ADDIS ABABA UNIVERSITY
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ORAL NEWCASTLE DISEASE VACCINATION TRIALS & STUDIES OF NEWCASTLE DISEASE IN ETHIOPIA

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JANUARY 1998

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A thesis submitted in partial fulfilment for the degree of
Master of Science in Tropical Veterinary Epidemiology
at the Freie Universität Berlin and Addis Ababa University

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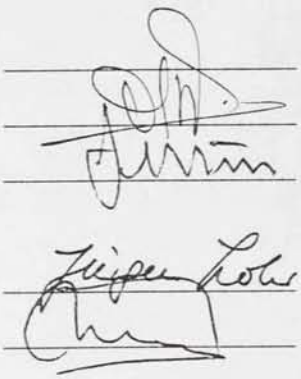

DEDICATION

This thesis work is dedicated to my beloved family and parents, my daughter Aida Mohammed, my wife Sr. Hannah Abebe, my mother Bedria Seid and my father Nasser Hassen.

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ABBREVIATIONS

AACMC	Australian Agricultural Consulting and Management Company
AAF	Amnioallantoic fluid
CAM	Chorioallantoic membrane
CI	Confidence interval
CL	Confidence limit
CMI	Cell-mediated immunity
EID_{50}	Embryonic infectious dose
ELD_{50}	Embryonic lethal dose
FAO	Food and Agriculture Organization
GMT	Geometric mean titre
HA	Haemagglutination
HB1	Hitchner B1
HI	Haemagglutination-Inhibition
ICPI	Intracerebral pathogenicity index
IVPI	Intravenous pathogenicity index
Mabs	Mouse monoclonal antibody
MDT	Mean embryonic death time
ND	Newcastle disease
NDV	Newcastle disease virus
NVI	National Veterinary Institute
PANVAC	Pan African Veterinary Vaccine Center
S.D	Standard deviation
SPF	Specific pathogen free
VNT	Virus neutralization test

ACKNOWLEDGEMENT

It would not have been possible to carry out this thesis work without the assistance of many people and organizations. Here I would like to express my gratitude to all those who assisted me in the course of my work.

advice and comments and also for providing some important literature on Newcastle disease. I would like to extend my thanks once again to Dr. Lohr and his family for their hospitality and accommodation during my stay at Aulendorf. I thank Professor Zessin and Dr. Ademe Zerihun for supervising my thesis work and for their advice regarding this research work.

Special thanks go to all staff of National Veterinary Institute, Faculty of Veterinary Medicine, Ethiopia, and to the staff of the Department of Postgraduate studies in Tropical Veterinary Medicine of the Freie Universität Berlin for assisting me during the course and the research work. I would like to thank especially Ato Berhanu, Ato Fantahun and other staff of the Foot and Mouth Disease Department of NVI, Ethiopia. Without their assistance the laboratory part of the thesis work would not had been possible.

I would also like to thank Dr. O. Werner and her staff of the Nationales Referenzlabor für Newcastle Disease und aviare Influenza at Insel Riems for characterizing the Ethiopian NDV field isolates.

I would further like to extend my sincere gratitude to National Veterinary Institute, Faculty of Veterinary Medicine and Poultry Development Enterprise, Ethiopia and Freie Universität Berlin for providing and allowing me to use the poultry houses, laboratory facilities, equipment and chemicals.

Last not least I wish to extend my sincere thanks to Dr. Schönefeld the course coordinator in Germany and the associate dean of faculty of veterinary medicine in Ethiopia, German Academic Exchange Service (DAAD), GTZ, and the German government who financed my project work and study in Germany and Ethiopia.

ABSTRACT

Three laboratory trials were conducted in commercial broiler chickens to determine the efficacy of the heat-resistant NDV-I_2 vaccine (similar to V4 vaccine), and to compare the NDV-I_2 vaccine with conventional vaccines (HB1 and LaSota). Sorghum and barley were tested for their suitability as vaccine carriers for oral administration. Further on eight field isolates of NDV were tested for their pathogenicity and nine ND outbreaks between 1983 and 1995 in state poultry farms in Ethiopia were studied retrospectively.

In the vaccination trials, chickens were vaccinated twice or three times with NDV-I_2 and conventional vaccines via the ocular and/or the drinking water route, and via feed with parboiled and untreated barley and sorghum as carriers. Sera were collected at weekly intervals up to the end of the trials and were tested for haemagglutination-inhibition (HI) activity. The protection level was assessed by challenging the chickens intramuscularly by a locally isolated velogenic strain of NDV.

Strain characterization was carried out by the standard methods for determining mean death time (MDT) in 9-day-old embryonated eggs, by calculation of the intracerebral pathogenicity index (ICPI) in one-day-old chicks and the intravenous pathogenicity index (IVPI) in six-week-old chicks. Further monoclonal antibodies (Mabs) was used for the differentiation of the field NDV isolates.

In the retrospective ND outbreak study, farm data were grouped according to study farms, season, type and age of the chickens, type and frequency of vaccination and concurrent diseases, and were analyzed for crude and specific mortality rates due to ND. Also, the possiblity of association between ND occurrences and husbandry factors were evaluated.

The results of the vaccination trials showed that conventional (HB1 and La Sota) and the thermostable NDV-I_2 vaccines gave similar antibody responses and protection against challenge when given via the ocular and the drinking water route. The oral application of NDV-I_2 was also shown to be effective with barley as vaccine carrier if barley was pretreated by parboiling. In contrast, untreated barley and both untreated and treated sorghum led to a rapid decrease of the virus titre and gave no serological response and protection against the challenge virus.

The characterization of the 8 field isolates by mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) demonstrated that all isolates, including the challenge strain (Alemaya) were velogenic strains of NDV. The monoclonal antibody test (Mabs) revealed that, all isolates differ from each other except Dembi 1 and Dembi 2, indicating that different velogenic strains of NDV are circulating in the country.

The retrospective study of 9 field outbreaks of ND which had occurred between 1983 and 1995, revealed that there was no seasonal pattern to the outbreaks, that the disease was mainly attributable to management and feeding faults, and that the ND symptoms were aggravated by concurrent diseases. Humans played a role in the spreading of the disease. The use of an inactivated vaccine substantially decreased mortality and egg production losses.

PART 1.0 INTRODUCTION AND OBJECTIVES

A large proportion of the human world population today is insufficiently supplied with quality protein in the diet. This is particularly true for a large part of the population in Ethiopia. Poultry occupies a unique position through its contribution to the supply of valuable food protein as well as by providing income to the families in developing countries.

One of the main factors impairing poultry production is disease. The current disease-related mortality from egg to adult chicken age is estimated between 20% and 50%. During some spectacular epidemics mortalities as high as 80% were recorded. Added to this must be the loss of production of the surviving sick birds (Alamargot, 1987).

In most developing countries Newcastle disease (ND) appears to be the most important avian disease. Virulent strains are widely distributed and seriously interfere with the development of the poultry industry. Farmers consider ND as a serious constraint to investing in the poultry business.

In Ethiopia it is apparent that village chickens are more important than modern intensive poultry production, both with regards to total numbers and to meat and egg production, despite the poor productivity of village chickens. They are usually kept in small numbers, they feed on leftovers, they require little attention and spend most of their time scavenging around the compound. They require no specific housing, breed naturally, receive no health care and are usually not vaccinated.

Newcastle disease in commercial or village chickens is a problem throughout the year although it is more serious at the beginning of the rainy season. The spread of ND in rural areas may be enhanced by selling or giving away sick birds, by carrier birds and by the roaming of chickens in search of feed. In contrast to commercial chickens, no efforts have been made in Ethiopia to control ND in village chickens.

The use of conventional vaccines for control of ND in village birds poses a difficult problem. Effective vaccination with conventional vaccines involves maintaining a cold chain, catching and handling each individual bird (which is time-consuming and laborious), using skilled vaccinators and repeating the whole procedure sufficiently often to ensure that every bird receives at least two doses of vaccines at different times. In addition, the vaccines themselves can produce complications which may reduce the birds' productivity or even result in clinical disease.

Protection or the immunogenic potential of the thermostable V4 strain of Newcastle disease virus (NDV) was reported by several workers (French *et al.*, 1967; Schalkoort and Spradbrow, 1980; Ibrahim *et al.*, 1981; Sagild and Spalatin, 1982; Westbury, 1984; Spradbrow, 1991).

Also many food stuffs have been tested as carriers of the vaccine and not all were found to be suitable, some have even proved unsuitable. However, some feed stuffs have not been studied in detail, such as sorghum, millet and other small grains (Spradbrow, 1992).

OBJECTIVES

The main objectives of this study were:

1 to study whether the heat-resistant NDV-I_2 strain of Newcastle disease vaccine gives adequate protection against velogenic strains of NDV.

2 to compare the heat-resistant NDV-I_2 Newcastle disease vaccine with the conventional vaccines (HB1 and LaSota) in terms of immunogenicity and protection.

3 to test the suitability of sorghum and barley as vaccine carriers.

4 to characterize field isolates of Newcastle disease viruses from Ethiopia.

5 to study retrospectively ND outbreaks which had occurred between 1983 and 1995 in State Poultry Farms in Ethiopia.

PART 2.0 LITERATURE REVIEW

2.1 Poultry Production in Ethiopia

Poultry raising has a long tradition in Ethiopia. Many people both in rural parts and urban centers of the country are engaged in keeping small numbers of chickens in their backyards to produce eggs and poultry meat for home consumption and to earn some occasional income (Alemu, 1985).

The most recent estimate of Ethiopian chicken population is about 58 million (FAO, 1993), out of which only one percent is found in large and small scale modern farms under industrial management. The other 99% of the flocks are raised under traditional or backyard conditions (Alamargot, 1987). Even though chickens are found in all parts of the country, densities differ from region to region. According to the 1994/5 census of the Central Statistics Office, the highest number of chickens are found in the regions of Oromia and Amhara.

The poultry sector in Ethiopia is based on chicken production, other domesticated birds (e.g. turkey, guinea fowl, pheasants, etc.) are almost unknown to the majority of the people. The sector is primarily concerned with egg production, while poultry meat production can be considered a byproduct of egg production. There are three main types of producers: the traditional subsistence producers, small scale and large scale commercial producers.

The backyard system is characterized by minimum inputs from the owners, with birds scavenging around the farm or belonging to households using no major inputs other than occasional grain feeds and household wastes (Alemu, 1985; Tadelle, 1996). The standard of housing varies greatly. In some areas primitive poultry houses are built from simple locally available materials, which in most areas the chickens share with their human owners at night (Alemu, 1995; Tadelle, 1996; Nasser, unpublished). Nesting materials are often provided to simplify the collection of eggs and to allow the control of brooding. Fertile eggs are hatched under broody hens and the hens attend the clutches of chicks, often without human intervention (Alemu, 1985; Nasser, unpublished).

From observations made so far, indigenous birds are very low producers of poultry meat and eggs. The annual production of a native hen is about 40-60 eggs with an average weight of 39-46 grams (Alemu, 1985). Tadelle (1996), after reviewing studies of research stations, stated that the average annual production of a native hen was 36-60 eggs under village conditions but this could be improved to 80-100 eggs under station conditions. The study at the Asela livestock farm revealed that the average production of local birds around Arsi was 34 eggs per hen per annum, with an average egg weight of 38 grams (Brannang and Persson, 1990). In regards to meat production, indigenous male birds reach an average weight of 1.52 kg in about one year (Alemu, 1985). Another study, conducted on five different local chicken strains, showed 1.38 kg within six months under research conditions (Teketel, 1987). Besides low genetic potential, malnutrition, diseases and poor management probably contribute most to this low productivity (Alamargot, 1987).

On the other hand, in Ethiopia the commercial poultry industry is still in its rudimentary stage. It is import-dependent for breeds, premixes, drugs and poultry equipment. Lack of quality food and of a market organization are also one of the present shortcomings (Habte, 1987; Nasser, unpublished). Small scale commercial systems keep mainly broilers and are found in the surroundings of the cities, especially around Debre Zeit, Addis Ababa and Nazreth. They are usually operations of about 100-10000 birds in simple open-sided houses with wire mash on two sides. In contrast large scale poultry farms are owned by the state and are integrated, combining breeding, hatching, pullet raising and egg and broiler production (Nasser, unpublished. Alemu, 1985).

In Ethiopia almost the entire production of eggs and poultry meat is derived from indigenous flocks. Rural poultry production represents a significant part of the national economy in general and of the rural sector in particular. It contributes 98.5% and 92.5% of the national egg and poultry meat production, respectively (AACMC, 1984). Annual output is estimated at 72300 metric tons of meat and 7800 metric tons of eggs (ILCA, 1993). According to the Watt poultry year book of 1991, Ethiopia in 1990 produced 1978 million eggs, 92 million chickens were slaughtered, and 74000 metric tons of poultry meat were produced with an average carcass weight of 0.8 kg. In 1981, Ethiopia produced 73800 metric tons of eggs and 6000 tons of poultry meat (FAO, 1981). The contribution of improved breeds was estimated to be 1000 tons of eggs and 500 tons of poultry meat. In 1986, 77300 tons of poultry meat were produced (Anon, 1989). Presently, poultry accounts for 15% of the total percapita meat consumption in the country (FAO, 1993).

Different estimates were made regarding the average number of chickens per household. AACMC (1984) estimated six birds, Tadelle (1996) 7-10 mature birds and FAO (1993) 6-10 birds. A small proportion of this is available for consumption by the family. The rest of the eggs and chickens is sold by the rural people to buy essential household items such as salt, soap, coffee, to pay school fees and to buy school materials (Mebratu, unpublished).

Different poultry diseases have been recorded in Ethiopia. The major economic losses are due to: Newcastle disease, salmonellosis, nutritional deficiency diseases, coccidiosis, mycoplasmosis, fowlpox, lymphoid leucosis (Alamargot, 1987, personal communication). Other diseases such as necrotic enteritis, omphalitis, external and internal parasites, and aspergillosis cause severe losses (Nasser, unpublished).

2.2 Newcastle Disease

2.2.1 Definition and history

Newcastle disease (ND) is a highly contagious and destructive illness in chickens, turkeys and many other birds (Hanson, 1978; Nonnewitz, 1986). It is characterized by the lesions in the respiratory tract, visceral organs and brain and causes moderate to severe mortality and morbidity in susceptible flocks.

The disease was first reported on the island of Java, Indonesia (Kraneveld, 1926) and Newcastle up on Tyne, England (Doyle, 1927). It is from this town that the disease has its name. According to Spradbrow (1987) and Alexander (1988) the disease was recognized in other parts of Asia (Korea, Philippines and India) in the same and subsequent years. However, several reports exist in the literature of disease outbreaks which predated the year 1926. Hanson (1978) and Alexander (1988) reviewing the literature, stated that ND first occurred in and around sea ports, apparently as a result of commercial activities by sea, and then spread to the interior of the countries much later.
Alexander (1988) described three panzootics of ND since its first recognition. Lancaster (1966) reviewed the worldwide spread of the disease from 1926-1964. Hanson (1978) grouped the theories on the origin of the ND virus into three categories: the first theory assumes mutation of the virulence of a related chicken virus, the second hypothesis is infection of chickens with a virus already virulent for them and originating from other avian hosts and the third theory suspects changes in the genetics and husbandry of hosts. None of the hypotheses were ever fully proved.

2.2.2 Etiology, classification and morphology

The causative agent of ND is a virus belonging to Avian Paramyxovirus type 1 (APMV-1) serogroups which, with viruses of the other eight APMV serotypes, have been placed in the Rubla virus, subfamily paramyxovirinae, family paramyxoviridae order mononga virales (Rima *et al.*, 1995). It is an enveloped RNA virus, which shows helical capsid symmetry. It possesses a non-segmented single-stranded genome of negative polarity, undergoes capsid assembly in the cytoplasm and is budded from the cell surface in an envelope of modified cell membrane (Alexander, 1991).
The shape of the virus is roughly spherical, although pleomorphic forms are commonly seen (Allan *et al.*, 1973). The virus particles are between 100 and 300 nm in diameter (Merchant and Packer, 1977). Alexander (1991) described the genome as very pleomorphic. Virus particles are generally rounded and between 100-500 nm in diameter. The envelope has spikes and contains the antigenic components that stimulate the host to produce haemagglutinin-inhibiting (HI) and virus neutralizing antibodies (Rott, 1964; Alexander, 1988).

2.2.3 Reaction to different physical and chemical agents

ND virus infectivity, immunogenicity and the ability to agglutinate red blood cells can be destroyed at varying rates by exposure to chemical and physical agents (Hanson, 1978; Alexander, 1991). The virus is readily inactivated by formalin, alcohol, lipid solvents and lysol. Thermostability varies among strains, the majority of strains resist 56°C for 30 minutes (Allan *et al.*, 1973). All activities of the virus are destroyed within one minute at 100°C. At 56°C, infectivity, haemagglutinity and immunogenicity are destroyed within 5 to 6 hours. At

37°C, hours and days are required to induce such changes (Hanson, 1978). The Newcastle disease virus is quite stable at a wide range of pH (Merchant and Packer, 1977).

2.2.4 Pathotypes

Although no significant serologic differences between strains exist, a wide range of pathotypes can be differentiated. Isolates may vary from being avirulent for a wide range of avian species to being highly lethal for fowls (Allan *et al.*, 1982; Spradbrow, 1987). Hanson (1978) described four forms of the disease: Doyle's, Beach's, Beaudette's, and Hitchner's. Nonnewitz (1986) classified NDV into five different pathotypes: Doyle, Beach, Mesogenic, Hitchner and atypical strains (psittacine strains).

NDV is conventionally classified into three types according to the virulence of the virus strain namely: lentogenic, mesogenic and velogenic (Anon., 1971a). Hanson and Brandly (1955) classified NDV into three groups on the basis of the time taken to kill chicken embryos, and according to the intracerebral index in day-old chickens and the intravenous index in six-week old chickens. Other tests include plaque formation in many cell cultures (Hanson, 1975). To the above three conventional pathotypes, avirulent strains of NDV can be added. Such strains rarely kill embryos and are not pathogenic in chickens or cytopathogenic in cell cultures (Spradbrow, 1987).

Schloer *et al.* (1975) in over forty cultures of a group of 1971-72 isolates of NDV, distinguished three to five subpopulations in monolayers of chicken embryo fibroblasts. Various strains of ND were shown to vary in virulence, in haemagglutinating activity against various mammalian erythrocytes and in heat stability of the haemagglutinating property (Merchant and Packer, 1977). Upton *et al.* (1953) reported immunological differences in the degree of cross-reactions in serum neutralization, haemagglutination-inhibition and neuraminidase-inhibtion tests. However, none of them showed variation in antigenic structure which would be significant in cross-protection studies. The development of the monoclonal antibody technology permitted a new approach to antigenic differentiation of NDV strains and isolates (Russel and Alexander, 1983).

