds were given the same amount of chlorine free water.

At day 42, the test birds in group III were vaccinated with the NDV-Komarov. Each bird received in 0.2ml of the vaccine in the thigh muscle. The control birds received

the same amount of sterile diluent. The vaccination schedule is shown in Table 15. Five chickens randomly picked from each of the test and control birds were bled for serum before each vaccination. Sera were tested for HI antibodies and the Geometric mean HI titre calculated.

6.2.1.5 Challenge Test

Ninety birds each were picked from the test and control birds of each group and 10 birds each were challenged with one of the velogenic strains at the appropriate day. Birds in group I were challenged day 21 post-vaccination while birds in groups II and III were challenged on days 42 and 63 respectively. The birds were given im 0.2ml of the challenge virus containing 10^{-6} ELD_{50} per ml.

6.2.1.6 Results

Between 0 and 3 (0-30%) of group I birds vaccinated with NDV-i/o and subsequently challenged with a velogenic strain died. All deaths occurred between 5 - 10 days post challenge. All the surviving birds in the vaccinated group had nervous signs and stunted growth. All the control unvaccinated birds died between 3-7 days. The GM titre of sera from

Table 15: Vaccination Schedule of Chickens with three Live NDV Vaccines

Vaccination Schedule	Day of Vaccination	Group I		Group II		Group III	
		T	C	T	C	T	C
NDV-i/o	0	+	-	+	-	+	-
NDV-Lasota	21	-	-	+	-	+	-
NDV-Komarov	42	-	-	-	-	+	-

\+ = Vaccinated

\- = Not Vaccinated

T = Test Birds

C = Control Birds

the NDV-i/o vaccinated birds at the time of challenge was 5_2 (Log $2^{5.6}$). No death was recorded in group II birds vaccinated with NDV-i/o and NDV-Lasota, however 40 out of 90 birds, had mild nervous signs, torticollis and retarded growth. All the control unvaccinated birds in this group died between 3 to 10 days post challenge. The GM titre of the vaccinated birds at the time of challenge was 294 (Log $2^{8.2}$). Group III birds that received all the three vaccinations survived challenge with no death or abnormal signs. All the control birds died between 6-12 days. The GM HI titre of sera from the vaccinated birds had risen from 294 to 1060 (Log $2^{12.5}$).

The control birds were negative for ND HI antibody titre. Results of challenge test are presented in Table 16. All death were confirmed as ND by necropsy and isolation of ND in embryonated eggs.

6.2.1.7 Discussion

Results obtained in this experiment have shown that birds vaccinated with NDV-i/o alone will succumb to clinical ND and a mortality ranging between 0-30% when exposed to virulent field velogenic ND strains. Although no death was recorded when the NDV-i/o was boosted with

Table 16: Result of Challenge of Vaccinated Chickens with Velogenic Nigerian Strains

Group	Type of Vaccination	Age at Vaccination	Age at Challenge (days)	Challenge Virus	Mortality: Control Unvaccinated	Mortality: Vaccinated	Geometric Mean HI Titre (Log_2^{-2})	Remarks
I	NDV/1/0	0	21	UI-104	10/10[A] (3-5[B])	3/10 (5-10	5.6	All the surviving birds in the vaccinated birds had nervous signs and stunted growth
				K-1090	10/10 (3-5)	2/10 (6-8		
				K-1084	10/10 (3-5)	3/10 (6-10		
				CM-1	10/10 (5-7)	1/10 6		
				KAD-1077	10/10 (4-6)	3/10 7		
				SLS	10/10 (3-7)	2/10 (5-6		
				NDV-P	10/10 (3-6)	2/10 (5		
				OJR-651	10/10 (5-5)	0/10		
				NY-1	10/10 (3-7)			
II	NDV/1/0 + Lasota	21	42	UI-104	10/10 (3-7)	0/10	8.2	Majority of surviving birds had mild nervous signs, torticollis and stunted growth
				K-1090	10/10 (3-7)	0/10		
				K-1084	10/10 (3-7)	0/10		
				CM-1	10/10 (5-10)	0/10		
				KAD-1077	10/10 (6-10)	0/10		
				SLS	10/10 (3-7)	0/10		
				NDV-P	10/10 (6-10)	0/10		
				OJR-651	10/10 (7-10)	0/10		
				NY-1	10/10 (7-10)	0/10		
III	NDV/1/0 + Lasota + NDV-Konarov	42	63	UI-104	10/10 (5-10)	0/10	12.5	No abnormal signs
				K-1090	10/10 (6-12)	0/10		
				K-1084	10/10 (6-11)	0/10		
				CM-1	10/10 (6-12)	0/10		
				KAD-1077	10/10 (6-10)	0/10		
				SLS	10/10 (6-11)	0/10		
				NDV-P	10/10 (6-9)	0/10		
				OJR-651	10/10 (6-12)	0/10		
				NY-1	10/10 (6-12)	0/10		

A = Number of dead chickens over total Number of chickens challenged.

B = Days when death were recorded.