2.2.5 Epidemiology

2.2.5.1 Susceptible species

According to Kaleta and Baldauf (1988) virtually all species of birds are susceptible to NDV. However, of the approximately 800 known avian species, only about 236 species have a record of NDV isolation. The disease is seen most frequently in domestic poultry, including guinea fowl, a species more susceptible than turkeys and peafowl (Allan *et al.*, 1973). Ducks, geese, partridges, and quail are relatively resistant (Higgins, 1971; Allan *et al.*, 1973). The most resistant species appear to be aquatic birds, while the most susceptible are gregarious birds forming temporary or permanent flocks (Kaleta and Baldauf, 1988). Most cases of infection in wild birds are the result of contact with infected poultry (Allan *et al.*, 1973). Recovered domestic chickens appear not to become long-term carriers of NDV in contrast to some psittacine birds (Spradbrow, 1987).

2.2.5.2 Transmission

NDV dissemination between flocks over long distances is frequently related to the movement of apparently normal poultry (Jungherr and Terrell, 1946) and poultry products (Dobsone and Simmon, 1951). It may also be accomplished by the introduction of diseased tissue such as a poultry offal in to feeds or by contamination of feed or water (Kaschula, 1952). Ingestion of faecal material from infected birds will also result in the transmission of the disease. This may be the method of bird-to-bird spread for avirulent enteric NDV and of the spread of the pigeon variant virus (Alexander *et al.*, 1984).

The primary mode of transmission of NDV within a flock is by aerosol (Beard and Hanson, 1984). While aerosol transmission between farms is of minor importance, air-borne transport of NDV within a farm is possible up to distances of 200 meters (Nonnewitz, 1986). Spread between countries is often mediated by birds, migratory birds, caged birds, racing pigeons or domestic chickens. Spread within a country is often attributed to the movement of birds, either domestic or wild (Spradbrow, 1987).
The introduction of ND in a village is most likely to occur when infected live chickens are introduced. Live bird markets are probably the major source of spread (Alexander, 1988), particularly since many village chicken owners take their birds to market as soon as they become sick in an attempt to salvage some value from them. Other means of spread include wild birds, infected birds being transported through the village, infected carcasses and movement of contaminated objects from an infected site (Alexander, 1988). Vaccination crews moving from farm to farm have been implicated in the spread of NDV (Utterback and Schwartz, 1973) and incomplete inactivation (Spalatin and Hanson, 1966) and contamination (Beard *et al.*, 1970) of vaccines may further contribute to the spread of NDV.

Airborne spread from flock to flock is probably unimportant among village chickens since a dense population of infected birds is necessary to generate a sufficient dose of aerosol for such transmission to occur (Martin, 1992).

2.2.5.3 Distribution

Spradbrow (1988), reviewing the 1985 animal health year book of the Food and Agricultural Organization of the United Nations (FAO), summarized that lentogenic or mesogenic strains of NDV were present in most countries of Asia, Africa, America and Europe, and in the USSR. More than one third of the countries of Asia and one fifth of the countries of the world acknowledged the presence of velogenic strain of the virus. In contrast, the countries of Oceania were relatively free of NDV. Some large countries recognized the presence of the avirulent strains of the virus, while many of the island states were apparently free from all pathotypes of NDV. Newcastle disease is also absent from some island states of America and in some European countries. Alexander (1991) stated that countries with predominantly village chicken flocks have greater problems than those where the industry consists almost exclusively of large commercial flocks reared in confinement.

2.2.5.4 Newcastle disease in Ethiopia

Newcastle disease is the most important cause of economic loss in poultry production in Ethiopia. The disease has different local names, but the most commonly used is Fengle (Tadelle, 1996; Mebratu, unpublished). The first documented evidence of ND in Ethiopia dates back to 1971 and was reported in Eritrea, which was then part of the northern part of the

country. The NDV involved was a velogenic strain and caused some 80% mortality. How the virus was introduced to the country is still a mystery. It is clearly known that the disease spread to the other parts of the country at a tremendous speed. In 1972, outbreaks had occurred in Addis Ababa, in 1974 in Harer in the poultry farm of the Agricultural University, and in the Shola and Debre Zeit poultry farms (NVI, 1974). Vaccinations against the disease were not practiced until 1974 (Lengerish *et al.*, 1991). Since 1991 the National Veterinary Institute (NVI) has produced more than 12 million doses of vaccine, half of which was sold to commercial poultry farms (Mebratu, unpublished).

Despite routine vaccinations with HB1 and LaSota strains in one of the biggest poultry farms (Dembi, Debre Zeit), at least nine outbreaks of ND have been reported since 1984 which affected more than half a million birds and killed more than 20% of them (Nasser, unpublished).

The epidemiology of ND in village birds is poorly understood. This is due to the failure on the part of the backyard owners to notify outbreaks. Farmers consider the losses as normal and natural (personal observation). Although chicken mortality in rural areas is as high as 80%, village people sell their chickens between the months of May and July, since they believe that Newcastle disease outbreaks occur mainly during the rainy season (Mebratu, unpublished). They attempt to sell all the birds on seeing the first signs of ND. The other reason for the high mortality is lack of understanding on the side of owners and lack of a disease monitoring capacity of the Department of Veterinary Services.

In 1995, ND outbreaks in the surrounding areas of Debre Zeit, Nazreth and Addis Ababa killed almost 50% of the local birds (Nasser, unpublished). A recent serological survey conducted in six villages around Dukem (non-vaccinated birds) revealed a high prevalence of NDV antibodies (Mebratu, unpublished).
ND outbreaks occur during the rainy season, i.e. at the end of May and beginning of June, but after the start of the villagization program in 1984 this remained a problem throughout the year, even though they are more serious at the beginning of the rainy season (Tadelle, 1996). Data on ND outbreaks in state poultry farms (personal data, unpublished) support these outbreak problems showing, that ND outbreaks are distributed throughout a year.

2.2.6 Clinical signs

Clinical signs of ND are extremely variable and depend upon the virulence and dose of the infecting virus, the degree of immunity of the birds, the age of the birds and on various physical conditions such as outside temperature (Coutts, 1981; McFerran and McCracken, 1988). Sainsbury (1980) stated that the incubation period of ND is around 2 to 7 days, although in some cases it can extend up to 3 weeks. According to Hanson (1978) it is 2 to 15 days or longer. Bains (1979), Beard and Hanson (1984) and Nonnewitz (1986) classified the clinical symptoms into five forms: 1) Doyle's form, an acute lethal infection of all ages, characterized by haemorrhagic lesions of the digestive tract. 2) Beach's form, an acute lethal infection of all ages characterized by respiratory and neurologic signs, 3) Beaudette's form, which is less pathogenic, where deaths are usually seen in young birds only, 4) Hitchner's form, characterized by a mild or inapparent respiratory infection, and 5) an Asymptomatic-enteric form of infection with the virus causing no obvious disease. The virus in the latter case appears to be located chiefly in the gut. McFerran and McCracken (1988) categorized clinical signs into five groups, namely: generalized, reproductive, respiratory, enteric and nervous

effects, while Allan et al. (1973) grouped them into subacute, acute and peracute signs, and the virus being either neurotropic or viscerotropic.

Reviewing clinical reports, birds are noticed to be quiet, dull, to stand huddled and with ruffled feathers. Where neurologic signs predominate, leg and wing weakness may be seen with muscular twitching, dropping first one, then both wings, twisting of the neck, and finally paralysis. A greenish diarrhea is almost always seen. In the pneumonic form there is generally difficulty of breathing and other respiratory signs, including gasping, gurgling and coughing, and there may also be mucoid nasal discharge. Recovered birds often show torticollis.

In laying birds any of the above signs may be accompanied by a drop in egg production. In birds with partial immunity or those affected by a mild virus, a production drop with soft-shelled and shell-less eggs and loss of egg shell pigmentation may be the only signs of ND.

2.2.7 Gross lesions

A number of well recognized pathological changes occur in ND, the severity of which vary according to immunity, strain of the infecting virus and other factors. As with clinical signs, none of the lesions found are pathognomonic for ND (Hanson, 1978; Coutts, 1981; Alexander, 1991).

Beard and Hanson (1984) described haemorrhagic lesions in the proventriculus, the small intestines and caeca. Gross lesions are not observed in the central nervous system of birds infected with NDV, regardless of the pathotype (McFerran and McCracken, 1988). Gross pathological changes are not always present in the respiratory tract, but, when observed, they consist predominantly of haemorrhagic lesions and marked congestion of the trachea (Alexander and Allan, 1974). Airsaculitis may be present even after infection with mild strains (Beard and Hanson, 1984). Coutts (1981) described inflammation of the trachea, haemorrhages in the heart fat and gizzard. In laying flocks, internal laying and egg peritonitis increases. On occasions the diphtheroid necrotic inflammation of the intestinal mucosa ("button ulcer") is considered to be suggestive of ND (Hanson, 1978).

2.2.8 Diagnosis

2.2.8.1 Clinical signs and lesions

None of the clinical signs or lesions seen in birds infected with ND can be regarded as a suspicious of ND (Allan *et al.*, 1973; Alexander, 1991), while Bains (1979) suggested that serological and pathological examination may suffice to confirm suggestive histories and signs in regions where ND is endemic. A final diagnosis can be made by virus isolation and identification or by serological means (Hanson, 1978; Allan *et al.*, 1982).

2.2.8.2 Virus isolation

a) Specimen

Virus can generally best be isolated from the trachea, other tissues, such as bone marrow and brain on occasion yield virus readily (Coutts, 1981). Bains (1979) indicated that swabs from the trachea and cloaca and specimens from other visceral organs and the brain yield virus on culture. Hanson (1980) pointed out that virus can be isolated easily by tracheal and cloacal swabs in almost all instances, sometimes from both, and from the brain even when the virus

has disappeared from the other sites. Hanson (1978) also mentioned that strains of low virulence or vaccinal strains can be recovered most readily from the trachea. In cases of more virulent strains successful isolation can be made from the trachea, lung, spleen, bone marrow and brain. Alexander (1989) stated that successful virus isolation can be made from faeces or faecal swabs and that the second most important site is the respiratory tract. Tracheal swabs and faecal material should be included in the sample, regardless of clinical signs. Allan *et al.* (1982) stated that isolation of virus is facilitated if specimens are taken during the viraemic phase (3 to 7 days after infection). In contrast, Heuschele and Easterday (1970), stated that explant procedures make replicable isolation of virus from trachea and brain tissue possible as long as 2 months after antibodies are detectable.

b) Embryonated eggs

Hanson (1975) pointed out that the embryonated chicken egg is preferable to the chicken or other animals for isolation. Alexander (1991) also stated that embryonated chicken eggs represent an extremely sensitive and convenient vehicle for the propagation of NDV and are to be used almost universally for diagnosis.

Fertile eggs from SPF (specific pathogen free) flocks are preferred, but eggs free from NDV antibodies can also be used (Alexander, 1991). Embryonated eggs, preincubated for 9-11 days at 37°C, are injected into the allantoic fluid with a filtered suspension of the suspected tissue. The eggs then are candled twice daily for 5 to 7 days (Alexander, 1989) or until death of all embryos. The allantoic fluid is then collected and tested for haemagglutination (HA), followed by the haemagglutination-inhibition (HI) test with NDV positive serum, in cases where the HA is positive.

c) Cell culture

NDV can replicate in a wide range of cells. Lancaster (1966) listed 18 primary cells and 11 cell lines as suitable. The most extensive cells used for isolation are either chicken embryo fibroblasts or chicken embryo kidney cells grown as monolayers on glass or plastic (Bankowski, 1964). Barahona and Hanson (1968) showed some relationship between plaque size and NDV strain. Alexander (1991) stated that cell cultures can be advantageous by being free of external agents. However, relatively poor growth of NDV in most cell systems means that they are generally impractical for primary virus propagation.

2.2.8.3 Serology

Serological tests for NDV may be used to demonstrate previous infection or to monitor vaccination results (Alexander, 1988). Allan *et al.* (1982) pointed out that serological diagnosis is practical in a non-vaccinated flock, but is difficult in vaccinated flocks, and, should be used as an adjunct rather than a primary means of diagnosis in such cases.

Alexander (1991), referring to different workers, summarized that NDV antibodies can be detected by a variety of tests (single radial immunodiffusion, single radial haemolysis, agar gel precipitation, virus neutralization in chick embryos and plaque neutralization). In recent years, enzyme linked immunosorbant assays (ELISA) have become popular, especially as a part of a semi-automated flock monitoring procedure. Conventionally, antibodies to NDV have been detected and quantified by the HI test.

Haemagglutination-inhibition test

The haemagglutination-inhibition test (HI) is the most frequently used serological test to demonstrate infection or to monitor vaccination (Miers *et al.*, 1983; Alexander, 1988). The most prominent features of the HI test are its simplicity and economy. Materials and technical requirements for the test are minimal and micro-methods to facilitate manual or semi-automatic processing of large numbers of samples are available (Allan and Gough, 1974). The HI test is also simpler and faster than the virus neutralization test (VNT) and also a measure of the immune response, while the latter is not routinely used for NDV serology because the VNT is more time consuming and costly than HI tests (Beard and Hanson, 1984). Several reports about the relationship between titre of the HI antibodies and the effect of challenge (Allan *et al.*, 1973) are available. However, even though the HI test is simple to perform, it is difficult to standardize among laboratories (Beard and Wilkes, 1985) and the test is not equally sensitive for antibodies to all strains of NDV.

In recent years ELISA tests have been developed (Miers *et al.*, 1983). Studies on the sensitivity and specificity of ELISA results and on the correlation with HI test results have produced variable results. Several workers showed that there is a high degree of correlation between mean ELISA and HI titres (Snyder *et al.*, 1983; Miers *et al.*, 1983; Xu, 1996). On the other hand, some workers indicated that the ELISA is able to detect much lower levels of antibodies than the HI test (Miers *et al.*, 1983). Still, with regards to protection, the ELISA does not give much more information than the HI test (Miers *et al.*, 1983).

2.2.8.4 Virus characterization

The isolation of NDV from birds showing disease signs does not confirm NDV as the causative organism even if the signs are typical for ND (Alexander, 1988). Allan *et al.* (1973) pointed out that, serologically NDV isolates do not vary in antigenic composition. The ability to agglutinate mammalian blood cells and the heat stability of the haemagglutinin at 56°C have been used to characterize individual strains but were not found to be confirmatory, neither was the pathogenicity of the virus on susceptible embryonated eggs or on chickens. Currently, common practice of virus characterization involves the determination of mean death time in 9-10-day-old embryonated egg (MDT), of the intracerebral pathogenicity index in day-old chicks (ICPI) test and of the intravenous pathogenicity index test in 6-weeks-old chicks (IVPI). Also, some modification of the above methods have been made, such as swabbing of the cloaca and the conjunctiva.

Other confirmatory methods involve in vitro tests, based on e.g. the ability of NDV isolates to form plaques in cell culture systems in the absence of trypsin (Rott, 1979). In recent years, mouse monoclonal antibodies (Mabs) are employed for characterization and group isolation, apart from their use in routine diagnosis (Russel, 1988). Virus properties such as structural polypeptide profiles, oligonucleotide fingerprints, amino acid sequences, polymerase chain reaction (PCR) and lecitin binding activities, have been used with success to demonstrate similarities or differences between NDV isolates (Alexander, 1991).

2.2.9 Immunity

All NDV strains are capable of provoking an antibody response in chickens, rabbits and other species into which they are introduced. The antigens inducing neutralizing and haemagglutinating antibodies are associated with the envelope of the virus (Hanson, 1978). For assessment of protection, especially for the response after vaccination, the HI test is

commonly used (Allan *et al.*, 1978). After natural infection or vaccination of chickens serum antibodies appear within 4-10 days (Hanson, 1978; Allan, 1982; Nonnewitz, 1986; Alexander, 1991) while signs of clinical disease are still apparent. Peak response occurs after 3-4 weeks (Hanson, 1978; Alexander, 1991). The decline in HI titre is initially slow but becomes more apparent after 3-4 months and detectable antibodies generally have disappeared in 8-12 months (Hanson, 1978). After recovery from infection with mesogenic viruses, HI antibodies may still be detected up to a year (Allan, 1978).

Locally produced antibodies in the respiratory and digestive tract appear at the same time as humoral antibodies. These antibodies have an effect on the protection against respiratory tract infections (Nonnewitz, 1986). The initial response to infection with NDV is cell-mediated and occurs as early as two or three days after infection with live vaccine strains (Ghumman and Bankowski, 1976). However, the importance of this local protection after vaccination is not clear and a strong secondary response to challenge, similar to that of hummoral antibodies, does not seem to occur (Timms and Alexander, 1977).

According to Spradbrow (1987), levels of HI antibody correlating with protection can be determined with standard tests. Chickens with high levels of antibodies will usually be refractory to infection, but no statement can be made for chickens with lower antibody titres. Some birds will resist infection even without detectable antibody. This occurs particularly in chickens which were recently vaccinated, but such resistance also is observed after vaccine-induced antibodies have waned. Chickens free of antibodies and with no exposure to virus are not protected.

Allan et al. (1973) pointed out that the level of passively acquired (maternal) antibodies in young chickens generally declines at a constant rate and has a half life of approximately 4.5 days. However, Spradbrow (1987) stated that NDV antibodies would persist longer in the slowly growing progeny of village hens than in that of commercial hens.

The exact function of local immunity is not clear (Holmes, 1979) it was proposed that the protection of the respiratory tract is independent of humoral immunity. Spradbrow (1987) suggested that the immune barrier at the mucosal surface probably involves cellular components of the immune system and secretory antibodies.

2.2.10 Prevention and control

Effective control of ND requires good sanitation, management, quarantine, an appropriate vaccination program, and monitoring and reporting system, including serotyping and pathogenicity testing of isolated virus (Meulemans, 1988). According to Hanson (1978) a minimum of 70% of flocks in high risk areas must be included in sanitary and combined vaccination programmes if control is to be effective.

2.2.10.1 Vaccination

Vaccination against ND can be performed using either live or inactivated vaccines (Meulemans, 1988). The effectiveness of ND vaccines in the control of the disease, whether under closed commercial, semi-closed intensive, or under free range rural systems in tropical countries, depends on the virulence of the field strain, the immunological state of the birds and the method of vaccine application (Meulemans, 1988).

2.2.10.2 Vaccination with live vaccine

Live virus ND vaccines have been used by the poultry industry for more than 30 years (Meulemans, 1988). ND live virus vaccine strains belong to two categories, lentogenic and mesogenic strains (Alexander and Allan, 1974).

a) Live lentogenic strains

The best known strains of live lentogenic vaccines are: HB1, F, and LaSota (Palaya and Rweyemamu, 1991). In recent years other lentogenic strains have been examined for use as vaccine, such as the Australian V4 and CT (Turner *et al.*, 1977; Kim and Spradbrow, 1978; Idris *et al.*, 1990), the Ulster 2C, the Iraqi AbuGrabi 68 and the Hungarian NDV-6 (Palaya and Rweyemamu, 1991).

The B1 and the F strains both are mild and can be used safely and effectively in all classes of chickens (Allan *et al.*, 1978; Sainsbury, 1980; Nonnewitz, 1986). The LaSota strain is more invasive and immunogenic than the HB1 strain and has a good booster effect but it also can cause adverse reactions (Sainsbury, 1980; Allan *et al.*, 1982; Nonnewitz, 1986). The strain can also spread from bird to bird within a house (Spalatin and Hanson, 1976). Allan et al. (1982) and Nonnewitz (1986) recommended the LaSota strains to be used for the second vaccination to boost immunity. Allan et al. (1973) reported that the F and B1 vaccines do not cause nervous disease in day-old chicks unless given intracerebrally, but may cause mild and transitory respiratory symptoms. The F strain causes the least reaction, the B1 strain generally has little or no clinical effect, whereas the LaSota strain causes more post-vaccinal respiratory symptoms.

Recently, a new live vaccine, which can establish long lasting immunity in the respiratory tract of newly hatched chickens, was developed (Zokay *et al.*, 1995). Furthermore, an in-ovo or in-egg type injectable vaccine was tested and found to be effective under laboratory conditions (Stone, 1996). A thermostable lentogenic Australian virus isolate V4 was selected and successfully used in Malawi, where LaSota and Komarov vaccines were inefficient in controlling ND (Sagild and Haresnape, 1987). The immunogenic potential of this vaccine was reported by several workers (French *et al.*, 1967; Schalkoort and Spradbrow, 1980; Tantaswasdi *et al.*, 1992).

Live vaccines can be administrated using individual or mass methods. Individual method of application produce more consistent protection in more birds than mass vaccination methods. However, individual methods are uneconomic in broilers and limited to the early vaccination of replacement layers and breeding stock, if necessary (Meulemans, 1988).

Live freeze dried vaccines can be stored at 4°C for a year without significant loss of the titre (Anon, 1971b), but they should never be held above 8°C for more than 1-2 hours before use. Care must be taken that during transportation of bulk supplies to local storage facilities the temperature does not exceed this limit.

b) Live mesogenic strains

The most widely used vaccines included in this category are: Roakin, Komarov, Hertfordshire (Herts), MK 107 and Mukteswar (Palaya and Rweyemamu, 1991). The Roakin, Komarov, Herts and Mukteswar strains are widely used throughout Africa, the Middle East, and South East Asia (Meulemans, 1988). These vaccines are administered by the parenteral route and are not recommended for immunization of chickens under 8-weeks of age nor for young pullets or

adults not previously immunized with lentogenic virus. They could produce serious problems in fully susceptible poultry (Meulemans, 1988).

2.2.10.3 Killed or inactivated vaccine

During the last twenty years, oil-based inactivated ND vaccines have been developed (Meulemans, 1988). The efficacy of oil-emulsion ND vaccines depends on the formulation (Brought and Siegel, 1978). The inactivated oil based vaccines give a long-lasting high HI antibody titre after priming with live vaccine virus (Nonnewitz, 1986).

2.3 Oral Newcastle Disease Vaccination

2.3.1 Thermostable Newcastle Disease Vaccines

Heat inactivation of conventional ND vaccines and the characteristical behaviour of village chickens hampered the delivery of conventional vaccines in tropical and sub-tropical countries so far (Spradbrow, 1992). The selection of varions for heat-resistance has been practiced for a long time (Spradbrow, 1993/4). Hofstad and Yoder (1963) noted that virus strains of NDV possess different heat-stability when prepared as freeze-dried vaccine. They postulated that it might be possible to select a stable V4 vaccine with heat stability that retained immunogenicity.

French (1964) conducted the first serological survey for NDV in Australia. After isolation of the V4 strain in 1966, he demonstrated the avirulence of the virus, its ability to infect chickens when delivered orally with food, and its potential usefulness as a vaccine (Spradbrow and Sabine, 1995). The first thermostable strain of NDV selected specifically for use as vaccine was probably the V4 selected by Schalkoort in 1979 (Spradbrow, 1993/4). V4 is not the only strain that responds to selection for heat stability. Spradbrow and Sabine (1995) carried out experiments with forty-five recently isolated strains of NDV and V4. Neither V4 nor any of the new vaccines produced detectable clinical signs. All viruses produced an antibody response and spread by contact and some of the newly isolated viruses produced a more rapid serological response than the V4 virus.

2.3.2 Oral Newcastle disease vaccination

Food baits seemed the most appropriate method for introducing heat-stable Newcastle disease vaccine to village chickens (Spradbrow, 1993/4). The V4 strain of NDV appears to be adapted to faecal-oral spread and transmission by the respiratory route seems to occur as well (Turner and Kovesdy, 1974). Spradbrow *et al.* (1988) compared the antibody response of food-borne vaccine to that induced by similar vaccine doses given by other routes, as its showed that vaccination via feed could produce satisfactory titres of HI antibody to ND. Although the feed vaccination does not produce high initial antibody levels, antibodies did persist, and vaccine viruses given via the drinking water or via the feed were apparently ahead in times of after the second application. Spradbrow *et al.* (1977) demonstrated that nearly all chickens vaccinated with a high doses of V4 vaccine by the oro-tracheal route and challenged soon after vaccination resisted challenge with a virus that produced 100% mortality in unvaccinated controls. Subsequent researchers showed that Australian strains used live vaccine conferred immunity in birds challenged under laboratory conditions (Turner *et al.*, 1977).

Comparative trials (Westbury *et al.*, 1984) with Australian strains against recognized vaccine strains as well as work by Spalatin and Hanson (1976) showed that the levels of protection were comparable to conventional vaccines. Moreover, it was found that the virus vaccine could give protection not only to directly vaccinated birds but also to birds in contact with them (Kim *et al.*, 1978; Idris *et al.*, 1987, Jayawaradene *et al.*, 1990). Jayawaradene and Spradbrow (1995) demonstrated in more detail that vaccination with V4 virus induces a mucosal immunity by producing virus specific IgA which was detected in the tracheal and intestinal washings, bile, lachrymal fluid as well as in serum. Ibrahim *et al.* (1992) undertook several experiments to test the efficacy of the feed pellet vaccine. Broilers were sufficiently protected after two applications and village chickens, which were kept under simulated conditions, were found substantially resistant to challenge after two vaccinations and under field conditions 60% of the village birds resisted artificial challenge.

2.3.3 Food carriers for oral vaccines

Spradbrow (1992), after intensively reviewing the literature on feed carriers for the delivery of oral Newcastle disease vaccines, stated that further work would be required on the treatment of feed grains to ensure the attachment of vaccine virus and its survival for a suitable length of time. Best results have so far been obtained with wheat based vaccines in Malaysia, even after prolonged storage, and in other countries with vaccine based on unhusked (paddy) rice. Protection was best when the vaccine-coated feed was fed soon after preparation. Trials with other types of grains are necessary and there have not yet been detailed trials with millet, sorghum or other small grains. Recently, Cumming (1992) and his group reported on methods for treating grain that allow recovery of virus from it after 18 hours and that reliably produce antibodies in chickens when fed after this time. Some well and rain water proved to be a suitable dilutent, and polyvinylprolidane was the most successful additive to protect vaccine virus after application to grain.

PART 3. GENERAL MATERIALS AND METHODS

3.1 Experimental Site

The experiments were carried out at Lemlem Poultry farm and the National Veterinary Institute (NVI) in Debre Zeit, Ethiopia. The distance between the two sites was 7 km. Separate houses each with an area of $12m^2$ for each treatment and challenge group were used.

3.2 Preparation of Experimental Houses

All experimental houses were thoroughly washed with water and detergent, soaked with 4% caustic soda, and the interior parts of the houses were disinfected with 1% formalin, starting from ceiling, walls and finally to the floor. The surrounding of the houses were also disinfected after cleaning. After allowing time to dry, clean new litter (*teff* straw) was spread to a depth of 7 cm over the floor area and a 1% formalin solution was sprayed over it. Equipment, including drinkers, feeders, buckets, etc. was cleaned and disinfected and introduced to the houses. Then the houses were closed until the arrival of the chicks.

3.3. Management of Chicks

All experimental chicks were brooded in one house until fourteen-days old. Then the chicks were randomly split into treatment groups. During brooding the room and the brooder temperature was maintained between 21 to 28°C and 28-35°C, respectively. An antibiotics (Oximid) and vitamins (Allvit MA) were supplied via the drinking water for the first five days of life and lateron every week for 3 days at the time of bleeding. Coccidiostat was given via the drinking water when a coccidiosis outbreak was suspected or diagnosed.

Water and feed was given *ad libitum*. Starter feed for the first 28 days (protein 21%, energy 3020 kcal) and finisher ration (Protein 19.2%, energy 3160 kcal) was supplied by the Bora Feed Processing Enterprise, Debre Zeit, Ethiopia.

Feed consumption and mortality were recorded daily while the average weight was measured every week for each group. Daily room temperature was recorded three times a day at 8 am, 12 am and 4 p.m.

Four different colors of leg tags were used for identification of each treatment group and individual birds were identified by their leg tag number. Individual and separate attendants and poultry equipment were assigned for each treatment group throughout the experiments.

3.4 Diagnosis and Sampling of Sick and Dead Birds

All experimental chickens were kept under the same management condition as described in section 3.3. The chickens were visited by the veterinarian every other day. Dead and sick birds were subjected to a clinical and/or postmortem examination. Depending on the tentative diagnosis or laboratory test results, treatments were carried out accordingly. In cases of suspected Newcastle disease a detailed postmortem examinations, particularly for lesions in the trachea, proventriculus, intestine, spleen, caecal tonsils and brain was carried out. Appropriate samples from dead or sick birds were collected for virus isolation, using standard techniques.

3.5 Vaccines

Two types of vaccines were used in these experiments. They were 1) conventionally used vaccines in Ethiopia and 2) the thermostable vaccine.

Conventional vaccines, used were: Hitchener B1 (HB1) and LaSota, live freeze dried vaccines in 500 and 100 dose vials, produced by NVI, Debre Zeit, Ethiopia. The thermostable vaccine NDV-I_2 was also a live freeze-dried, produced in 500 dose vials and was kindly granted by the Pan African Veterinary Vaccine Center (PANVAC). This vaccine is the same as the heat-resistant V4 Newcastle disease vaccine. (The I_2 seed virus was obtained from Queensland University, Brisbane, Australia).

.3.6 a Preparation of Ten-Fold Dilution Series.

Nine ml of PBS was added to each of 10 bottles using a 10 ml pipette. Each bottle was marked from 10^{-1} to 10^{-10}.

One ml of the virus material to be assayed then was transferred into the first bottle, using a 2 ml pipette. The contents of the bottle were mixed using an agitator or a pipette filling and emptying ten times. Using the same pipette, 1 ml of the virus dilution (1:10) was transferred from the first bottle to the second, mixed well as described above, between each ten fold dilution step the pipette was changed. This serial operation was repeated to the last bottle (10^{-10}).

3.6 b Source of Eggs and incubation procedures

Fertile local chicken eggs were purchased from the local market. Before incubation, the eggs were cleaned with 70% alcohol and checked for the size of the air sac and for dead embryos.

Initially, eggs were incubated in an incubator at a temperature of 38-39°C and a relative humidity of 60-70%. The eggs were turned four times daily for proper development and to prevent adhesion of the embryonic membrane. Eggs were usually incubated for 9-10 days before being inoculated. After 5-6 days of incubation the eggs were candled to separate fertile from non-fertile eggs. Before inoculation, eggs with weak embryos or with abnormal air sac size or position were discarded. Candling was performed in a dark room, using a locally made candling box.

3.7 Sample Preparation

Tissue samples were prepared as a 10-20% suspension by grinding by mortar and pistile. Swabs were diluted in as small a volume as required for testing (3-4 ml) and then were mortared to expel as much virus material as possible from the swab fibers. Swab and tissue suspensions were centrifugated at 1500g for 20 minutes in a refrigerated centrifuge (4-10°C) to sediment tissue debris and most bacteria. The supernatant was then aseptically removed and placed in two appropriate vials for egg inoculation and storage. Antibiotics were added to the specimens which were kept at room temperature for 1-2 hours before egg inoculation.

Specimens, considered to be heavily contaminated with bacteria that could not be controlled by antibiotics or centrifugation, were filtered through a 450-nm millipore filter.

3.8 Inoculation of Embryonated Eggs and Harvesting of Allantoic Fluid

3.8.1 Inoculation

Before inoculation, all embryos were candled and checked for viability and normal position of air cells. The site of inoculation was disinfected with a cotton swab with a solution of 70 % ethyl alcohol containing 3.5% and 1.5% sodium iodide. The eggs were placed in an egg flat, air cell up and cleaned and disinfected. A small hole was drilled through the egg shell along the center axis at the top of the egg using a pointed stent. Using 2 ml sterile syringe fitted with a 25 gauge 1/8 inch needle, 0.1 or 0.2 ml of the suspension was inoculated per egg by

inserting the needle vertically through the hole, the entire length of the needle. The hole was then sealed by wax and the egg returned back to the incubator.

3.8.2. Collection of allantoic fluid from inoculated eggs.

The egg-shell over the air cell was cracked by tapping the egg shell with the blunt end of sterile forceps. Then the egg-shell covering the air cell was removed. Different forceps were used to tear the egg shell membrane, the chorioallantoic membrane (CAM) and the amniotic membrane by depressing the membrane over the yolk sac with forceps. The amnioallantoic fluid (AAF) was allowed to collect above the forceps. Using syringe and needle, the fluid was aspirated and placed into a vial. The AAF was clarified by centrifugation at 1500g for 10 minutes and tested for evidence of virus using the haemagglutination (HA) test. A loopful of the AAF was cultured for bacteria, using blood or nutrient agar plates.

3.9 Serology

3.9.1 Serum sample collection

Prior to vaccination, in order to obtain base line information on maternal immunity and the declining levels of passively acquired immunity, bleeding of 40 chicks was done at the age of 1 and 7 days.

From thereon, bleeding was carried out at weekly intervals, bleeding was carried out from all experimental chickens until the end of the experiment. This was to get information on the development of immunity over time after the series of vaccinations, and on the level of immunity of surviving chickens 14 days after challenge.

3.9.2 Methods of serum collection

Bleeding was done by exposing and plucking a few feathers from the ventral surface of the humeral region of the wing. Then the skin was wetted with 70% alcohol and the needle inserted into the wing vein. One ml of blood was collected and placed in a clean tube which was laid nearly horizontally until the blood clotted. After clotting the tube was returned to a vertical position to permit the serum to collect. Then the sample was held at 37°C for several hours or the sample was left overnight before the serum was removed. If the serum was not tested immediately, it was filled into serum storage bottles (microcentrifuge polypropylene with snap-top cups) labeled (tag number and treatment group) and stored at -20°C until the haemagglutination-inhibition test was performed.

3.9.3 Preparation of 1% RBCS

Blood was collected from four or five chickens older than 3-weeks and serologically negative to ND. Bleeding was done with a syringe containing Alsever's anticoagulant solution (equal volume). After gentle mixing, the blood was transferred to a large conical centrifuge for washing. The red blood cells were washed three times by gentle centrifugation in physiological saline (1500g revolutions per minute for five minutes), and by pouring off the supernatant and by adding 20 to 30 volumes of physiological saline to the packed cells. This procedure was repeated once or twice until the supernatant was clean. The final suspension

was stored at 2 to 8°C if not used immediately. Then a 1% erythrocyte working suspension was made in PBS by mixing 1 ml of the packed cells with 99 ml PBS.

3.9.4 Preparation of haemagglutinating antigen

0.1 ml of the LaSota strain virus suspension was inoculated into the allantoic cavity of nine-day-old embryonated eggs. The eggs were candled twice daily and all embryos dead during the first 24 hours were discarded. Seventy two hours post inoculation the eggs were chilled overnight at 4°C in a refrigerator. The amnio-allantoic fluids (AAF) were harvested aseptically taking care to avoid contamination with albumen or yolk. The antigen (AAF) then was tested for its titre as described under 3.9.5 and stored at 4°C or -20°C.

3.9.5 Titration for haemagglutinating activity of the antigen

50µl antigen was added in well one and 25µl PBS in the subsequent wells 2 to 12. Dilution in two-fold dilution steps was done by transferring 25µl of the antigen from well 1 to the other wells of the first row and by thoroughly mixing each dilution step approximately 10 times between each transfer. Finally, 25µl from the last well was discarded. Then 25µl PBS was dispensed into each well (1-12) and lastly 50µl of the 1% erythrocyte suspension was added to each well.

Two or more wells were selected as erythrocyte controls which only contained 50µl PBS and 50µl 1% erythrocyte suspension, but not antigen. The plate was kept in the refrigerator for 30 minutes and was read 3-5 minutes after the plate was taken from the refrigerator and left standing at room temperature. A magnifying mirror was used for reading the reaction.

Interpretation
The plates were tilted and the erythrocytes left to flow. Sedimented erythrocytes (i.e. not haemagglutinated) flow to the bottom of the wells' edge forming a"nose" with a cross bar. Haemagglutinated erythrocytes do not flow. The HA titre is the reciprocal of the last antigen dilution with complete haemagglutination.

3.9.6 Control of the HA units

25 µl PBS was dispensed into all wells of the first 3 columns (A,B,C), with the exception of wells 5 to 8 of the first column (A5, A6, A7 and A8), which received 33µl, and the four last wells of the same column (A_9, A_{10}, A_{11} and A_{12}) which received 40µl. 25µl PBS was dispensed in 2 other wells (H_{11}, H_{12}), which served as RBC controls. Then the antigen was added into the wells of the first column, 25µl in the first 4 wells. (A_1, A_2, A_3 A_4) 17 µl in the next 4 wells (A_5, A_6, A_7, A_8), and 10µl in the remaining 4 wells (A_9, A_{10}, A_{11}, A_{12}).
The contents of the first column (A) were mixed well and 25µl were transferred to the neighbouring well situated in the second column (B). This was continued in the same way between B and C columns. 25µl was discarded from the last dilution. This gave a two-fold dilution

1/2, 1/4 and 1/8 in the first row (1)
1/3, 1/6 and 1/12 in the next row (2)
1/5, 1/10 and 1/20 in the last row (3)

25 µl of the RBC suspension was dispensed to each well, the contents were gently mixed by handshaking and the plate was kept at a room temperature for 30 minutes.

Interpretation
The adjustment was accepted if all wells containing ≥ 1/4 (A_1 to A_4 and B_1 to B_4) showed total haemagglutination.
The antigen adjusted to 4 HA units was stored (2 to 8°C) in a refrigerator.

3.9.7 Test sera

Test sera were obtained from experimental chickens at weekly intervals as described in section (3.9.1) and stored at 2 to 8°C for some days or at -20°C for longer periods. Decomplementation of the sera was not done.

3.9.8 Control sera

Negative control sera were prepared by collecting sera from NVI SPF chickens and were also obtained from Germany. The SPF chickens were known to be negative for ND antibodies.

Positive control sera were prepared by collecting sera from surviving chickens that had survived natural outbreaks or were from the challenged chickens. Reference positive sera were also obtained from Germany. Both negative and positive control sera were kept at 2-8°C.

3.9.9 Titration of the haemagglutination inhibition

50µl of test serum was added to row A_1 column 1-11, of a microtiter plate with U-shaped bottom. Row A1, column 12, received 50µl working haemagglutinin. Walls 12 G/H remained free for red blood cell controls. Using a multi-channel pipette 25µl PBS was added into all wells of rows B-H. Using a micropipette 25µl was transferred from each serum or haemagglutinin dilution, respectively, from top to bottom, leaving 12 G and 12 H wells for red blood cell controls. 25µl diluted serum and haemagglutinin was discarded from the last well of each dilution.