Table 16: Result of Challenge of Vaccinated Chickens with Velogenic Nigerian Strains

Group	Type of Vaccination	Age at Vaccination	Age at Challenge (days)	Challenge Virus	Mortality: Control Unvaccinated	Mortality: Vaccinated	Geometric Mean HI Titre($\log_2$)	Remarks
I	NDV/1/0	0	21	UI-104	10/10[A] (3-5[B])	3/10 (5-10)	5.6	All the surviving birds in the vaccinated birds had nervous signs and stunted growth
				K-1090	10/10 (3-5)	2/10 (6-8)		
				K-1084	10/10 (3-5)	3/10 (6-10)		
				OM-1	10/10 (5-7)	1/10 (6)		
				KAD-1077	10/10 (4-6)	3/10 (7)		
				SLS	10/10 (3-7)	2/10 (5-6)		
				NDV-P	10/10 (3-6)	2/10 (5)		
				OJR-651	10/10 (5-5)	0/10		
				NY-1	10/10 (3-7)			
II	NDV/1/0 + Lasota	21	42	UI-104	10/10 (3-7)	0/10	8.2	Majority of surviving birds had mild nervous signs, torticollis and stunted growth
				K-1090	10/10 (3-7)	0/10		
				K-1084	10/10 (3-7)	0/10		
				OM-1	10/10 (5-10)	0/10		
				KAD-1077	10/10 (6-10)	0/10		
				SLS	10/10 (3-7)	0/10		
				NDV-P	10/10 (6-10)	0/10		
				OJR-651	10/10 (7-10)	0/10		
				NY-1	10/10 (7-10)	0/10		
III	NDV/1/0 + Lasota + NDV-Lasota	42	63	UI-104	10/10 (5-10)	0/10	12.5	No abnormal signs
				K-1090	10/10 (6-12)	0/10		
				K-1084	10/10 (6-11)	0/10		
				OM-1	10/10 (6-12)	0/10		
				KAD-1077	10/10 (6-10)	0/10		
				SLS	10/10 (6-11)	0/10		
				NDV-P	10/10 (6-9)	0/10		
				OJR-651	10/10 (6-12)	0/10		
				NY-1	10/10 (6-12)	0/10		

A - Number of dead chickens over total Number of chickens challenged.

B - Days when death were recorded.

NDV-Lasota, 40 out of the 90 birds showed mild clinical disease when challenged with the velogenic strains. NDV-Komarov conferred a solid immunity on the birds. Although anamnestic response was noticed after vaccination in each case, it would appear that the level of immunity produced by both the NDV-i/o and NDV-Lasota is not enough to protect the birds from NDV infection when challenged by the virulent velogenic strains. The condition may even be worse on the field where post vaccination titre may not be as high as those obtained under the ideal experimental condition described above. The choice of ND vaccines varies from one country to another. In countries where the disease is under control the vaccine of choice would be a lentogenic strain, but in countries like Nigeria where the disease is endemic more virulent strains are used as vaccines. The finding of this study clearly supports this. It is therefore suggested that in order to build up enough antibody level to withstand the virulent velogenic strains the mesogenic vaccine should always be used alongside the two lentogenic vaccines.

6.3.0 Experiment II

Preparation of Experimental Inactivated Oil Emulsion (OE) Vaccines from selected Clones of Nigeria Velogenic Strains and Two Vaccine Strains.

6.3.1 Materials and Methods

6.3.1.1 Viruses

Two clones UI-104L and UI-104S obtained from the parent strain UI-104 and two vaccine strains NDV-Komarov and NDV-Lasota were used for the preparation of the inactivated vaccines. Procedure for obtaining the clones and the properties of the clones have been described in Chapter Four, Section 4.2.3.

6.3.1.2 Chickens

Newcastle disease susceptible chickens were supplied from the Egg production Unit of the National Veterinary Research Institute, Vom.

6.3.1.3 Reagents

6.3.1.3.1 Beta-propiolactone (BPL)

Beta-propiolactone was obtained from Sigma Chemicals Company, and was used at a dilution of 0.1% in PBS as the inactivating agent.

6.3.1.3.2 Arlacel A (Mannide Monooleate)

Arlacel A obtained from Sigma Chemicals Company was used as the oil phase emulsifier.

6.3.1.3.3 Liquid Paraffin

Liquid paraffin was obtained locally and it provided the source of mineral oil.

6.3.1.3.4 Tween 80 (Monooleate Sorbitan)

Tween 80 was obtained from Sigman Chemicals Company, and was used as the aqueous phase emulsifier.

6.3.1.4 Preparation of Antigen

Antigens were prepared from allantoic fluids of 9-10 day old embryonated eggs. The eggs were inoculated with 0.1ml each of the clones or vaccine strains containing approximately 10^6 EID_{50}. Eggs were incubated at 37^oC and candled twice daily. Dead and moribund eggs were chilled at 4^oC overnight before ready for harvesting. Allantoic fluids were harvested, tested for haemagglutinin, and titrated in embryonated eggs. The different allantoic

fluids were diluted to contain approximately 10^{10} ELD_{50} per ml. The antigens were inactivated with 0.1% beta-propiolactone in PBS for 2 hours at 37^{o}C. Inactivation was tested and confirmed by three blind passages in embryonated egg. The allantoic fluid was certified free of live virus after the third blind passage, if no haemagglutination activity was detected.

6.3.1.5 Preparation of Oil Emulsion Vaccine

The OE vaccines were prepared according to the method described by Stone et al (1978). An oil phase was first prepared by mixing 72 ml of liquid paraffin with 8ml of Arlacel A, while the aqueous phase was prepared by mixing 19.2ml of the inactivated allantoic fluid antigen with 0.8ml of the Tween 80. Both phases were then combined by adding dropwise to constantly stirred oil phase the aqueous phase. This mixture contained 20% of the AF antigen and resulted in a final concentration of Log $10^{9.3}$ ml of each virus in the emulsion. Emulsification was done by combining dropwise the aqueous phase with the oil phase to give a final aqueous to oil ratio 1:4. Emulsification was done in a "Virtis 45" homogenizer (The Virtis Company, Gardiner, New York) first at a speed of 30 for 30 seconds and completed

at a speed of 50 for 60 seconds. The procedure for the preparation of the emulsion vaccine is presented in Table XVII .

6.3.1.6 Determination of Emulsion Type, Viscosity and Stability of the Vaccines

The physical characteristics of emulsions were determined by the drop test according to the method described by Cessi and Nardelli (1973). Two drops of the vaccines were dropped separately on a clean microscope slide. One drop was mixed with one drop of oil and the other with one drop of water. The drops were observed for some few seconds. A water-in-oil emulsion should blend readily with oil but not with water.

The relative viscosity was determined by the pipette method. A 1ml serological pipette was filled with the vaccine to the zero mark, and the time taken for the emulsion to discharge 0.4ml was taken. This was repeated thrice with different pipettes, and the mean of three determinations was taken.

Emulsion stability was determined by accelerated aging at $37^{o}C$ in close glass tubes. Tubes were observed daily,

at a speed of 50 for 60 seconds. The procedure for the preparation of the emulsion vaccine is presented in Table XVII .

6.3.1.6 Determination of Emulsion Type, Viscosity and Stability of the Vaccines

The physical characteristics of emulsions were determined by the drop test according to the method described by Cessi and Nardelli (1973). Two drops of the vaccines were dropped separately on a clean microscope slide. One drop was mixed with one drop of oil and the other with one drop of water. The drops were observed for some few seconds. A water-in-oil emulsion should blend readily with oil but not with water.

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Emulsion stability was determined by accelerated aging at $37^{o}C$ in close glass tubes. Tubes were observed daily,

Table 17: Procedure for the Preparation of 100ml of Inactivated Oil Emulsion Vaccine according to Stone et al, 1978.

Step 1:	Prepare oil phase:		
	(a)	Liquid paraffin	72.0ml
	(b)	Arlacel A	8.0ml
	(c)	Mix thoroughly A and B	
Step 2:	Prepare aqueous phase:		
	(d)	Antigen	19.2ml
	(e)	Tween 80	0.8ml
	(f)	Mix thoroughly D and E	
Step 3:	Combine aqueous and oil phases:		
	(g)	Add aqueous phase dropwise to constantly stirred oil phase.	
Step 4:	Emulsify combined aqueous and oil phases:		
	(h)	Homogenise mixture to obtain the emulsion vaccine.	

and the time it took for the emulsion to separate into the aqueous and oil phases were observed. The emulsion was regarded as stable if it did not separate into the two phases of oil and water.

6.3.1.7 Vaccination of Susceptible Chickens with the Oil Emulsion Vaccines

Four hundred 3-week old NDV-susceptible chickens were divided into 5 groups each of 80 birds. Birds in groups A and B were inoculated with vaccine prepared from clone 104-L and clone 104-S respectively. Those in group C received vaccine prepared from NDV-Komarov while group D birds received vaccine prepared from Lentogenic strain Lasota. The last group served as the control group. Each bird received 0.5ml of the appropriate vaccine subcutaneously in the neck region, thus bringing the amount of virus injected into each bird from the Log $10^{9.3}$ml of virus contained in the vaccine to Log $10^{9.0}$. Group E birds were inoculated with 0.5ml of normal allantoic fluid.

Birds were housed in semi-isolation rooms and were bled at weekly intervals for 10 weeks, starting from the second week. The immune response of individual chicken was measured by the HI test and the geometric mean titre of each group of birds calculated from the results.

6.3.1.8 Challenge Test

The vaccinated birds were challenged at 10 and 20 weeks post vaccination. On each occasion, 20 birds from each of the vaccinated group was challenged with either NDV-Herts or KAD 1077. Each bird received intranasally 0.2ml of the challenge viruses containing $10^{6.0}$ EID_{50} per ml. Birds were observed for 15 days for signs of Newcastle disease.

6.4.0 Results

6.4.1 Viruses

All the viruses grew to a high titre in embryonated eggs when inoculated at a low multiplicity. Infectivity titre ranged from 11.1 (104-L) to 13.2 (Lasota). The HA titre of the respective allantoic fluid in log_2 titres are NDV-Lasota, 11.0, NDV-Komarov, 9.3, 104-S, 10.8 and 104-L 8.9 (Table 18).