25µl working haemagglutinin was added to column 1 and 25µl PBS instead of working haemagglutinating antigen in column 2.

The plates were shaken by hand and air bubbles at the well bottom were removed by stilette, then they were incubated at room temperature for 10 minutes and 50µl of PBS was added to the red blood cell controls in wells 12 G and 12 H.

Finally, 50µl of the red blood cell suspension was added to each well, including 12 G and 12 H. Known positive and negative control sera were included. Then the microplate was incubated for 30 minutes in a refrigerator.

Interpretation
The plates were tilted the erythrocytes were left to flow and the flow of sedimented erythrocytes (i.e. no haemagglutinated) to the bottom of the well's edge forming a ''nose'' with a cross bar was observed. The HI titre was the reciprocal of the last serum dilution that completely prevented haemagglutination. The test was read within 5-10 minutes after removal of the plate from the refrigerator.

The test was not interpretable if the following conditions were not met:

1. Total sedimentation of control RBCs
2. No alteration of the images or RBC agglutination with control serum.
3. The negative control serum had a titre of < 1/8
4. The known positive control serum showed a titre near to the already registered titre.

3.10 Challenge

3.10.1 Challenge virus selection

Among the NDV isolates of Ethiopia, a virulent strain was selected according to the mean embryonic death time (MDT) in 9-day-old embryonated eggs and to the intravenous pathogenicity index (IVPI) in six-week old non-vaccinated chickens.

3.10.2 Titration of the challenge virus (EID_{50})

The test procedure followed the recommendations by Allan *et al.* (1978):

1. Seven universal bottles were filled with 9 ml PBS containing antibiotics and were marked. -1 to -7
2. Four more bottles were filled with 8 ml PBS containing antibiotics and marked -7.7,-8.4, -9.1 and -9.8
3. The selected virus material (allantoic fluid) was transferred from bottle one to seven, diluting the virus ten-fold with each transfer
4. From bottle 7, 2 ml were removed and transferred to bottle 7,7 (this will make a 1:5 dilution for which the log value is $10^{-7.7}$)
5. The five-fold dilutions were continued by serially transferring 2 ml upto and including the test bottle -9.8
6. 28 eggs were marked and 0.1 ml of the dilutions of -7.7, -8.4, -9.1, and -9.8 were inoculated into allantoic cavity using seven eggs per dilution. The eggs were sealed, incubated and the death was recorded.
7. The Spearman-Karber formula was used for calculation of the end point. (Annex, page 115).

3.12.3 Challenge procedure

In experiment one, three weeks after the second and the third vaccinations, 25 chickens from each treatment groups and all experimental chickens three and two weeks after the second vaccination during the second and the third experiments were infected intramuscularly by inoculating each chicken with a dose of 10^6 EID_{50} of the locally isolated velogenic NDV (Alemaya strain).

The challenged birds were observed for a period of two weeks for the development of clinical signs of the disease and/or mortality during each challenge. The time of death for each bird was also recorded. The disease was diagnosed tentatively on the basis of clinical signs and postmortem lesions. Trachea, proventriculus, spleen and brain samples were taken for virus isolation and for identification of the virus.

3.13 Statistical Analysis

3.13.1 Feed based Newcastle Disease vaccine trials

Prechallenge mean HI antibody titres were compared by the Kruskal-Wallis one way analysis of variance on ranks. Where the results were significant, Dunn's or Student Newman-Keules multiple comparison tests were done to establish differences in antibody response between pairwise treatments. The Mann-Whitney rank test was used for comparison of results between individual weeks. Mortality after challenge between treatment groups was analyzed by the chi-square test. Kruskal-Wallis one-way-analysis of variance on ranks was done to test differences between treatment groups for the mean death time from challenge to death.

3.13.2 Retrospective studies of Newcastle Disease outbreaks in state poultry farms in Ethiopia

Pearson moment correlation was used to analyze the correlations between age and mortality rates and between frequencies of vaccination and mortality rates. The student t- test was used to compare production losses between chickens vaccinated either with live or inactivated vaccines. The Mantel-Haenszel test for odds ratios was carried out to evaluate the strength of protection afforded by the inactivated vaccine over the live vaccine and to control the effects of confounding factors (concurrent diseases) on the mortality rates.

PART 4 . ORAL FOOD BASED NEWCASTLE DISEASE VACCINE

4.1.0 Introduction and Objectives

Newcastle disease is the most important avian pathogen in many countries, including Ethiopia. Virulent strains are widely distributed and seriously interfere with the development of the poultry industry. Control of ND in commercial chickens is done by the use of vaccines. However, conventional vaccines and conventional ways of vaccine delivery methods make ND vaccination inaccessible to village chickens, because the vaccines requires cold-chain, cold storage and handling of individual chickens.

The recently developed heat-resistant V4 vaccine does not need a cold chain. It can be delivered by the oral route and can be applied by unskilled personnel, under laboratory and field conditions. Several workers confirmed that the V4 vaccine is highly protective when given by conventional routes. The efficacy of V4 vaccine as oral feed vaccine was tested in many countries under laboratory and field conditions on different grains as carriers. However, contrary to conventional application methods, the results with feed as vaccine carriers were variable. Some grains were found to be suitable vaccine carriers, others not. The recent report by Cumming and his group (1992) indicated that short heating, washing, drying and cracking led to the recovery of the vaccine virus from grains as late as 18 hours after mixing.

The present three trials were undertaken considering the situation of ND in village chickens in Ethiopia, advantages of the V4 vaccine and the positive experience of some countries in the control of ND in village chickens by using the V4 vaccine. The objectives of this study were:

1- to study whether the heat-resistant NDV-I_2 gives adequate protection against velogenic field strains of Newcastle disease viruses in Ethiopia.
2- to compare the immunogenicity and protection potential of NDV-I_2 with that of conventional vaccines (HB1 and LaSota).
3- to test the suitability of sorghum and barley as NDV-I2 vaccine carriers.

4.2.0 Materials and Methods

4.2.1 Experimental design

The experimental design was a complete random design. The complete random design was preferred because of its simplicity in regards to the layout, but also because randomization gives every group of the experimental material an equal probability of receiving treatment.

The trial was conducted to compare the heat-resistant NDV-1_2 vaccine strain of NDV with the conventional vaccines to test different ways of vaccination with the NDV-I_2 vaccine, and to assess the suitability of barley and sorghum as vaccine carriers as well as the stability of the vaccine virus in these feed carriers.

4.2.2. Estimating the EID_{50} of the thermostable (NDV-I_2) vaccine

Titration of the thermostable vaccine was performed in 9-day-old embryonated eggs. For this test, thirty viable 9-day-old embryonated eggs, 5 each, were marked from 10^{-5} to 10^{-10}. Five further embryonated eggs were kept as a negative control.

0.1 ml of the virus dilution was inoculated per egg starting from the 10^{-10} dilution using sterile 1-ml all glass tuberculin syringes. The control groups received PBS and were inoculated last.

Following inoculation, all eggs were sealed with wax and incubated at 37°C. They were candled daily and embryos dead within the first 24 hours were discarded. Because the thermostable virus causes little mortality in chicken embryos, the 50% embryo lethal dose (ELD) could not be calculated. Instead, infected eggs were harvested after 4 days of incubation and the allantoic fluid was examined for NDV haemagglutinin, using chicken red blood cells. The fifty percent embryo infectious dose (EID_{50}) was calculated by the method of Spearman-Karber for determination of the end point (Annex !).

4.2.3 Stability of the thermostable vaccine (NDV-I_2)

The stability, infectivity and haemagglutination activity of the NDV-I_2 vaccine was tested after reconstituting the vaccine with distilled water and mixing it with parboiled and untreated sorghum and barley.

The vaccine mixed feed and the reconstituted vaccine solution were kept at room temperature for periods of 0, 1, 6 and 14 hr. The infectivity was assayed by mixing 10 g of the feed /vaccine mixture with 10 ml of PBS. This was centrifuged at 1000 rpm for 30 minutes. The supernatant was collected and filtered through a 0.45μ millipore filter. 0.1 ml of the supernatant was inoculated into five 9-day-old embryonated eggs per group and incubated at 37°C. Four days post inoculation the allantoic fluid was collected and tested for HA and HI by known antiserum.

4.2.4 Preparation of parboiled sorghum and barley

This was done according to the recommendations of Cumming 1992.

1. Boil for 2 minutes i.e. bring water to boil, put in grain at 1 kg per 3 liters of water - bring again to boil, then leave for 2 minutes.
2. Cool down let water go off, boil and when cool enough to move (in about 5 minutes) cool down under running tap water.
3. Dry again-i.e. get as much water of as possible by straining and put grain in plastic on bench in as much sunlight as possible and allow to dry. This takes up to 48 hr.
4. Crack grain using a house hold blender. Blend for 3 seconds about 100 g at a time.
5. Soak grains-place grains either cracked or whole into a container at 100 g /400 g of water. Soak all day - pour off twice with running water and repeat overnight.
6. Dry as in step 3 above.

4.3.0 Experiment A

4.3.1 Experimental chickens and vaccination

Four hundred day-old broiler chicks purchased from the local hatchery (ALEMA) were brooded together for 14-days in one house. After 14 days three hundred of them were randomly split into five equal treatment groups. Each bird was tagged and identified by a tag number.

Group A I.
Chickens in this group were vaccinated with conventional vaccines (HB_1 and LaSota) by intra-ocular and drinking water methods as recommended by the producers of the vaccines.

The first vaccination was carried out at the age of 14-days with HB1 by eye drop. 100 doses of HB1 vaccine were reconstituted in 2.5 ml of distilled water and one drop (25µl) of the vaccine solution was instilled into one eye.

The second and the third vaccinations were done at the age of 35 and 63-days of age respectively. 100 doses of the LaSota strain vaccine were reconstituted in 2 liters of well water and 20 ml of the vaccine solution were given to each bird accounting for the number of birds during the vaccination procedure. The birds were deprived of water overnight before vaccination. The approximate dose of the vaccine was 10^7 EID_{50} per chicken.

Group A II
The first vaccination was carried out by the intra-ocular method at 14-days of age. For this, 500 doses of the NDV-I_2 thermostable Newcastle disease vaccine were reconstituted in 12.5 ml of distilled water (The approximate dose per chicken was 10^7 EID_{50}). One drop (25µl) of the vaccine solution was instilled into one eye.

The second and the third vaccination were done at 35 and 63 days of age respectively. The 500 doses of vaccine were reconstituted in 10 liters of well water and 20 ml of the vaccine solution were given per chicken. Before vaccination, the birds were deprived of water overnight.

Groups A III and A IV

Birds in these groups were vaccinated by the thermostable vaccine NDV-I_2 mixed with husked ground barley and ground sorghum. To vaccinate chickens, 500 doses of the vaccine were reconstituted in 12.5 ml of distilled water (25µl of the vaccine solution per bird which is approximately 10^7 EID_{50} virus per chicken). Further 2 ml of the reconstituted vaccine solution were diluted with 198 ml of distilled water, then the diluted vaccine solution was vigorously mixed in a polythene bag with 800 gms of previously prepared husked ground barley or ground sorghum respectively. The vaccine feed /mixture were kept overnight at room temperature (14 hr.) before feeding to chickens fasted overnight. The vaccine was administrated accounting for the number of birds in the experimental group calculated dose was 10^7 EID_{50} per 10 g of feed vaccine per bird.

Second and the third vaccinations were carried out in the same manner as the first vaccination with 10g of vaccine /feed mixture per bird.

Group A V

This group was kept unvaccinated, but the birds were fed barley and sorghum during every vaccination procedure and half of the group received 25µl of distilled water into one eye during the 1st vaccination of the treatment groups.

4.4.0 Experiment B

4.4.1 Experimental chickens and vaccination

160 day-old broiler chicks, purchased from the local hatchery, were brooded together in one house, until 14-day-old. Later on, 120 chicks were randomly divided into six treatment groups and vaccinated. The remaining 40 chicks were transferred to NVI from the experimental site and were reared till six weeks of age until the intravenous pathogenicity index (IVPI) test was carried out.

Group B I and B III

Birds in these groups were vaccinated by mixing the NDV-I_2 vaccine solution with husked and ground untreated barley and sorghum for group I and III, respectively. To vaccinate the birds, the vaccine solution was reconstituted with distilled water as described in section 4.2.5.1. Then 2 ml of the vaccine solution was reconstituted in distilled water as described in section 4.2.5.1. The diluted vaccine solution was vigorously mixed with 800 gms of sorghum or barley in a polythene bags. 10 g of the vaccine feed/mixture (10^7 EID_{50} per 10 gm) per bird were fed immediately to chickens which had fasted overnight. The second vaccination was carried out in the same manner 3 weeks after the first vaccination.

Group B II and B IV

These groups were vaccinated with the NDV-I_2 vaccine mixed with sorghum and barley, respectively. To vaccinate the chickens the vaccine-feed-mixture was prepared like for group I and III, but feeding was done after keeping the vaccine feed-mixture for 14 hr. at room temperature.

Group B V

This group received the 1st and the 2nd vaccinations via the ocular route. 1 drop (25µl) of the vaccine solution (NDV-I_2) was instilled into the eye as described in section 4.3.1.

Group B VI
This group served as unvaccinated control, it only received pure sorghum and barley orally and distilled water via the ocular route at the time when the other groups were being vaccinated.

4.5.0 Experiment C

4.5.1 Experimental chickens and vaccination

One hundred day-old non-vaccinated broiler chicks were purchased from the local hatchery and reared in isolation until the first vaccination. The parent stock was vaccinated against ND. Parboiled sorghum and barley were prepared as explained in section 4.2.4. Vaccine concentrate containing 500 doses per vial was diluted in 500 ml of distilled water, then 20 ml of the vaccine solution was further diluted in another 100 ml of distilled water. The 120 ml of diluted vaccine solution then was mixed with 200 gms of either parboiled barley or sorghum in a plastic bowl. The 10 gm of vaccine-mixed-feed per bird represented approximately 10^7 EID_{50} virus per chicken. This feed was fed immediately or six hours after mixing to chickens which had fasted overnight.

Group C I and C III
These groups of chickens received vaccine-mixed parboiled barley immediately after mixing and six hours after mixing , respectively.

Group C II and C IV
These groups of chickens received vaccine-mixed parboiled sorghum immediately after mixing and six hours after mixing, respectively.

Group C V
This group remained unvaccinated control, chickens received pure parboiled barley and sorghum during times of the vaccination procedure of the treatment groups.

4.6 Serology

Prior to vaccination, in order to obtain some baseline information on maternal immunity status, blood samples were collected from 40 chicks at the age of 1, 7 and 14 days in experiment A, and at 7 and 14 days of age in experiments B and C.
After vaccination, sera were collected from all experimental chickens at weekly intervals up to the end of the experiments and 14 days after challenge of the surviving chickens of all three experiments.

4.7 Challenge

In experiment A two challenges were carried out three weeks after the second and the third vaccinations, each time 25 chickens from each treatment group were challenged. In experiments B and C, 20 chickens from each treatment group were challenged three and two weeks after the second vaccinations, respectively. The challenge virus was applied intramuscularly.

4.8 Results

4.8.1 Titration of the vaccine virus and the challenging virus

The titre of the vaccine was $10^{9.5}$ EID_{50} per ml, while the titre of the challenge virus was 10^{9} ELD_{50} per ml.

4.8.2 Vaccine virus stability

Results of assessments on the stability of the NDV-I_2 vaccine virus that is mixed with non-boiled and parboiled sorghum and barley as well as the vaccine suspension kept at room temperature for various periods before inoculation are shown in Table 1.

Table 1. Survival of NDV-I_2 vaccine virus on non-boiled and parboiled barley and sorghum kept at room temperature for various periods of time before inoculating into embryonated eggs.

Storage time (in hours)	Non-boiled			Parboiled		
	sorghum	barley	virus suspension	sorghum	barley	virus suspension
0	yes*	yes	yes	yes	yes	yes
1	no*	no	yes	yes	yes	yes
6	no	no	yes	yes	yes	yes
14	no	no	yes	yes	yes	yes

*yes = recovered (survived)
*no = not recovered (not survived)

Boiling, washing and drying of the grains significantly extended survival of the virus on the parboiled grains and recovery of the virus from the allantoic fluid was possible as late as 14 hours when kept at room temperature. Recovery of the virus from unboiled grains was possible only immediately after mixing (0 hour).

4.1.8.3 Experiment A

The geometric mean HI antibody titres induced by vaccination with conventional vaccines via the ocular and drinking water routes of the thermostable vaccine (NDV-I_2) via the ocular and drinking water routes, and of the vaccine mixed sorghum and barley as carriers and applied by the oral route are shown in Table 2 and Fig 1.

Table 2 Antibody responses (geometric mean titre (GMT) ± standard deviation (S.d)) of chickens vaccinated with conventional vaccines, with NDV-I_2 and with NDVI_2 feed mixes (sorghum & barley) via oral or ocular and drinking water routes (HI-Test).