Table 18: Log^{10} ELD_{50} and Log_2 HA Titres of the Allantoic Fluids used in the Preparation of Oil Emulsion Vaccine.

Clones	Titre of allantoic fluid in Embryonated Egg (Log_{10} ELD_{50})	HA titre of harvested allantoic fluid (Log_2 HA_{50})
UI-104-L	11.1	8.9
UI-104-S	13.0	10.8
ND-Komarov	11.6	9.3
NDV-Lasota	13.2	11.0

6.4.2 Physical Characteristics of Vaccines

The prepared vaccines were of the water in oil types. All the vaccines readily blended with oil. A water-in-oil emulsion vaccine will readily blend with oil. It took between 8.0 to 8.3 seconds for the vaccine to discharge 0.4ml from a 1ml pipette. The standard average for emulsion vaccine is between 3 to 15 seconds. The vaccines were stable at 37°C and room temperature for 11 weeks and 8 months respectively. A stability of 6 weeks and above at 37°C is generally acceptable. The vaccines were tolerated by the birds and were easy to inject (Table 19).

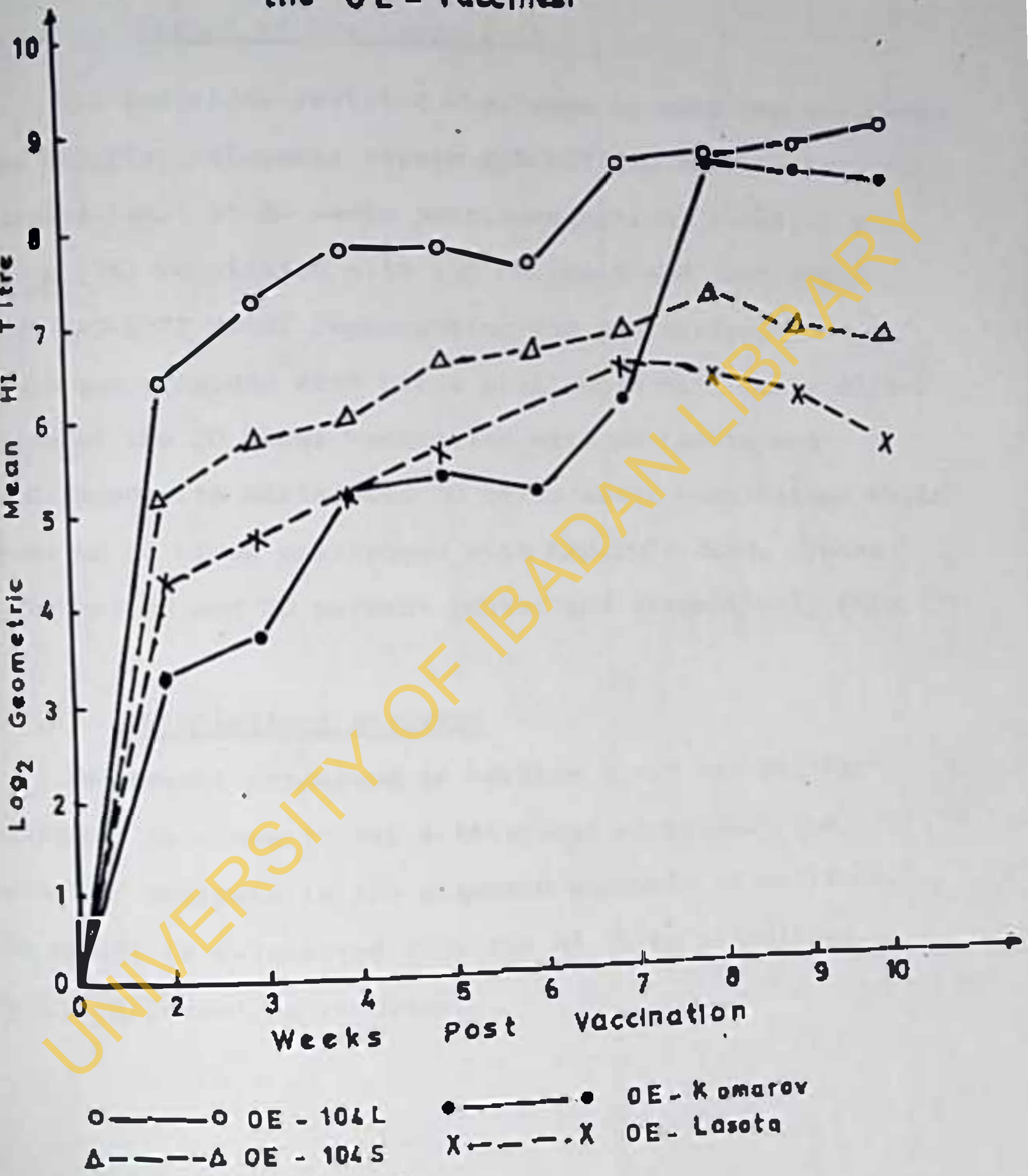
6.4.3 Result of Vaccination

The GM titre 2 weeks after vaccination ranged from Log $2^{3.3}$ for OE-Komarov to Log $2^{6.4}$ for OE-104L. Peak titres were obtained at 10 weeks (Log_2 9.1) for OE-104L, 8 weeks (Log_2 8.7) for OE-Komarov 8 weeks ($Log_2 7.3$) for OE-104-S and 6 weeks (Log $2^{6.6}$) for OE-Lasota. The weekly GM titres are shown in Fig. 4.