	Maternal immunity		First vaccination			Second vaccination				Third vaccination			
Treatment groups	1	2	week 0	week 1	week2	week 3	week 4	week5	week 6	week 7	week 8	week 9	week 10
Conventional (HB1 & LaSota)	4.8 (±1.1)	3.4 (±1)	1.2 (±1.1)	3.2 (±1.2)	4.8 (±0.9)	4.4 (±1.3)	4.1 (±1.1)	3.9 (±1.2)	3.5 (±1.2)	2.8 (±1.4)	3.7 (±1.2)	4.5 (±0.8)	4.3 (±1.2
NDV-I_2	4.8 (±1.1)	3.4 (±1)	1.1 (±1.2)	2.3 (±1.1)	3.9 (±1.2)	4.6 (±1.2)	3.9 (±1.2)	3.9 (±1.4)	3.4 (±1.4)	2.3 (±1.0)	3.0 (±1.1)	3.3 (±1.3)	3.4 (±0.9
Sorghum	4.8 (±1.1)	3.4 (±1)	1.2 (±1.2)	0.2 (±0.4)	0.1	0.1	0.0	0.0	0	0	0	0	0
Barley	4.8 (±1.1)	3.3 (±1)	1.2 (±1.2)	0.1 (±0.3)	0.0	0.0	0	0	0	0	0	0	0
Control	4.8 (±1.1)	3.4 (±1)	1.3 (±1.1)	0.1 (±0.3)	0.1	0.0	0	0	0	0	0	0	0
Tested chickens per group	40	40	60	60	60	60	60	60	60	30	30	30	30
Age at bleeding (days)	1	7	14	21	28	35	42	49	56	63	70	77	84

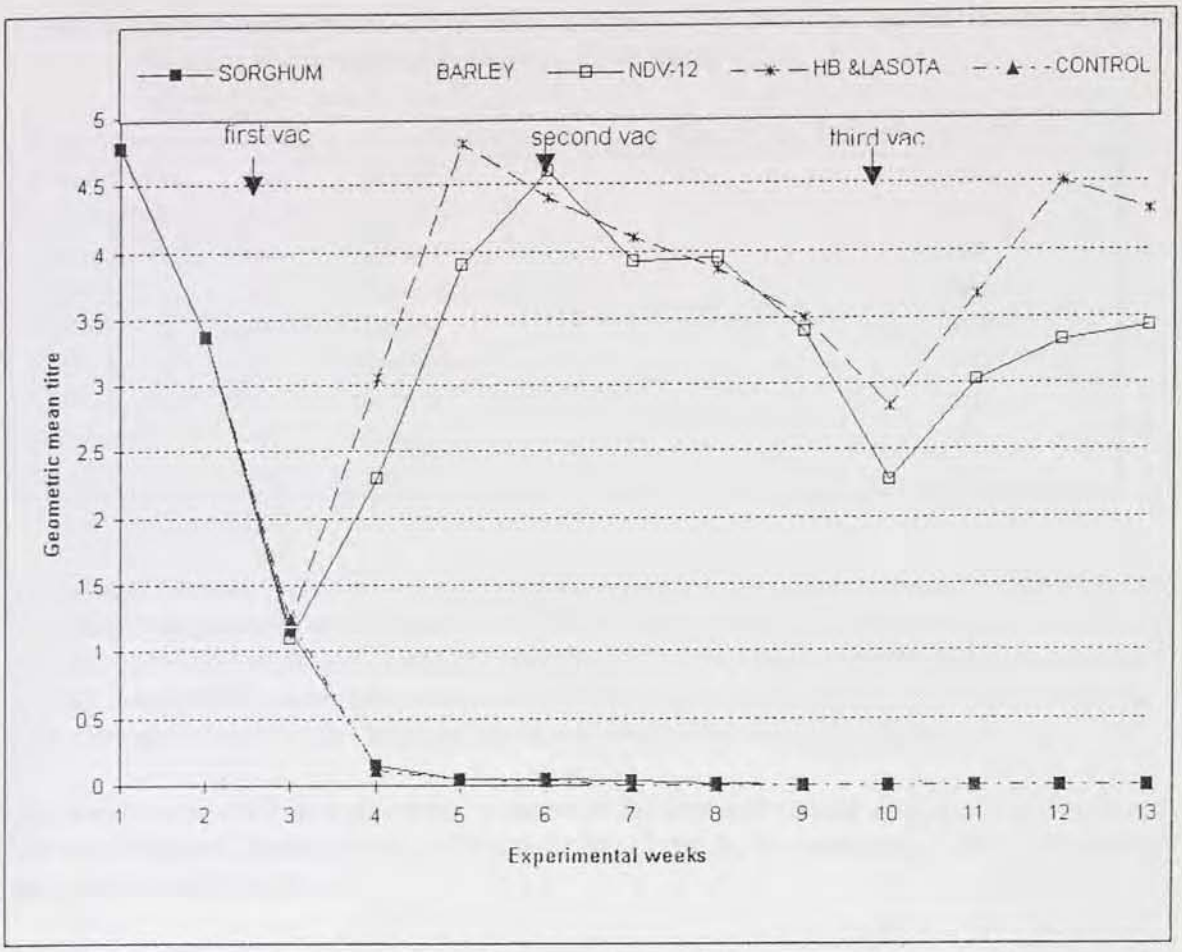

Figure 1. Antibody response of chickens (geometric mean titre) vaccinated with conventional and NDV-I_2 vaccines via the ocular and drinking water route and vaccinated orally with the NDV-I_2 vaccine mixed with untreated sorghum and barley as vaccine carriers (HI Test).

Day-old chicks started with a maternal antibody titre of $\log_2^{4.8}$ which dropped to $\log_2^{1.2}$ by the third weeks of age. As from there, antibody titres of unvaccinated control chicks and of chicks vaccinated with untreated sorghum and barley as NDV-I_2 carriers dropped to zero from the second week after the first vaccination, they were susceptible to the first and the second challenge infection (Table 4). Vaccinations with NDV-I_2 and HB1 and LaSota strains in contrast led to an increase of the HI-antibody titre, beginning one week and reaching peaks two weeks after the first vaccination for conventional vaccines (HB1 and LaSota) and three weeks for NDV-I_2 ocular route. Thus, there was no HI antibody response in the groups vaccinated with NDV-I_2 orally on untreated sorghum or barley.

For NDV-I_2 and conventional vaccines, antibody titres decreased after the second vaccination until the third vaccination (booster dose) was given. As from there, the HI antibody titres gradually increased reaching a maximum HI titre of $\log_2^{3.4}$ for NDV-I_2 and $\log_2^{4.3}$ for conventional vaccines at week ten.

The Kruskal-Wallis one-way of analysis of variance on ranks revealed a statistically significant difference ($p < 0.05$) in the median values among the treatment groups. In order to isolate groups that differed from others, pairwise multiple comparison test (Student Newman Keuls) was used. The results are shown in Table 3. There was a significant difference ($p= 0.05$) between the treatment groups except for conventional vs. NDV-I_2.

Table 3. Pairewise comparisons of vaccination results (HI Test geometric mean titres) between the treatment groups in experiment A
(Student Newman Keuls Test)

Experimental vaccination and control groups			Significant difference at $p<0.05$
Conventional	vs.	Sorghum	yes
Conventional	vs.	Control	yes
Conventional	vs.	Barley	yes
NDV-I_2	vs.	Conventional	no
NDV-I_2	vs.	Barley	yes
NDV- I_2	vs.	Sorghum	yes
NDV- I_2	vs.	Control	yes
Sorghum	vs.	Barley	no
Sorghum	vs.	Control	no

The Mann-Whitney rank test shows that the geometric mean titres at week 1 after the first vaccination was significantlly higher ($p <0.0001$) than at week 0 for the conventional vaccines and the NDV-I2 ocular and drinking water group, but not the sorghum, barley and control groups. Also (GMT) vaccination titres differed statistically significant ($p =0.0001$) at week 7 after first vaccination when compared to titres 6 weeks after the first vaccination.

The distribution of HI antibody titres at times of the first and second challenges expressed by the percentage of chickens with an HI titre $\geq \log_2^3$ and by the percentage of birds protected are recorded in Table 4.

Table 4 Distribution of HI titres at times of first and second challenges three weeks after second and third vaccinations and the degree of protection conferred.

			HI titres ($\log_2$)								
Challenge	Treatment groups	No. chicks tested	0	2	3	4	5	6	GMT	% HI $\geq\log_2^3$	Protection %
	Conventional	25	-	2	11	10	1	1	3.5	92	100
	NDV-I_2	25	-	4	12	9	-	-	3.2	84	100
First	Sorghum	25	25	0	0	0	0	0	0	0	4
(56 days)	Barley	25	25	0	0	0	0	0	0	0	0
	Control	25	25	0	0	0	0	0	0	0	0
	Conventional	25	-	-	9	7	3	6	4.2	100	100
	NDV-I_2	25	-	4	10	7	4	0	3.4	84	100
Second	Sorghum	25	25	0	0	0	0	0	0	0	12
(84 days)	Barley	25	25	0	0	0	0	0	0	0	8
	Control	25	25	0	0	0	0	0	0	0	0

84-92% of chickens vaccinated with NDV-I_2 via the ocular and drinking water routes and of the conventional vaccine groups had HI antibody titres $\geq \log_2^3$ during the first challenge and 84-100% of the chickens of these groups had titres $\geq \log_2^3$ at the time of the second challenge. Chickens in the control group and in the untreated sorghum and barley groups had no measurable HI antibodies at the times of the two challenges.

The level of immunity, indicated by the HI titre ≥ $\log_2^3$ does not correspond to the protection obtained by the two challenges. It was noted that chickens with an HI titre < $\log_2^3$ and some chickens without detectable HI antibodies from vaccinated groups survived the challenges.

Table 5. Results of challenges and mean death time at 56 and 84 days of age

Challenge	First		Second	
Treatment groups	Surv/Challenge d (%)	MDT (days)	Surv/Challenge d (%)	MDT (days)
Sorghum untreated	1/25 (4)	4.85	3/25 (12)	5.5
Barley untreated	0/25 (0)	5.16	2/25 (8)	5.5
Control unvaccinated	0/25 (0)	4.64	0/25 (0)	5.3
NDV-I_2	25/25 (100)		25/25 (100)	
Conventional	25/25 (100)		25/25 (100)	

MDT = mean death time (from challenge to death)

100% of the chickens vaccinated by the conventional vaccines and by NDV-I_2 ocular and drinking water route were protected against the two challenges with locally isolated virulent Newcastle disease virus. In contrast, all chickens in the control group and 88-100% of chickens of the untreated barley and sorghum groups died after the first and the second challenges.

The comparison of treatment groups in regards to mortality showed statistically significant differences between treatment groups for the first and second challenge, (X^2 =121, $p < 0.0001$ and X^2 = 106.8, $p < 0.0001$) respectively. The level of protection of the sorghum and barley groups appeared to be relatively better during the second challenge than during the first challenge, but the Mann-Whitney rank on sum test ($p < 0.05$) revealed that this difference was not statistically different from the first challenge. Also, the Kruskal Wallis one-way analysis of variance by ranks showed that the levels of protection obtained at first and second challenges in the untreated barley, sorghum and unvaccinated control groups were not statistically significant ($p = 0.12$ and $p = 0.75$ respectively, for the first and second challenges). The longest mean death times were 5.16 and 5.54 days, the shortest mean death times 4.64 and 5.3 days for first and second challenge respectively. The Kruskal-Wallis one-way analysis of variance on ranks indicated that there were no a statistically significant differences ($p=0.12$) and ($p =0.754$), respectively, in regards to MDT for first and second challenge between the sorghum barley and the unvaccinated control experimental groups.

4.8.4. Experiment B

The geometric mean weekly HI titres of chickens vaccinated twice with NDV-I_2 via the ocular route, with the NDV-I_2 vaccine on untreated sorghum and barley as carriers of the NDV-I_2 vaccine and of the unvaccinated control group are shown in Table 6.

The 7-day old chickens started with a GMT of $\log_2^{3.5}$ maternal immunity level, this value dropped to almost zero by 4 weeks of age in all treatment groups, except for the NDV-I_2 group vaccinated by the ocular route. The groups receiving vaccine with immediately after mixing (barley 0) or barley and sorghum, 14 hours after mixing (barley 14, sorghum 14) and the unvaccinated control groups did not develop antibodies up to the end of the experiment (56-days of age).

Increase in antibody titres for the NDV-I_2 ocular group in contrast were observed starting from the first week after first vaccination and continuous to increase to reach the peak two weeks after the second vaccination. From then on thus slightly dropped by week 6 after the first vaccination.

The Kruskal-Wallis one-way analysis of variance on ranks showed that the median values of the HI antibody titres among the treatment groups was statistically significant ($p<0.05$).

Table 6. HI antibody response (geometric mean titre ± standard deviation) of chickens vaccinated twice with the NDV-I_2 vaccine by different routes and of the unvaccinated control group,

Treatment groups	Maternal immunity	First vaccination			Second vaccination			
		week 0	week 1	week 2	week 3	week 4	week 5	week 6
Sorghum 14 **	3.5 (±1)	1.1 (±0.9)	0.2 (±0.4)	-	-	-	-	-
Barley 14 **	3.5 (±1)	1.3 (±0.8)	0.2 (±0.4)	0	0	0	0	0
Barley 0 *	3.5 (±1)	1.4 (±0.8)	0.7 (±0.8)	0.1 (±0.3)	0.1 (±0.3)	0	0	0
Sorghum 0 *	3.5 (±1)	1.3 (±0.9)	0.8 (±0.9)	0.1 (±0.2)	0.1 (±0.3)	0	0	0
Control	3.5 (±1)	1.4 (±0.8)	0.2 (±0.4)	0.1 (±0.3)	0	0	0	0
NDV-I_2 ocular	3.5 ±	1.4 (±0.5)	2.2 (±0.9)	3.2 (±1.1)	3.3 (±0.8)	3.9 (±0.8)	4.3 (±0.7)	3.4 (±0.9)
Chickens tested	40	20	20	20	20	20	20	20
Age at bleeding	7 days	14-days	21-days	28-days	35-days	42-days	49-days	56-days

**= untreated, given orally 14 hours after mixing with the NDV-I_2 vaccine

* =untreated, given orally immediately after mixing with the NDV-I_2 vaccine

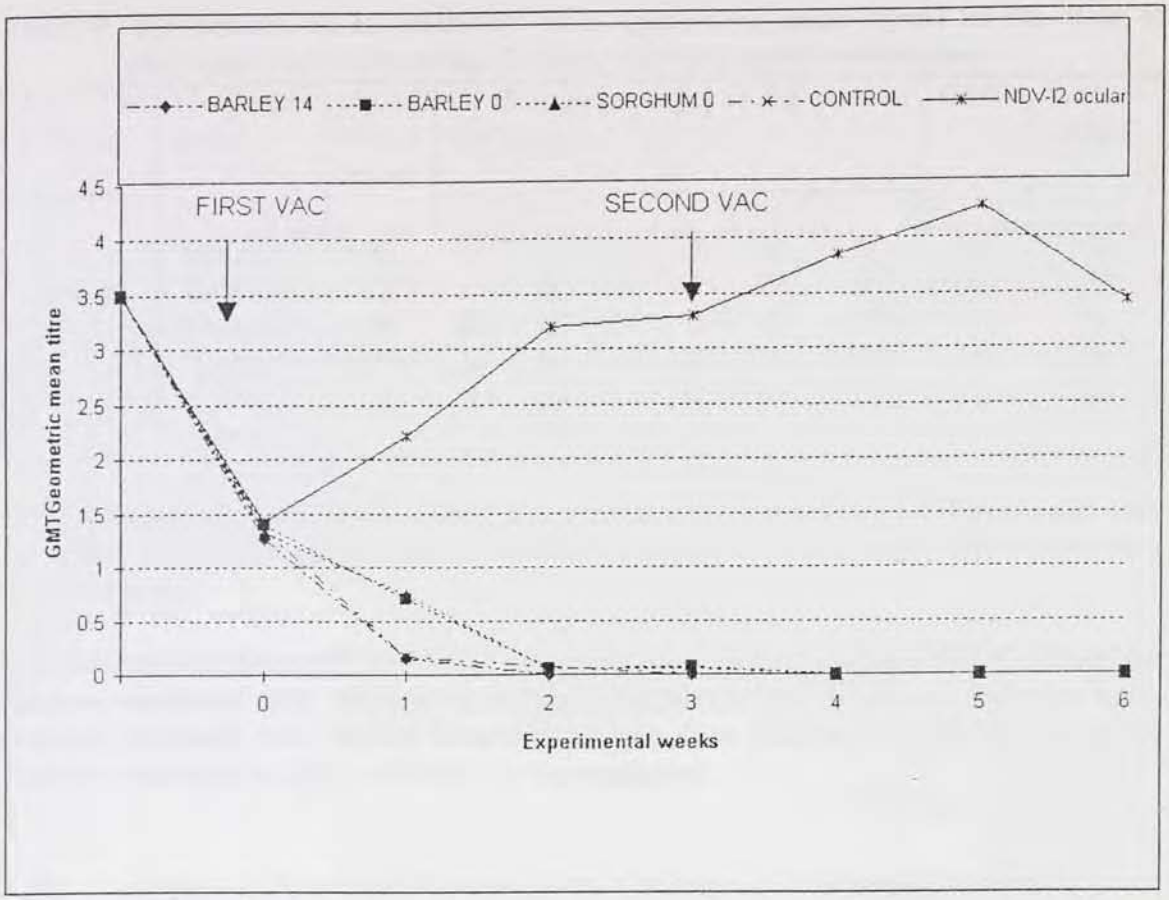

Figure 2. HI antibody response (geometric mean titres) of chickens vaccinated with NDV-I_2 via the ocular, of NDV-I_2 orally on untreated sorghum and barley as vaccine carriers and of the unvaccinated control group.

Further analysis of the geometric mean HI titres by Dunn's multiple comparison test inorder to isolate the group or groups which differed from others, indicated a statistically significant difference ($p < 0.05$) between the NDV-I_2 and the other treatment groups (Table 7).

Table 7. Comparison of vaccination response of treatment groups vs. the NDV-I_2 ocular route group.

(Dunn's multiple comparison test)

Comparison groups			significant difference at $p < 0.05$
Barley 14	vs.	NDV-I_2 ocular	yes
Control	vs.	NDV- I_2 ocular	yes
Sorghum 0	vs.	NDV-I_2 ocular	yes
Barley 0	vs.	NDV-I_2 ocular	yes

Except for the NDV-I_2 ocular group, where 94% of the chickens had attained an HI titre of $\geq \log_2 3$ at the time of challenge, all chickens of the other treatment groups showed no HI antibodies at all (Table 8).

Table 8. Distribution of HI antibody titres (geometric mean titres) at the time of challenge, expressed as $\log_2$ (21 days after the second vaccination).

Age at bleeding	Treatment group	No. of chicks tested	HI distribution $\log_2$ at the time of challenge.						GMT	% HI $\geq \log_2^3$
			0	1	2	3	4	5		
56 days	Barley 14	20	20	0	0	0	0	0	0	0
	Barley 0	20	20	0	0	0	0	0	0	0
	Sorghum 0	20	20	0	0	0	0	0	0	0
	Control	20	20	0	0	0	0	0	0	0
	NDV-I2 ocular	19	0	0	1	11	4	3	3.5	94

Table 9 shows the results of the challenge, carried out at 56 days of age, i.e. three weeks after the second vaccination, and of the mean death time (days from challenge to death) for each treatment group.

The challenge test showed that 100 % of the unvaccinated control chickens and 90-95% of the chickens vaccinated with untreated barley and sorghum as NDV-I_2 carriers died after intra-muscular challenge with virulent Newcastle disease virus (Alemaya strain). 94.7% of the chickens vaccinated ocularly with NDV-I_2 were protected.

Table 9 Results of Newcastle Disease virus challenge of 56-day-old vaccinated and unvaccinated chickens, three weeks after second vaccination and mean death time (MDT) from challenge to death.

Treatment groups	No. survival/no. challenged	% survival	MDT in days
NDV-I_2 ocular	18/19	94.7	6
Barley 14 **	1/20	5	5.2
Barley 0 *	2/20	10	5.1
Sorghum 0 *	2/20	10	5.1
Control (unvaccinated)	0/20	0	4.8

* = Untreated, given orally immediately after mixing with the NDV-I_2 vaccine.

** = Untreated, given orally 14 hours after mixing with the NDV-I_2 vaccine.

In regards to mortality differences between treatment groups were statistically significant ($X^2 = 68.2$, $p<0.05$). The shortest mean death time was 4.8 days (unvaccinated control group), the longest was 5.18 days (barley 0 group). However, the Kruskal Wallis one-way analysis on ranks test showed that the differences between the median values of the mean death times among the treatment groups of sorghum 0, barley 0, barley 14 and unvaccinated controls were not significant ($p=0.57$).

The level of immunity, indicated by an HI titre $\geq \log_2^3$ (Table 8) does not correspond to the protection shown by the challenge results (Table 9). Some chickens without a detectable antibody titre from the vaccinated groups survived the challenge.