Table 19: Physical Characteristics of the Oil Emulsion Inactivated Vaccine

Vaccines	Viscosity Test in (Secs.)	Emulsion Type	Stability Test (Weeks at 37°C)
OE-104L	8.0	Blend readily with oil	11
OE-104S	8.3	Blend readily with oil	11
OE-NDV-Komarov	8.0	Blend readily with oil	11
OE-NDV-Iasota	8.0	Blend readily with oil	11

Figure 4 – Weekly HI Response of Chicken Vaccinated with the OE - Vaccines.



6.4.4 Result of Challenge Test

All the birds resisted challenge by both the NDV-Herts and Nigerian velogenic strain KAD-1077 10 weeks after vaccination. At 20 weeks post vaccination, 1 out of 20 birds (5%) vaccinated with the OE-104-S and challenged with KAD-1077 died, representing 95% protection. No death was recorded with birds challenged with Herts 33/56. Three of the 20 birds vaccinated with OE-Lasota and challenged with Herts died 20 weeks after vaccination while 5 out of 20 birds challenged with KAD-1077 died. These represent 85 and 75 percent protection respectively (Fig. 5).

6.4.5 Statistical Analysis

The result discussed in section 6.4.3 was further subjected to conventional statistical analysis. The method of analysis is the standard analysis of variance. The result is calculated from the HI titre stimulated by the different OE vaccines.

	OE-104-L	OE 1045	OE-K	OE-L
	320	160	320	80
	140	80	640	80
	320	80	320	160
	160	40	80	160
	160	40	80	40
	320	160	320	20
	640	160	640	160
	160	160	320	20
	320	20	80	-
	640	40	160	-
	640	320	-	-
Mean $= \bar{X}_i = \frac{1}{n} \sum_{j=1}^{n_i} x_{ij} =$	392	114	296	90
Variance $= S^2_i = \frac{1}{n_1 - 1} \sum_{j=1}^{n_1} (x_{ij} - \bar{x}_1)^2 =$	42822.4	7847.6	44160	3885.7

grand mean $= \bar{X} = \frac{1}{n} \sum_{i=1}^{1} \sum_{j=1}^{n_1} = \frac{1}{n} \sum_{i=1}^{1} n\bar{X} = 231.7$

SS Error $= \sum_{i=I}^{1} (n_1 - 1) S^2_1 = 931340$

SS Treatment $= \sum_{i-I}^{1} n_i (\bar{x}_i - \bar{x})^2 = 637019$

$F = \frac{SST/(I-1)}{SSE/(n-I)} = \frac{(637019,20)/3}{931340/36} = \frac{212339.6}{25870.5} = 8.20$