4.8.5. Experiment C

The weekly geometric mean HI antibody titres induced by vaccination of chickens at 14 and 35 days of age with the NDV-I_2 vaccine and parboiled sorghum and barley as carriers and of un-vaccinated control chickens are shown in Table 10 and Fig 3.

Maternal antibody levels of both vaccinated and unvaccinated groups dropped from HI titre of $\log_2^{2.2}$ at seven days of age to $\log_2^{0}$ by 3 weeks of age. Chickens which had received the NDV-I_2 vaccine mixed with parboiled barley immediately after mixing (barley 0) and 6 hours after mixing (barley 6), responded positively to the vaccination after the booster dose had been given, while the sorghum 6 group and the unvaccinated controls did not develop antibodies up to the end of the experiment (49 days of age). In the sorghum 0 group, 3 to 5 chickens showed only very low HI antibody titres throughout the experiment. Peak HI antibody titre for the barley 0 and barley 6 vaccination groups were observed two weeks after the second vaccination (Table 10, Fig 3).

Table 10. HI Antibody responses of chickens (Geometric mean titres ± standard deviation) vaccinated twice with the NDV-I_2 vaccine mixed with parboiled sorghum and barley and of unvaccinated controls.

Treatment groups	Maternal immunity	First vaccination			Second vaccination		
	week -1	week 0	week 1	week 2	week 3	week 4	week 5
Barley 0 *	2.2	0.85 (±0.7)	0.2 (±0.4)	0.3 (±0.4)	0.4 (±0.6)	2.6 (±1.2)	3.4 (±1.5)
Barley 6 **	2.2	0.8 (±0.6)	0.2 (±0.4)	0.2 (±0.4)	0.3 (±0.4)	1.2 (±0.9)	2.5 (±1.1)
Sorghum 0 *	2.2	0.9 (±0.6)	0.3 (±0.3)	0.1 (±0.3)	0.1 (±0.3)	0.3 (±0.4)	0.2 (±0.4)
Sorghum 6 **	2.2	0.6 (±0.5)	0.2 (±0.2)	0	0	0	0
Control unvaccinated	2.2	0.8 (±0.7)	0	0	0	0	0
Chickens tested	20	20	20	20	20	20	20
Age at bleeding	7	14	21	28	35	42	49

* = Given orally immediately after mixing with the vaccine.

**= Given orally 6 hours after mixing with the vaccine.

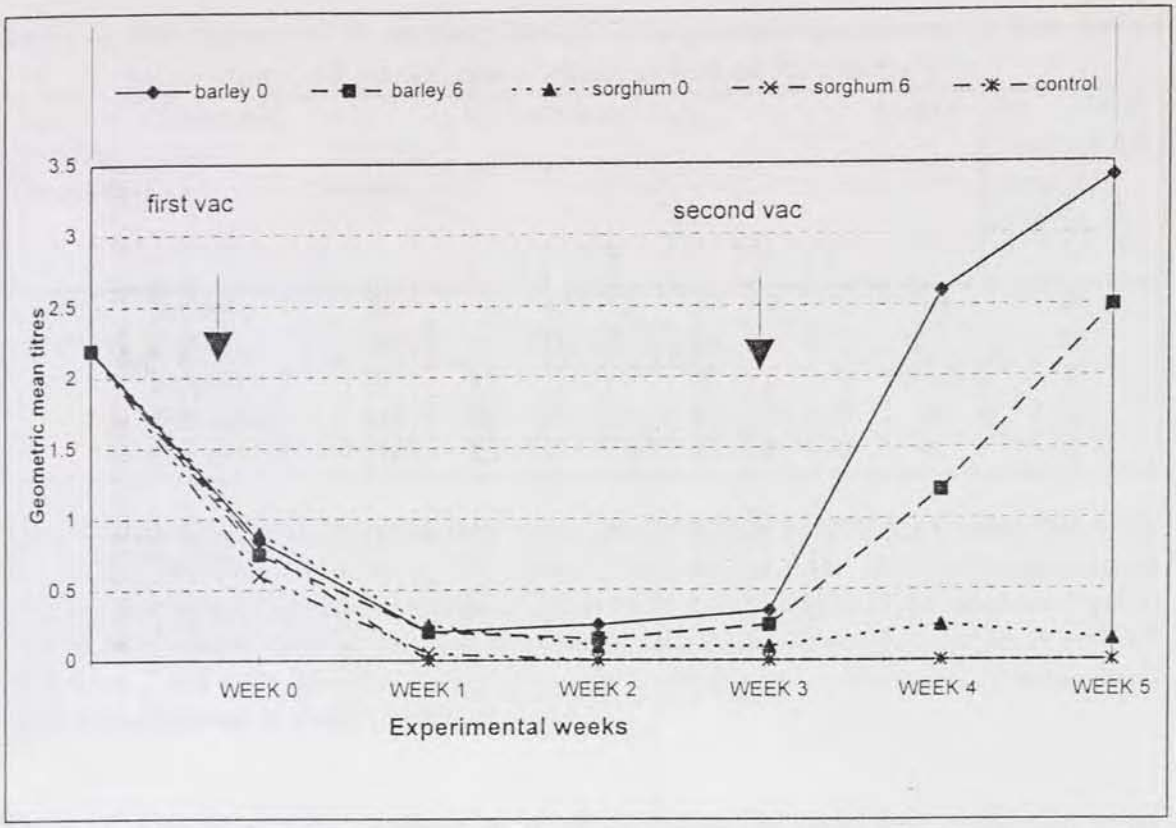

Figure 3. HI Antibody response of chickens (Geometric mean titres) vaccinated orally with the NDV-I_2 vaccine mixed with parboiled sorghum and barley, and of unvaccinated controls.

The Kruskal-Wallis one way analysis of variance on ranks showed that there was a statistically significant difference ($p = 0.0294$) in the median values among the treatment groups with respect to GMT. In order to isolate the groups that differed from each other, Dunn's multiple comparison test was carried out (Table 11).

Table 11. Pairwise comparison of treatment groups.
(Dunn's multiple comparison test)

Comparison groups			Significant difference at $p < 0.05$
Barley 0	vs.	Control	yes
Barley 6	vs.	Control	yes
Sorghum 0	vs.	Control	no
Sorghum 6	vs.	Control	no
Sorghum 6	vs.	Barley 0	yes
Barley 0	vs.	Barley 6	no

The GMT of the groups barley 0 vs. barley 6, sorghum 0 vs. control and sorghum 6 vs. control groups were not statistically significant ($p<0.05$), but differences significant were between the groups barley 0 vs. control, barley 6 vs. control and sorghum 6 vs. barley 0.

The distribution of HI antibody titres at the time of challenge (two weeks after the second vaccination) and the percentages of chickens with HI antibody titres ≥ to $\log_2^3$ were shown in Table 12.

Table 12. Distribution of HI antibody titres at time of challenge (two weeks post second vaccination) and percentages of chickens with an HI ≥ to $\log_2^3$.

Age at bleeding (in days)	Treatment group	No. of chickens tested	HI distribution $\log_2$						GMT	% birds with an HI titre ≥ to $\log_2^3$
			0	1	2	3	4	5		
49	Barley o	20	2	-	2	6	4	6	3.4	80
	Barley 6	20	1	2	7	6	4	0	2.5	50
	Sorghum 0	20	17	3	0	0	0	0	0.15	0
	Sorghum 6	20	20	0	0	0	0	0	0	0
	Control	20	20	0	0	0	0	0	0	0

Table 12 shows that 80% of the chickens in the barley 0 and 50 % of barley 6 group had an HI titre ≥ $\log_2^3$ at the time of challenge. In contrast, the sorghum 6 and the control groups were without detectable HI antibodies and three out of the twenty chickens in the sorghum 0 group showed very low HI antibody titres at the time of challenge. However, some birds with HI titres ≤ $\log_2^3$ still were protected against challenge as the challenge results and the mean death times from challenge to death (Table 13).

Table 13. Results of NDV challenge of chickens vaccinated twice and challenged at 49 days of age, and the mean death time from challenge to death.

Treatment groups	No. survived /no. challenged	% survival	Mean death time in days
Barley 0 *	20/20	100	-
Barley 6 **	19/20	95	6
Sorghum 0 *	6/20	30	4.9
Sorghum 6 **	0/20	0	5
Control (unvaccinated)	0/20	0	4.8

* = Given orally immediately after mixing with the vaccine.
**= Given orally 6 hours after mixing with the vaccine.

95-100 % of the chickens from the barley 0 and barley 6 groups were protected against challenge with virulent NDV. 30 % protection was also obtained in the sorghum 0 group, while birds from the unvaccinated control and sorghum 6 groups were not protected.

The comparison between all treatment groups showed statistical significant differences (X^2 =64.1 and $p < 0.05$), but no significant differences were detected between barley 0 and barley 6 and also between sorghum 0, sorghum 6 and control groups in respect to mortality. The shortest mean death time was 4.8 days (control) and the longest was 6 days (sorghum 6). However, no statistical significant differences in mean death time between sorghum 0, sorghum 6 and the unvaccinated control groups could be established.

In all birds that died after challenge, gross pathological lesions such as haemorrhages and necrosis in the proventriculus, trachea, intestine and caecal tonsil, and the formation of

annular ring plaque in the intestine were found at necropsy. Homogenates of brain, trachea and liver samples from these dead chickens were positive for NDV in embryonated eggs.

4.9 Discussion

The objectives of these trials were to evaluate whether the heat resistant NDV-I_2 vaccine gives adequate protection against velogenic strains of NDV, to compare this vaccine with conventional (HB1 and LaSota) vaccine types and to assess the possibility of using barley and sorghum as carriers of NDV-I_2 vaccine for oral application.

The results indicate that chickens vaccinated with the thermostable NDV-I_2 vaccine via the ocular and drinking water routes or orally with parboiled barley as vaccine carrier were substantially protected against challenge with a virulent NDV strain equally as with the HB1 and LaSota strain via the ocular and drinking water route. This confirms results of previous workers (Ibrahim *et al.*, 1980, 1981; Bell *et al.*, 1991; Copland and Spradbrow, 1997), who reported about the efficacy of the V4 vaccine strain, which is identical to the NDV-I_2 strain. They determined that this vaccine, applied by intranasal, intraocular route or given as aerosol, spray or via the drinking water, resulted in seroconversion and protected the vaccinated birds against challenge with virulent NDV.

In our work we also compared the efficacy of the NDV-I_2 vaccine with the conventional HB1 and LaSota vaccines. The results indicate that with respect to seroconversion and protection against challenge with virulent NDV there is no statistically significant difference between the two vaccine types when given via the drinking water and the ocular route. Three weeks after first vaccination a peak titre of approximately $\log_2^{4.6}$ was recorded for the ocularly vaccinated groups. This corresponds to the peak of $\log_2^{5.0}$ reported by Idris *et al.* (1987) and Bell *et al.* (1995) 2 weeks after a single vaccination with a V4-UPM heat-resistant clone. A similar peak titre within 2-4 weeks after vaccination was reported by Spradbrow and Samuel (1987). In contrast, Westbury (1984) indicated that the V4 vaccine was less effective than the HB1 and LaSota vaccines, based on the protection afforded by aerosal vaccination under laboratory conditions against increasing challenge virus doses injected intramuscularly. Similarly, Biswas *et al.* (1996) also observed slightly better protection with conventional vaccines than with the thermostable vaccine NDV4HR. Since Allan and Borland (1980) observed differences in the immunogenicity of different commercial vaccines based on the virus strains used, it is well possible that the immunogenicity of the HB1 and LaSota preparations used by Westbury (1984) differed from those of commercial vaccines available nowadays. Our results certainly show the potential of the NDV-I_2 vaccine when applied via the ocular and drinking water routes are in fully agreement with the findings of Kim and Spradbrow (1978), Ibrahim *et al.* (1981), and Bell *et al.* (1995).

Chickens vaccinated with the NDV-I_2 or HB1 and LaSota strains and surviving the challenge must nevertheless have been infected with virulent virus because they showed rising antibody titres. Turner and Kovesdy (1974), Spradbrow *et al.* (1980), Ibrahim *et al.* (1981) and Westbury (1984) reported that surviving chickens showed no respiratory illness during 14 days of observation and yet exhibited a marked rise in the antibody titres following challenge. This indicates that they had been infected by the challenge virus. This agrees with Spradbrow (1993/4) statement that oral Newcastle disease vaccine will prevent disease but not infection.

Applying the NDV vaccine orally via vaccine-treated feed appears to be an attractive alternative to the conventional application methods in situations where poultry are roaming freely most of the time, such as in the case of village chickens in South East Asia and Africa. Samuel *et al.* (1992) and Cumming (1992) found that uncooked grains as vaccine carriers were not entirely satisfactory and showed that vaccine, washed off immediately after having been added to grains, had lost at least 90% of its initial virus titre. They assumed that these grains probably contained water soluble antimicrobials, possibly phenolic compounds, which are necessary to protect the grain seeds after planting in moist soil until successfully germinating. Cumming (1992) therefore suggested that short boiling and washing the grain would significantly extend the survival of the virus on the grain and that coarse cracking would extend its survival time. A possible mechanism might be that this treatment leads to a better attachment or absorption of virus to the interior parts of the grain, rather than to the outside of the grain where the antimicrobials are concentrated.

The present work shows that uncooked barley and sorghum were unsuitable vaccine carriers, vaccine virus could not be recovered in embryonated eggs, except immediately after (0 hours) of mixing with the grains. In contrast, vaccine virus was recovered even after 14 hours, if the barley had been pretreated by parboiling. Also, birds vaccinated with non-parboiled grains did not seroconvert and were unprotected, while chickens vaccinated with parboiled barley seroconverted and were well protected against challenge with virulent NDV. On the other hand, vaccine added to parboiled sorghum, did not stimulate any production of antibody and birds were susceptible to challenge. Cumming (1992) also found a high correlation between recovery of virus from eggs and seroconversion in chickens as well as and successfully stimulated seroconversion only when the sorghum was consumed within two hours of mixing. Copland and Spradbrow (1997) reported that the I_2 vaccine delivered by the ocular and the drinking water in Tanzania indicated about 70% efficacy under field condition in contrast when the vaccine delivered on boiled sorghum was less effective.

A previous work carried out in Ethiopia by Rushton (1995) showed that vaccine mixed V4 with barley, resulted in seroconversion and protected 100% of the vaccinated chickens after the third vaccination. This finding was not confirmed by the present work. Vaccine virus mixed with untreated barley was not recovered one hour after mixing and chickens neither seroconverted nor were protected against challenge with virulent NDV virus.
The study thus shows that chickens vaccinated with the HB1 and LaSota vaccines and with the NDV-I_2 vaccine via the ocular and drinking water route, only ocularly, and also orally after mixing NDV-I_2 with parboiled barley were substantially protected against challenge with virulent strains of NDV. It was, however, surprising that some chickens with a low or even an undetectable NDV-HI titre after vaccination nevertheless were protected against challenge with the velogenic NDV challenge strain used. Furthermore, a number of chickens in the vaccinated groups showed low antibody titres, below $\log_2^3$, but, were fully protected against challenge three weeks after the second or the third vaccination while all birds of similar age from the unvaccinated groups died after challenge. Ibrahim *et al.* (1981) concluded that no correlation existed between the HI antibody titres due to NDV4 vaccination and resistance to challenge. Similar observations were also reported by other workers (Winterfield *et al.*, 1957; Spradbrow *et al.*, 1977; Westbury, 1984; Bell *et al.*, 1991). Timms and Alexander (1977) suggested that local immunity and cell mediated immunity plays a role in the protection of vaccinated chickens against velogenic ND viruses. Ghumman and Bankowski (1976) stated that the initial response to infection with NDV is cell mediated and occurs as early as two or three days after infection with live strains. Spradbrow and Samuel (1991) postulated that oral vaccination may induce the production of secretory

immunoglobulins by stimulating the intestinal lymphoid tissue. Holmes (1979), stated that the exact function of humoral immunity is not clear and it was proposed that protection of the respiratory tract is independent of hummoral immunity and Jayawarredane and Spradbrow (1995) pointed that there was no correlation between HI antibody titres and CMI response. The latter would contradict the statement made by Allan *et al.* (1978), who claimed a strong association between HI titre and challenge protection. It would, however, agree with the conclusions by Beard and Brugh (1975) that antibody titres only reflect the total immune response to vaccination. Thus, the present results seem to indicate that a low NDV-HI antibody titre does not necessarily mean susceptibility to virulent NDV, especially not in older chickens, where age resistance might also be involved. High antibody titres, on the other hand, usually indicate protection (Ibrahim *et al.*, 1981).

The results of experiment A show that in both treatment groups (conventional and NDV-I_2 vaccines) the booster dose given three weeks after first vaccination did not lead to a rise in antibody titres. Instead, antibody titres gradually declined until third vaccination. The reason for this observation might be that revaccination with lentogenic vaccines in the presence of high levels of residual antibody did not stimulate immunity. Allan *et al.* (1978) also stated that if the time interval between primary and secondary vaccination is less than 21 days, the antibodies produced by the first vaccination are likely to interfere with the multiplication of the second dose of vaccine virus. This phenomenon is well known to occur after vaccination against other avian diseases, such as Marek's Disease, Infectious Bursal Disease. Generally, the serological response to repeated applications of lentogenic live vaccine thus is poor. Therefore, in countries with a high prevalence of virulent ND, mesogenic strains of NDV are generally performed for booster vaccinations. However, experience with the latest velogenic NDV outbreak in Europe had shown that the available lentogenic vaccine strains offer sufficient protection against clinical disease, as long as they are applied correctly and frequently (Lohr, pers. communication).

In these trials the GMT of the NDV-I_2 vaccinated chickens via the ocular and drinking water routes were approximately one two-fold dilution step lower than are generated by the conventional vaccines. Also, the GMT in the barley-6-group was one two-fold dilution step lower than in the barley-0 group. However, this difference was statistically not significant and there was no difference in protection.

In experiment C, no HI antibody response was observed until the third week after the first vaccination and in the barley group a rising NDV-HI antibody titre was only observed after the second vaccination. Similar results were also reported by other workers (Idris *et al.*, 1987). Spradbrow (1993/4) stated that protective immunity is often not apparent until after the second dose of oral vaccination. This delayed response might be explained by the varying amounts of feed eaten by the chicks and by the time required to consume the feed. The chicken age at the time of vaccination in this instance might play an important role. It was observed that during the first vaccination it took young birds more than two hours to consume the vaccine-treated feed, while during second and third vaccinations it was consumed in less than 30 minutes. The prolonged exposure of the vaccine/feed mix at first vaccination might affect the survivability of the vaccine virus, particularly under extreme environmental conditions. After all, the amount of vaccine virus taken per bird is a critical factor. Furthermore, the results of this study indicate that generally a single oral vaccination might not be sufficient to confer immunity because a high percentage of birds were without detectable immunity after first oral vaccination. This finding agrees with previous results (Ibrahim *et al.*, 1981; Idris *et al.*, 1987), who observed that a high percentage of chickens

vaccinated only once died from the challenge. In another study of Spradbrow (1987) on the other hand birds were given vaccine-coated food pellets and were reported to have developed high levels of HI antibodies within two weeks after first vaccination.