Calculated F = 8.20

Tabulated $F_{(3, 36, 0.05)} = 2.86$

Calculated F > Tabulated F at 0.05 significant level

The Null hypothesis (Ho) states that

$$Ho = Mean_{104L} = Mean_{104S} = Mean_{OE-K} = Mean_{OE-L}$$

The Alternative hypothesis states that

$$H_1 = Mean_{104L} \neq Mean_{104S} \neq Mean_{OE-K} \neq Mean_{OE-L}$$

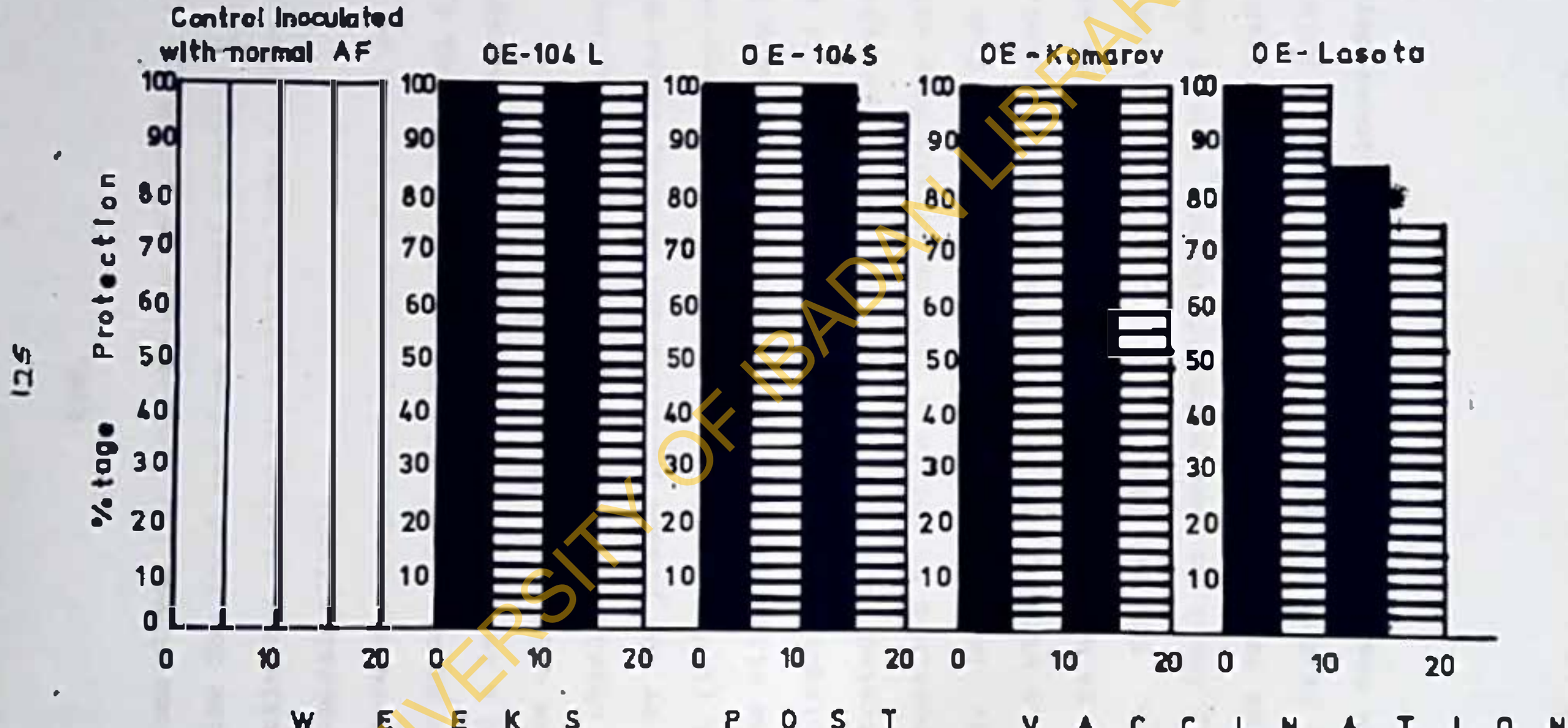
Reject Ho that the means are equal and accept the alternative hypthesis that the means are not equal.

Result of statistical analysis confirms the result expressed in Section 6.4.3 and represented in Fig. 4 that the differences in the HI titres stimulated by the OE vaccine are significant.

6.5.0 Discussion

There has been an increased interest in the use of inactivated oil-emulsion vaccines in recent years in Europe and America where both field and laboratory evidence suggests that these vaccines induce high levels of protection against clinical VVND (Hofstad 1963; Box and Furminger, 1975; Stone et al, 1978 and Edison, et al, 1980). However the use of such vaccines has not been introduced into Nigeria. There is the urgent need to consider the use of inactivated OE vaccines considering the rapid expansion of poultry industry in Nigeria. Control of the disease in Nigeria is by vaccination using the three live vaccines produced at the National Veterinary Research Institute. The use of these vaccines have not been without problems. Post vaccination reactions such as drop in egg production and outbreak of overt clinical disease are often associated with these vaccines especially in partially immuned chickens. Apart from the diversity in antigenecity and the differences in pathogenecity and virulence, one other factor that may be responsible for the failures of these vaccines in the field may be due to loss of potency of the live vaccines due to the hot tropical weather and the improper storage. However recent findings by Patrick (1986) have shown that the vaccines are relatively stable under field

Figure 5— Percentage Protection of Birds vaccinated with 4 Types of OE Vaccines Following Challenge with NDV-Herts and KAD-1077.



conditions. These two problems may be solved by (1) preparing a vaccine for local use from a local strain. '(2) preparing an inactivated vaccine that will be able to withstand the hot tropical climate.

There are at present a number of methods of obtaining vaccine strains from wild ND strains. Reeve et al (1974) derived a less virulent but comparatively immunogenic strain from the wild Essex 70 by the limiting dilution. Lomniczi (1975) obtained a less virulent clone from the Hertfordshire strain by the plaque purification method, while Smith and Parsell (1977) obtained a temperature sensitive mutant from the wild Essex 70. Thiry (1964) chemically induced small plaque mutants by using $NaNO_2$. In all, it has been demonstrated that through some laboratory manipulations, it is possible to obtain a vaccine strain from wild field strains. In this study, two clones isolated from a Nigerian velogenic strain, and two vaccine strains currently in use in Nigeria were used to prepare inactivated oil emulsion vaccines. The two clones were found to stimulate high antibody level in vaccinated chickens. One of the good qualities required of a strain to be used as inactivated vaccine is the ability of the strain to grow into a high titre in the embryonated egg. The two clones derived from

the Nigerian velogenic strain grew to high titres (11.1 and 13.0 ELD_{50}) in the embryonated egg. No further concentration of the AF was needed before use in the preparation of the OE vaccine. This was achieved by repeated passages of the clones in embryonated eggs at low multiplicity dose.