It could be summarized that a single oral vaccination is insufficient and that the immune response needs to be boosted with at least one further oral vaccination. The question, of the duration of immunity after oral vaccination and thus the frequencies of revaccinations required to maintain full immunity has to be observed by further research.

PART 5. STRAIN CHARACTERIZATION OF FIELD NDV-ISOLATES IN ETHIOPIA

5.1 Introduction and Objectives

The purpose of this study was to characterize field NDV-isolates from Ethiopia. Differentiation of NDV isolates is important because strains vary in pathogenicity. Also, some ND viruses of low virulence for commercial poultry are prevalent enzootic in feral birds and live vaccines are almost universally used. In addition, more specific strain identification can be usefully employed in the epizootological tracing of outbreaks.

Several pathogenicity tests for the identification of high and low virulence in the laboratory have been devised. The tests employed in this study were the mean death time (MDT) in embryonated eggs, the intravenous pathogenicity index (IVPI) in six-week-old chickens, and the intracerebral pathogenicity index (ICPI) in day-old chicks. Furthermore, Dr. O. Werner from the Bundesforschun gsanstalt, Riems Germany, used Mouse monoclonal antibodies (Mabs) to further differentiate eight field isolates of NDV from Ethiopia.

5.2 Materials and Methods

5.2 1 Samples

Samples from trachea, proventriculus, intestines, caecal tonsils, spleens, brains, and swabs from trachea and cloacas from birds showing clinical signs of acute ND. were collected aseptically in sterile equipment. Samples from previous years from the collections of the NVI were also investigated.

5.2.3 Origin of the samples

Half of the samples were collected from the 1996-1997 outbreaks around Debre Zeit and other parts of the country. The other samples were older isolates from the Eastern part of the country and from around Debre Zeit. Places and years of collection of the isolates are given in Table 14.

Table 14 Origins and years of collection of Newcastle disease field virus-isolates

Serial No.	Sample	Place of origin	Year of collection
1	Lab	Koka	1997
2	Walayeta	Maji	1997
3	Dembi 1	Debre Zeit	1987
4	Dembi 2	Debre Zeit	1996-97
5	Lemlem	Debre Zeit	1996
6	Kebele 2	Debre Zeit	1984
7	Alemaya	Harar	1984
8	Market	Arsi	1997

5.2.4 Embryonated Egg culture

Tissue and organ samples were finely minced and placed in cell culture medium (Dulbecco or RPMI) to which Penicillin (100 IU/ml) and streptomycin (100 mg/ml) were added. The suspension was held at room temperature for about two hours before centrifugation at 1000 g for 10 minutes. A volume of 0.1 to 0.2 ml of the supernatant from the sample was then inoculated into the allantoic cavity of 9-day-old embryonated chicken eggs. The eggs were placed at 37°C and candled twice daily. Embryos that died during the first 24 hours were discarded. Eggs dead or dying at a minimum of four and a maximum of seven days after inoculation were chilled at 4°C and the allantoic fluid was harvested and tested for HA activity. All HA positive fluids were tested for sterility from bacteria and fungi by culture. An HI test using known positive ND serum (NVI-Debre Zeit) was performed with each HA positive allantoic fluid to exclude influenza virus and other haemagglutinating agents.

5.2.5 Mean death time in embryonated chicken eggs. (MDT)

Tenfold dilutions of freshly infected allantoic fluid were made in sterile physiological saline containing antibiotics as described in section (3.6a). Dilutions ranging from 10^{-1} to 10^{-9} were prepared. Thirty 9-day-old embryonated eggs of equal size were candled and marked for each virus isolate. Ten eggs were marked for each dilution in the series of 10^{-7}, 10^{-8} and 10^{-9}. Five of the eggs were marked "A" for the morning inoculation and the remainder "B" for the afternoon inoculation.

At 8:00 a.m. groups of five eggs were inoculated into the allantoic cavity using 0.1 ml of each dilution. The hole in the shell was sealed with wax and the eggs were incubated at 37°C. Following these inoculations the remaining virus dilutions were maintained at +4°C, and in the late afternoon the "B" series of eggs was inoculated. All eggs were candled twice daily at 8:00 a.m. and 6:00 p.m. The time of death of each embryo was recorded. Eggs with dead embryos were examined for the presence of Newcastle disease haemagglutinin.

The test was continued for seven days, and at the end of the time all remaining eggs were chilled and tested for haemagglutinin.

The minimum lethal dose was the highest dilution that killed all the embryos in both sets of eggs, and the mean death time of embryos was recorded.

Interpretation
NDV strains were classified on the basis of MDT as described by Allan et al. (1978), according to which velogenic strains have a mean death time of less than 60 hours, mesogenic strains of 60 to 90 hours, and lentogenic strains of more than 90 hours.

5.2.6 Intravenous pathogenicity index (IVPI)

5.2.6.1 Chickens

Forty one-day-old broiler chicks were purchased from a local hatchery and reared in isolation for six weeks. At weekly intervals blood samples were collected from all chickens to determine the HI antibody levels.

At the end of the sixth week all birds were confirmed completely negative for HI antibodies were divided randomly into three treatment groups. Each group comprised of 10 chickens for the tests, 2 chicks per group served as negative controls.

5.2.6.2 Treatment groups

From the eight field isolates of NDV three were selected for this test based on the origin of the isolates, they were namely: The Alemaya strain from the Eastern part of Ethiopia, the Welayeta (Maji) strain from the southern part of Ethiopia and the Kebele 2 (Debre Zeit) strain from the central part of Ethiopia.

5.2.6.3 Test procedure

For the test, a freshly infected allantoic fluid was diluted to 10^{-1}. 0.1 ml of this virus dilution was injected intravenously into each of ten six-week-old chickens for each treatment.

The inoculated chickens were observed daily at the time corresponding to the time of inoculation for ten days, and the results were recorded. For this test the birds were rated as healthy, sick, paralysed, or dead. Dead birds were recorded cumulatively. Birds were classified as sick if they huddled together, were disinclined to move, feed or drink, but did not show any marked signs of wing or leg paralysis and as paralysed if they were prostrated and showed clear incoordination of wings or legs. In this test a normal bird was weighted zero, a sick bird 1, a paralysed bird 2, and a dead bird 3, (Allan *et al.*, 1978). The IVPI was calculated as the sum of the weighted values over the number of observations.

Interpretation
Virulent strains of NDV give IVPI-values close to three, while viruses of low and intermediate virulence give values of 0.

5.2.7 Intracerebral pathogenicity index.

0.05 ml of fresh infected allantoic fluid diluted to 1:10 with sterile physiological saline without antibiotics was injected interacerebrally in each of ten one-day-old chicks. The virus dilution under test was plated out for bacterial sterility. Two additional chicks were included as a negative control and were inoculated with sterile diluent only. The inoculations were carried out with a fine hypodermic needle (a needle 0.45 mm in diameter and 5 mm in length). The inoculum was injected into the caudal aspect of the cranium.

The test was read daily by inspection of the chicks at the same hour of the day as the original injection. Chicks were scored or rated as normal (alert, moving without incoordination), sick (including chicks that exhibited signs of paralysis or were prostrate, but excluding chicks that were only dull), and dead. Care was taken to distinguish between chicks recorded incorrectly on which thus disinclined to move and chicks affected by the virus.

The test was continued for eight days, and the results recorded as described by Allan *et al.* (1978). The ICPI was expressed as the weighted mean over the number of observations made.

5.8 Results

For characterization of the eight field isolates of NDV in Ethiopia, the mean death (MDT) time in 9-day-old embryonated eggs, intracerebral pathogenicity index (ICPI) in day-old chicks, a mouse monoclonal antibody test (Mabs) (the latter two were performed in Germany) and the intravenous pathogenicity index (IVPI) in six-week-old chickens were established. The mean death time was performed in duplicate. The results are shown in Tables 15 and 16.

Table 15. Mean Death Time (MDT), Intracerebral Pathogenicity Index (ICPI) and Intravenous Pathogenicity Index (IVPI) of NDV field isolates from Ethiopia.

Field isolate	Origin	Date of collection	MDT	ICPI	IVPI
Lab	Koka	1997	49.9	1.80	n. d
Welayeta	Maji	1997	54.2	1.86	2.4
Dembi 1	Debre Zeit	1997	52.8	1.83	n. d
Dembi 2	Debre Zeit	1987	56.1	1.79	n. d
Lemlem	Debre Zeit	1996	52.5	1.64	n. d
Kebele 2	Debre Zeit	1984	55	1.73	2.33
Market	Arsi	1997	55.4	1.79	n. d
Alemaya	Harer	1984	51.1	1.84	2.51

n. d = not done

Table 16. Results of examination of NDV field isolates from Ethiopia in the Monoclonal Antibody in the HI test.

Isolate	Reaction with Mabs in HI test					
	10	27	33	39	51	617
Lab	-	+	+	+	+	+
Welayeta	-	±	+	+	-	+
Dembi 1	-	-	-	-	-	-
Dembi 2	-	-	-	-	-	-
Lemlem	-	+	-	-	+	+
Kebele 2	-	+	-	-	+	-
Market	+	+	+	+	+	+
Alemaya	+	+	-	-	+	-
Pigeon type PMV-1	-	-	+	+	-	+
German NDV 1993/94	-	-	-	-	-	-
German NDV 70ies	+	+	-	-	+	-

Performed by Dr. Werner, Bundesforschungsanstalt, Riems.

The MDT ranged from 49.9 to 56.1 hours. The shortest MDT was calculated for the Lab isolate (Koka) and the longest for Dembi 2 isolate. Market, Kebele 2, Dembi 2, and Welayeta isolates had MDT between 54.1 to 56.1. Alemaya, Lemlem and Dembi 1 had MDT between 51.1 and 52.8 and MDT for the Lab isolate was 49.9 hours. The MDT for test 1 and test 2 and also for morning and afternoon sets of eggs showed very little variation for each isolate.

The IVPI for Alemaya , Welayeta and Kebele 2 isolates was 2.51, 2.48 and 2.33 respectively. All chickens inoculated with the Welayeta, Alemaya and Kebele 2 isolates died within 4, 5 and 5 days, respectively. Out of 10 chickens from each group, 8, 9 and 10 chickens from Alemaya, Kebele 2 and Walayeta, respectively, were paralyzed before death. The two control chickens from each group which had received PBS did not show any clinical symptoms until the 8th day post inoculation.

The ICPI values for all tested isolates were above 1.5 ranging from 1.64 to 1.86. The highest ICPI value was for the Welayeta isolate (1.86) and the lowest for the Lemlem isolate (1.64). Reaction with Mabs in the HI test indicated that all isolates differ from each other except Dembi 1 and Dembi 2.

5.9 Discussion

The isolates used in this study were collected from birds clearly showing disease signs, or they were from old specimens from NVI. Preliminary cell and egg cultures and lateron HA and HI tests carried out at NVI Debre Zeit, Ethiopia, and in Germany at Nationales Refernzlabor, für Newcastle Disease und aviäre Influenza Insel Riems, confirmed that all the isolates contained avian paramyxo viruses of the APMV-1 serotype. All eight NDV isolates had MDTs of less than 60 hours ICPIs values of greater than 1.5, and the three isolates Alemaya, Kebele 2 and Welayeta had IVPI values greater than two. Based on the common criteria for the classification of NDV as published by Hanson and Brendly (1955) and Allan *et al.* (1978), the present isolates have to be classified as velogenic strains. The present result confirms previous work on the Alemaya-isolate by Lefevre and Martel (1975). The Mabs test showed that only Dembi 1 and 2 seems to be identical strains while the others differed.

Apart from the results of pathogenicity tests, the history of the eight isolates was that they were highly virulent to chickens. The Dembi 1 and Lemlem isolates caused high mortality in vaccinated chickens (Nasser, unpublished) and isolates Koka, Lab and Market, caused 100% mortality in local experimental chickens (Nasser, unpublished). The Welayeta strain also caused high mortality in unvaccinated local chickens around Maji (Aweke, personal communication). The isolates Dembi 2 and Kebele 2 were responsible for the 1984 and 1987 ND outbreaks at Dembi farm and had caused 37.4% and 65.8% mortalities in vaccinated and unvaccinated chickens, respectively. The Alemaya isolate finally was used as challenge virus in our feed-based vaccination trial and caused 100% mortality in unvaccinated control chickens. For comparison, the MDT ICPI and the IVPI values of some known ND virus strains are shown in Table 17.

Table 17 Examples of pathogenicity indices of strains of NDV.

Virus strain	Pathotype	MDT	ICPI	IVPI
Hitchner B1	lentogenic	120	0.2	0
LaSota	lentogenic	103	0.4	0
Muketswar	mesogenic	46	1.4	0
Roakin	mesogenic	68	1.45	0
G.B Texas	velogenic	55	1.75	2.7
Milano	velogenic	50	1.9	2.8
Alemaya		51.1	1.84	2.51
Welayeta		54.2	1.86	2.48
Kebele 2		55	1.73	2.33

MDT = Mean death time (in hours)
ICPI = Intracerebral pathogenicity index
IVPI = Intravenous pathogenicity index

The isolates groups Alemaya and Lab, Dembi 1 and Lemlem, and Welayeta, Dembi 2, Market and Kebele 2 showed similar MDT values, but none of the isolates had similar ICPI values and the Mabs test showed except Dembi 1 and Dembi 2 all differ from each other. Also the test result with Mabs indicated that the Ethiopian ND outbreaks were caused by different strains of NDV. The Alemaya isolate seems to be identical to the German NDV isolates from the 70ies and Dembi 1 and Dembi 2 seem to be identical to German NDV isolates from the 1993/94, but none of the isolates resemble the pigeon type PMV-1 (Table 16).

PART 6 RETROSPECTIVE STUDIES OF NEWCASTLE DISEASE OUTBREAKS IN STATE POULTRY FARMS IN ETHIOPIA

6.1 Introduction

The poultry sector provides relatively cheap animal protein to the human population. Hence, efforts to promote it have received attention in most countries. In Ethiopia, in order to improve the genetic make-up of indigenous breeds, upgrading work has been undertaken since 1950 (Alemu, 1985). Modern commercial poultry production began most probably between 1960-1970 (Habte , 1987). At present, the poultry industry is represented by two main sectors, namely the private sector and the Government sector.

The governmental Poultry Development Enterprise was established to render services to the residents of Addis Ababa, and to the main cities in the country to convert cereal grains and byproducts into eggs and poultry meat. However, the enterprise did not achieved its targets, because it depended on imports for breeds, premixes, drugs, poultry equipment, and there was also a lack of quality feed and market organization and, above all, recurrent diseases that could not be controlled. Among the epidemic diseases, Newcastle disease appears to be most important and re-occurring every year. Although vaccination against ND, using HB1, LaSota and sometimes an inactivated vaccine is practiced, outbreaks of ND were reported almost every year.

The objectives of this retrospective study were:

1. to asses the importance and describe the occurrence of Newcastle disease in terms of season, age, strains of affected birds, vaccine types used, vaccination frequency and concurrent diseases.
2. to assess the control methods used.

6.2 Materials and Methods

6.2.1 Study farms

6.2.1.1. Dembi poultry farm

Dembi Poultry farm was established in 1983 by a loan obtained from the Agricultural and Industrial Development Bank of Ethiopia. The farm is a complex comprising of

a) a breeding unit consisting of broiler and layer parent stock, recently changed to a rearing unit.
b) a rearing unit consisting of 9 houses entirely used for rearing of commercial layer replacements for Dembi and Shola farms.
c) a commercial layer unit consisting of 8 battery houses. This unit produces only table eggs in caged houses.
d) a hatchery this unit produces day-old layer and broiler chicks.

Capacities:

Rearing = 17,000 x 2 cycles = 234,000 pullets

Layers = 18720 x 2 blocks = 37,440
18240 x 6 blocks = 109,440
Total = 146,880 layers

Hatchery = 1.7 million chicks per year.

No physical separation exists between units. The total area is 26 acres.

6.2.1.2 Shola poultry farm

The Shola poultry farm was established in 1966 under the Animal Production Division within the former Ministry of Agriculture. The objectives of the farm were then to train interested farmers in improved methods of poultry keeping and production and to distribute improved breeds to peasants and urban families. In addition, it was intended to serve as a trial station for observing adaptability, disease resistance and production efficiency of foreign breeds under Ethiopian conditions.

Shola poultry farm specializes in table egg production, it consists of 4 houses with a capacity of 13680 layers/house, thus a total of 54720 layers.

6.2.1.3 Lemlem poultry farm

Lemlem poultry farm was established by two former Alemaya College graduates Ethiopian nationality before the revolution, then was nationalized in 1977. The expansion of this farm

took place in 1983. It is specialized in broiler production and the capacity is more than 400,000 broilers per annum using 12 poultry houses. The area of the farm is 7 acres. Additionally, the farm was also used for rearing of parent stock. A manual slaughter house is located on one of the sides of the premises.

6.2.1.4 Location of the farms

Shola farm is located about 10 km from Addis Ababa city, along the Dessie road. Lemlem and Dembi farms are located in the outskirts of Debre Zeit and both of them are situated 42-43 km from Addis Ababa.

6.2.1.5 Climate

The poultry farms are located at elevations ranging from 1932 to 2408 meters above sea level with an annual rainfall ranging from 850.7 to 1185.8 mm. The mean annual temperatures of the three farms with their minima and mean maxima are summarized below.
The mean minimum and maximum temperature for Debre Zeit and Addis Ababa 11.1, 11.4°C and 23, 26.2°C, respectively.

6.3 Management Conditions

6.3.1 Breeds, breeding and source of chicks

The birds maintained to date at the state poultry farms include egg types such as Yarkon, Starcross or Shaver 288, Shaver 585, Isa Brown, Babcock, Bovans white and brown. The meat types include Starbro, Anake, Isa vedette, Avian Farms, and Hubbard the above birds were imported from Israel, England, Holland, and Egypt.

Some years ago the Poultry Development Enterprise used to keep egg and meat type parent stock at one of the three farms but mainly at Dembi farm. However, due to repeated outbreaks of Salmonellosis, the maintenance of parent stock was stopped and egg-type commercial layers as well as eggs for hatching were imported from the above mentioned countries. Broiler breeders are maintained mainly at Lemlem poultry farm.

6.3.2 Feeding

Poultry maintained on the farms were fed concentrated feed, containing mainly maize, oil seed meals, meat meal, bone meal and various milling by-products.

These mixed feeds, which were produced by the former Poultry Development and Feed Processing Enterprise and/or by private feed processing plants, usually were not correctly formulated due to discontinuities in supplies and variations in composition of ingredients, shortages of aminoacids, vitamins, minerals and premixes. Feed were permanently deficient in energy and protein in relation to the nutritional requirements of the types of poultry concerned.

6.3.3 Keeping system and hygiene

Except for Dembi Poultry farm, the two other farms are constructed near the main road. All farms lack proper isolation from the surrounding villages. Dogs and other wild animals enter the farms freely. Also, there is no physical isolation between units. Dead birds are disposed in pits located within the farms.

The three farms do not practice an all-in-all-out system. Multi-age groups have been managed in one farm. Different age groups and male and females were sometimes managed in the same house.