The immunogenecity of a strain of NDV as a live vaccine is dependent on the ability of that strain to multiply and subsequently contain viral particles sufficient to stimulate elevated immune response. In contrast, the immungenecity of inactivated vaccines is dependent on the antigenic mass of the NDV strain introduced into the organism. However, the ability of each antigenic mass of the different strains to stimulate immune response further depends on the stability rather than the virulence of the antigenic material (Gough and Allan, 1974). This probably explains the differences in the immune response obtained in this study where equal amount of virus was inoculated into the chickens. Earlier reports have shown that the haemagglutinin of the virulent strains are more stable than the less virulent strains (National Aca. Science, 1971).

Results obtained from the antibody response of the chickens following vaccination showed that UI-104L, NDV-Komarov and UI-104-S stimulated HI titre significantly higher than NDV-Lasota. Result of stability test carried out on the viruses in Chapter 3 Section 3.2.6.4 showed that the haemagglutinin of UI-104 and NDV-Komarov were stable for 120 minutes at 56^{o}C while the Lasota was not stable at all. In a similar work, Gough and Allan (1974) found that the Ulster 2C strain was superior in activated vaccines than the F, B1 and Lasota strains because of the relative stability of its antigenic material. Nedelicu *et al* (1982) also observed differences in the antibody level stimulated by four strains of ND virus. The difference in their result was attributed to the "intrinsic immunogenic value of a fixed number of viral particle", i.e. the ability of the viral particle to remain immunogenic.

The result obtained in this study indicated that the UI-104L obtained from a Nigerian velogenic strain is better than NDV-Lasota as an OE vaccine. Antibody response developed rapidly, a titre of $Log_2$6.2 in 2 weeks, in chickens vaccinated with OE-104L. This property will make it a

vaccine of choice during an epizootic. One of the greatest disadvantages of using virulent strains in inactivated vaccines may be the possibility of introducing the disease into a flock as a result of incomplete inactivation. This problem can however be taken care of by thorough and well tested inactivation.

There is a similarity in the result of protection tests with both the three live vaccines and the four inactivated vaccines. With both live and inactivated vaccines chickens reponded with higher antibody response following challenge with virulent strain than with non-virulent strains. In live vaccines, the moderately virulent strains multiply faster and attain higher titre in the organism, while in inactivated vaccines, the haemagglutinin which is responsible for the antigenecity seems to be more stable with the virulent strains.

It can be seen that from the challenge test with the live vaccine that birds vaccinated with NDV i/o and Lasota, if exposed to very virulent strain of NDV may likely respond with clinical NDV. In a similar result, all the birds vaccinated with the four inactivated vaccines were protected when challenged with two velogenic strain 10 weeks after vaccination; however between 5% and

25% mortality was recorded among birds vaccinated with OE 104-S and OE-Lasota 20 weeks after vaccination. It is likely that the immune response stimulated by both OE-Lasota and OE-104-S had already declined below the protective level at 20 weeks while the immune response stimulated by OE-104L and OE-Komarov was still very high at the same time (Fig.4). Comparative studies on the duration of immunity of such vaccines will be necessary in future. Patrick (1986) using the existing live ND vaccines in Nigeria observed that the moderately virulent Komarov strain elicited a higher antibody response (Log $2^{9.7}$) than the Lasota strain (Log $2^{6.8}$). He however recorded a mortality of 10% when the live Komarov was used alone as a result of post vaccination reaction. While the result obtained with the inactivated vaccines is similar to what Patrick (1986) observed in this study, the inactivated vaccine however had an advantage over the live vaccines as far as post-vaccination reactions are concerned.

At present it seems there is no satisfactory information on the duration of immunity of either the live vaccines or inactivated vaccines to the Nigerian velogenic strains of NDV. This is an area that requires further investigation.

CHAPTER SEVEN

7.0 GENERAL DISCUSSION AND CONCLUSION

Newcastle disease will remain a big threat to poultry industry in Nigeria for a long time to come. The disease is endemic in the country, and the prevalent ND strains in the field are the virulent velogenic strains to which the exotic birds are exposed. These virulent strains are maintained by the free roaming Nigerian local chickens (Nawathe et al 1975, Adu et al, In Press).

It has been established through this study that the most prevalent strains of NDV in Nigeria are the highly virulent velogenic strains. Serological and pathogenecity tests have also shown variations in antigenecity and virulence between virulent strains and the vaccine strains in use in Nigeria. It is likely that the high virulence and extreme velogenic nature of the Nigerian strains has contributed to the difficulty in establishing the required degree of protection in vaccinated flocks. These may be one of the major contributing factors of vaccine failures often reported from the field. Allan et al (1978) while relating the HI level of vaccinated

chickens to response to challenge with Herts 33/56 found that birds having Log 2^2 or less will suffer 100% mortality while birds with HI titre of between Log 2^2 to Log 2^5 will record a mortality of 10%. Birds with titre of Log 2^6 to Log 2^8 may not suffer any mortality but serious drop in production may be recorded. There is every likelihood that when vaccinated chicks are challenged on the field by these virulent strains, the extremely velogenic nature of the challenge virus causes a drop in the antibody level below the protective level referred to by Allan (1978) and since the challenge virus is still available to attack the chicken, the clinical disease often results. This postulation was further strengthened by the finding of Beard and Brush (1975) who claimed that although vaccination can confer sufficient flock immunity to protect against lethal infection with virulent viscerotropic NDV, however such birds may shed large amounts of virus and come down with nervous signs 18 to 21 days after exposure. A similar situation may be occurring in the field in Nigeria. Field experience has shown vaccine failures in NDV vaccinated birds in recent times. Three reasons may be easily deducted from this (1) either faulty preparation

of vaccine or (2) the vaccines do not stimulate enough immune response as a result of many factors or (3) antibody titre in these birds had dropped below the acceptable protective level as a result of challenge by these extremely virulent strains. In this study it was found that a titre of Log $2^{5.6}$ did not protect between 0-30% of the vaccinated chickens when challenged with the Nigerian velogenic strains. While 0% mortality was recorded among birds with titre of Log $2^{8.2}$, 40 (44%) of the birds showed mild clinical signs of NDV. This is in agreement with the findings of Beard and Brush (1975) and Allan et al (1978). In view of the above, and in order to control ND effectively, a reappraisal of the existing vaccines and vaccination programmes in Nigeria may have to be urgently looked into. This is where the introduction of inactivated OE vaccines will be of advantage especially when combined with the live vaccines. When combined with live vaccines, OE vaccines will be able to maintain the high antibody level needed to resist challenge with the virulent field strains, because of their adjuvanted nature and gradual release of their antigenic mass.

It is also very necessary to undertake a study on the duration of immunity conferred by the existing vaccines against the Nigerian velogenic strains.

Significant antigenic variations have been established in this study between the velogenic strains and the vaccine strains. This difference was however not detected in the in vivo test. The factors leading to natural selection of a given virus are largely unknown. Although NDV is known to be a single serotype, there remains a

possibility that the viral glycoproteins may undergo some minor antigenic drifts that escapes detection by the conventional neutralization and haemagglutination inhibition tests. Therefore, it will be of interest to elucidate the detailed relationship between the changes in primary sequence of each glycoprotein and its antigenic structure employing more discriminating serological probes such as the monoclonal antibody technique. Since it has been suggested that structural changes in the external glycoproteins might have a definite correlation with the evolution of strains with different pathogenecity, the monoclonal antibody technique may as well be the perfect tool for use in the development of a specific glycoprotein related vaccine for future use in Nigeria.

Result obtained during the course of this study has shown that the NDV i/o and NDV-Lasota vaccines are not immunogenic enough to confer the degree of immunity required to protect birds against the Nigerian velogenic strains of NDV. It is therefore recommended that the National Veterinary Research Institute, Vom recommendation on vaccination programme should be strictly adhered to meanwhile. It is therefore suggested that on no occasion

should the lentogenic vaccines be used alone. While the live Komarov vaccine elicits higher antibody level than the two lentogenic vaccines, the adverse post vaccination effects frequently noticed in vaccinated chickens has limited this advantage. It therefore seems logical that the immediate answer to the problem may be the introduction of inactivated oil emulsion vaccine prepared from a moderately virulent or more virulent strain. The ability of oil emulsion vaccines to remain immunogenic for a long period and to continue to stimulate high immune response as a result of the gradual release of the antigenic mass will be of great advantage in situations where high antibody level is required to protect against highly virulent wild strains. There is therefore an urgent need to introduce inactivated oil emulsion vaccine for use in Nigeria.

In this study, the following findings were established:-

1. The prevalent strains of ND in Nigeria is the velogenic strain.
2. There is antigenic variation between the vaccine strains in use and the prevalent velogenic strains.
3. The lentogenic vaccines used for vaccination in Nigeria are not immunogenic enough to confer the

degree of immunity required for ptoection against the prevalent velogenic strains.

4. A clone of low virulence (UI-104-S) was isolated from a Nigerian velogenic strain.

5. An experimental inactivated oil emulsion vaccine prepared from UI-104L, a clone isolated from a Nigerian velogenic strain stimulated higher antibody response in vaccinated chickens than the corresponding vaccines prepared from the two vaccine strains in use. This vaccine also conferred 100% protection on chickens when challenged at 20 weeks with both NDV-Herts 33/56 and a Nigerian velogenic strain KAD-1077.

The following recommendations are therefore put forward:

1. Because of the prevalence of the viscerotropic velogenic strains of NDV in Nigeria, it is suggested that a reappraisal of the current vaccines and vaccination procedures now practised in Nigeria should be re-examined.

2. Birds should not be vaccinated with the lentogenic vaccines alone, but should always be accompanied with vaccination from a more virulent strain.

3. There is the urgent need to introduce inactivated vaccine prepared from a more virulent strain in commercial basis in Nigeria. It is therefore recommended that a nationwide test of such vaccine conducted on a large number of birds be embarked upon in the nearest future.

4. There is the urgent need to determine the duration of immunity of NDV vaccines against the Nigerian velogenic strains of NDV following vaccinations.

5. A further pathgenecity test should be done on the UI-104-S clone with the aim of finally obtaining a vaccine strain from it.

6. A further serological tests to determine the detailed relationship between the strains of the virus in Nigeria should be undertaken using a more discriminating method like the monoclonal antibody technique. This may enable the isolations of specific glycoprotein that will be of future use in vaccine production in the country.

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