Layer replacements are reared on the floor. Density varied from 11 to 16 chicken/m^2. Brooding is done for 30 days and feed is given by automatic feeders. Commercial layers are kept in batteries. Eggs are collected by hand, feeding and watering is done automatically. Broilers are entirely kept on the floor, feeding is done by automatic feeders, brooding is done for 30 days.

Managers and supervisors frequently visit the farms and farm unit without changing boots and overalls between units. Overalls and boots are only supplied twice a year. The attendants freely move from one site to the other without restriction. Many of the workers have local chickens at their homes.

6.3.4 Vaccination (Preventive measures)

Even though many different poultry diseases have been recorded on the farms, the major causes of economic losses are Newcastle disease, nutritional deficiency diseases, coccidiosis, and salmonellosis.

Preventive measures against Newcastle disease, fowl pox and Marek's disease by vaccination are carried out.

With regard to the ND vaccination program, layers are vaccinated 5 times during the rearing period and every 3 months during the laying period. Recently, an inactivated ND vaccine was occasionally used for layers, parent stock and also for broilers.

Vaccination program for ND

- at day old	HB_1
- at 21 days	LaSota
- at 35 days	LaSota
- at 60 days	LaSota
- at point of lay	LaSota

Thereafter every three months

Application of Vaccines

For day old chicks the vaccine is delivered via the ocular or via the drinking water route after depriving the birds of water overnight. The other vaccinations are always done via the drinking water, except for the inactivated vaccine, which is given subcutaneously.

6.4 Study Materials and Methods

6.4.1 Characterization of the data

Daily mortality records, monthly, quarterly and yearly reports, individual flock records and laboratory diagnostic results were used in this study.

The records and reports provide tentative diagnoses made at the farm level by the farm veterinarian and also contain laboratory results. Whenever a disease like Newcastle disease is suspected, it is always obligatory to send specimens to the laboratory for confirmation. Only when the disease was confirmed, it also was put in the records.

The data were grouped for individual farms, i.e. Dembi, Lemlem, and Shola, for type and age of birds, season or month of the outbreak, type of vaccine used, number of vaccinations given, concurrent diseases, and other management failures.

6.5 Analysis of the Data

The data were analyzed after calculations of crude and specific mortality rates. Associations between particular rates were calculated as follows (Thrusfield, 1995).

$$\text{Attack rate (A.R)} = \frac{\text{Total no. of animals that develop disease during a specified time period following exposure}}{\text{Total number of animals exposed}} \times 10^2$$

$$\text{Crude mortality rate} = \frac{\text{Total death in a time period}}{\text{Average population at risk in time period}} \times 10^2$$

$$\text{Age specific mortality rate} = \frac{\text{No of deaths among animals in specified age group}}{\text{Average no. in specified age group}} \times 10^2$$

$$\text{Cause-specific mortality rate} = \frac{\text{No. of deaths from a specific cause}}{\text{Average population at risk}} \times 10^2$$

6.6 Temporal Pattern of the Outbreak

The temporal pattern of disease event is represented by an epidemic curve for each outbreak. The curves illustrate the magnitudes of the problem and the rapidity with which the epidemics progressed. The frequencies of new cases were plotted on the ordinate (y-axis) over a time scale on the abscissa (x-axis) for each outbreak.

6.7 Spatial Distribution

The distribution of the disease or the spread of the disease within one outbreak has plotted graphically for the farm, indicating the flock size of the birds affected and the distribution of the disease with time.

6.8 Results.

From 1983 to 1995, nine Newcastle disease outbreaks had occurred in the three farms of interest (Dembi, Lemlem and Shola). Years of outbreaks, affected farms, flocks and affected flock size, mortalities, functional and affected houses during the outbreaks are shown in Table 18.

Table 18. Demographic data of Newcastle Disease outbreaks at Dembi, Shola and lemlem poultry farms in Ethiopia between 1983 to 1995, showing affected flock size, mortality, year and month of the outbreaks.

Year of outbreak	Month of outbreak	Affected farm	Functional houses during the outbreak	Affected houses absolute and (%)	Flock size during the outbreak	Size of affected flock and (%) of flock involved	Mortality absolute and (%)	95% C.I * for mortality
1983	Novembe r	shola	3	3 (100)	38,165	38,156 (100)	2,370 (6.2)	0.24
1984	March	dembi	12	2 (16.7)	91,181	40,454 (44.4)	26,615 (65.8)	0.46
1987	Decembe r	dembi	11	1 (9.1)	120,000	15,376 (12.8)	5,759 (37.4)	0.76
1988	May	dembi	10	1 (10)	130,000	14,422 (11.1)	281 (1.9)	0.23
1989	July	dembi	14	11 (78.6)	197,000	133,450 (67.7)	12,057 (9.03)	0.15
1991	March	dembi	12	10 (83.3)	122,535	101,079 (83)	4,651 (4.6)	0.13
1992	January	dembi	8	4 (50)	90,309	30,289 (33.5)	18,384 (60.7)	0.55
1994	April	dembi	13	10 (76.9)	150,213	124,190 (82.7)	12,275 (9.9)	0.17
1995	June	lemlem & shola	17	17 (100)	124,296	124,296 (100)	10,037 (8.1)	0.15
Total					1,063,699	621,721 (58.5)	92,429 (14.9)	

*C.I = confidence value

Out of 1,063,699 chickens, 621,721 (58%) were affected and among these, 92,429 (14.9%) died during the outbreaks. From 100 functional houses, 58 were affected. The attack rate varied from 11.1 to 100%, while mortality rates ranged from 1.98 to 65.8%. If individual houses are considered, the mortality approached 100 % during the 1992 outbreak, when chickens were concurrently severely affected by avitaminosis and coccidiosis. From the nine outbreaks, seven, two and one outbreak were recorded at Dembi, Shola and Lemlem poultry farms, respectively.

459,260, 74,870 and 87,591 chickens were affected in Dembi, Shola and Lemlem farms, respectively, during the outbreaks. 80,022, (17.4%) , 2,598 (3.47 %) and 9,809 (11.2 %) died in the above respective farms. The highest rate of mortality was recorded during the 1984 outbreak (65.8%). In this outbreak, 78.4 % of unvaccinated and 39.1% of vaccinated birds died. The lowest mortality rate was in 1988 when only 281 (1.98%) birds of the affected flock died and the disease did not spread to other houses. Age-specific mortality in Table 19, and Fig 4 shows the mortality rates with respect to the ages of the egg-type chickens during the outbreaks .

Table 19. Age-specific mortality rates for broilers and egg type birds during the 1983 to 1995 ND outbreaks in state poultry farms in Ethiopia.

Type of chickens (age)	Production type of chickens	Affected flock size	Mortality (no. birds)	Mortality (%)	95% C.I for mortality
Chicks (1-60 days)	egg type	154,598	61,765	40	0.24
Pullets (61-140)	egg type	162,768	13,951	8.6	0.14
Layers (> 140 days)	egg type	216,664	6,904	3.2	0.07
Starters (1-28 days)	meat type	21,356	2,479	11.6	0.43
Finishers (> 28 days)	meat type	61,378	7,213	11.8	0.26
Breeders	meat type	4,857	113	2.3	0.42

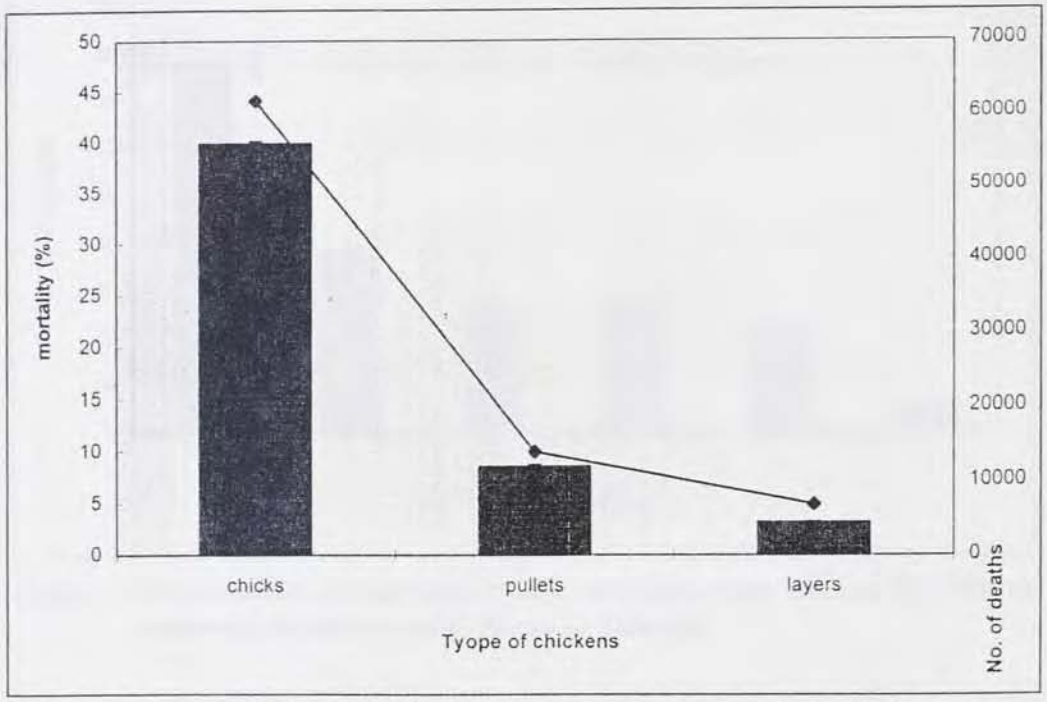

Figure 4. Age-specific mortality (%) during the 1983 to 1995 ND outbreaks in state poultry farms in Ethiopia.

Almost 40% of chicks, 8.6% of pullets, 3.2% of layers and above 11.6% of broilers died during the outbreaks. The highest rate of mortality was in chicks and the lowest was in breeder stock, where only 2.3% died. There was no difference in the mortality rate between starters and finishers. In egg-type chickens the mortality rate decreased with increasing age. Mortality was negatively correlated with age (r = -0.926). The comparison of frequency of vaccination *vs.* mortality is shown in Table 20 and Fig 5.

Table 20. Frequencies of vaccination vs. mortality rates during ND outbreaks in state poultry farms in Ethiopia between 1983 and 1995

Frequency of vaccination (no. vaccination)	Affected flock size	Mortality (%)	95% C.I for mortality
0	27,466	21,531 (78.4)	0.49
1	12,988	5,084 (39.1)	0.84
2	31,375	8,364 (26.7)	0.49
3	134,832	36,471 (27)	0.24
4	17,348	3,890 (22.4)	0.62
5	191,998	9,782 (5.1)	0.10

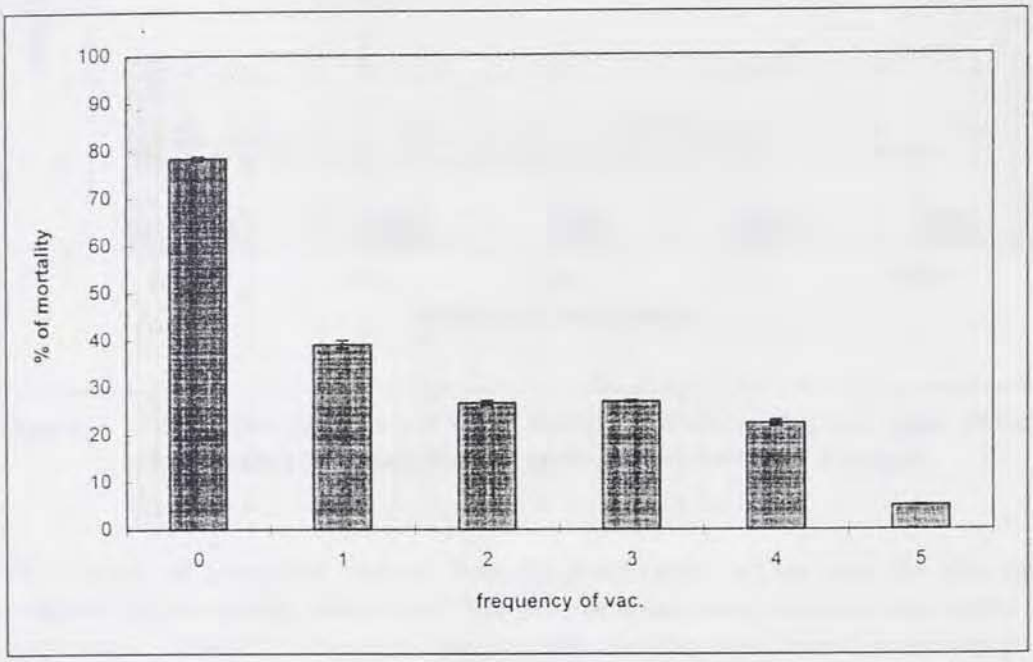

Figure 5. Frequencies of vaccinations and mortality rates during the 1983 to 1995 ND outbreaks in state poultry farms in Ethiopia.

The highest number of outbreaks had occurred in chickens which had received 3 vaccinations (14 outbreaks). The highest rate of mortality was encountered in unvaccinated chickens, where 78.4% of the animals of the affected flock died. The lowest mortality rate in contrast, was recorded in chickens which had received 5 vaccinations. Unvaccinated chickens and

chickens vaccinated only once fell into three age groups and vaccination was carried out very late. Generally, mortality rates decreased with increased vaccination frequency, (Pearson moment correlation r = 0.95). No differences in mortality rates between chickens which received two or three vaccinations were established.

The effect of live or inactivated vaccines on mortality and production is shown in Fig 6. Mortalities and losses in egg production (egg/hen/week) were higher in chickens which were vaccinated with live vaccines, and it took longer to re-assume previous production levels after outbreaks. The t-test used indicates a statistically significant difference ($p < 0.05$) for production loss between groups vaccinated with live and inactivated vaccine. The ages of birds of the two vaccine groups were very similar.

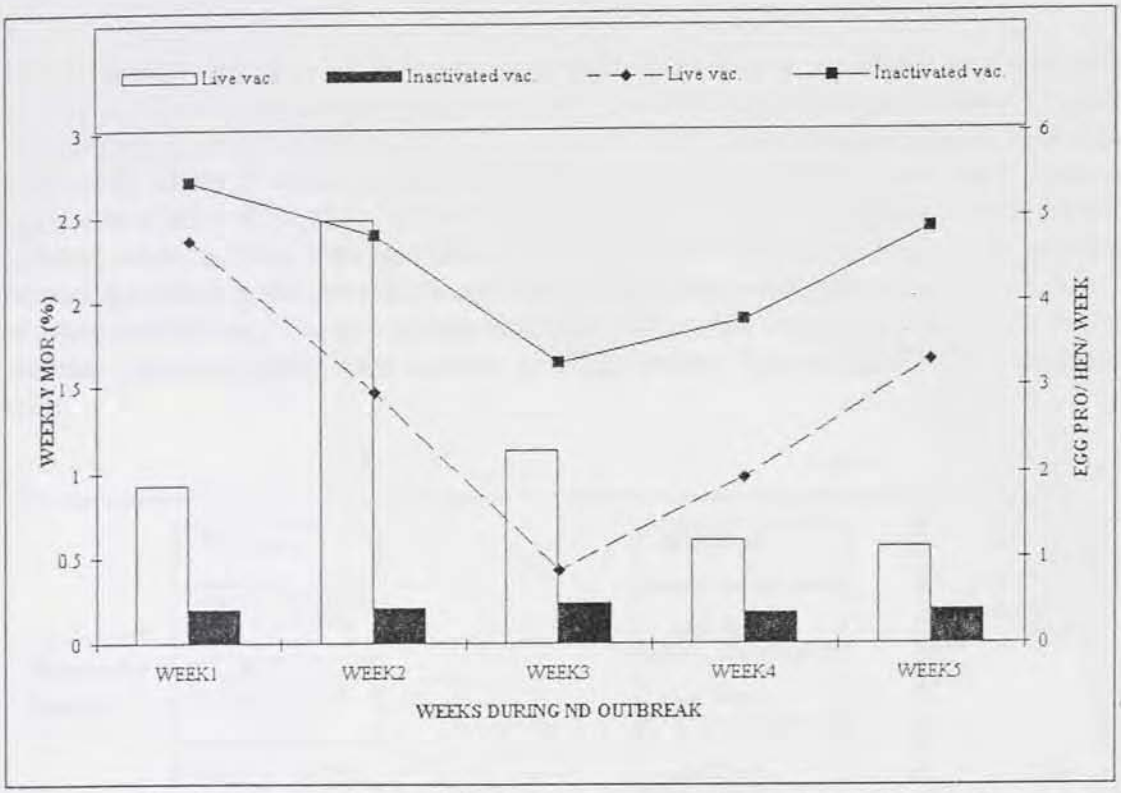

Figure 6. Production per hen per week, weekly mortality rates and types of vaccine used during ND outbreaks in state poultry farms in Ethiopia.

The strength of protection inferred from the inactivated vaccine over the live vaccine was compared by calculating Odds Ratio (Mantel-Haenszel) test for broilers and layers the results are shown in Table 21. The pooled analysis indicates that birds from live vaccine groups were 3.6 times more likely to die than birds from inactivated vaccine groups. In specific cases broilers and layers at Shola and layers at Dembi, receiving live vaccines, showed Odds Ratios (ORs) of 2.7, 10.6 and 4.3, when compared to groups which had received inactivated vaccine. Non vaccinated groups were 5.6 times more likely to die than birds from vaccinated groups. (ORs 5.6 and confidence limit = 5.39-5.90), regardless of the type of vaccine used in the latter groups.

Table 21. Mortality rate and Odds ratios for mortality of chickens vaccinated with live or inactivated vaccines during the 1983 to 1995 ND outbreaks in state poultry farms in Ethiopia.

	Live vaccine				Inactivated vaccine				Mantel-Haenszel	Odds ratio (C.I.)	Chi-square value
Type of chicken	total	dead (no. birds)	mortality (%)	C.I	total	dead	mortality (%)	C.I	pooled	3.64 (3.5-3.8)	72.3
Broilers	36,58	6,40	17.5	0.4	46576	3356	7.2	0.2	broilers	2.77 (2.6-2.9)	138.2
Layers Shola	38,65	2,370	6.2	0.2	36705	228	0.6	0.1	layers shola	10.6 (9.2-12.1)	94.7
Layers Dembi	58,44	2,798	4.8	0.2	69519	742	1.1	0.1	layers dembi	4.30 (4.3-5.1)	95.5

The influence of concurrent diseases on mortality during the outbreaks was analyzed by calculating Odds Ratio (Mantel-Haenszel test). Salmonellosis was more likely to increase mortality rates 2.62 fold (ORs 2.62, C.L = 2.186-2.342) while avitaminosis and coccidiosis were more likely to have increased mortality 9.28 times. These values were statistically significant (ORs = 9.29., C.L = 8.97-9.62). During 1983 and 1995, all functional houses were affected, while in 1984, 1987 and 1988 the outbreak was localized only in one or two houses without spreading to the other functional houses. The other outbreaks covered more than 50% of functional houses. The spread from one farm to the other was noted only during the 1995 outbreak spread occurred from Lemlem to Shola poultry farm, which is more than 50 km apart.

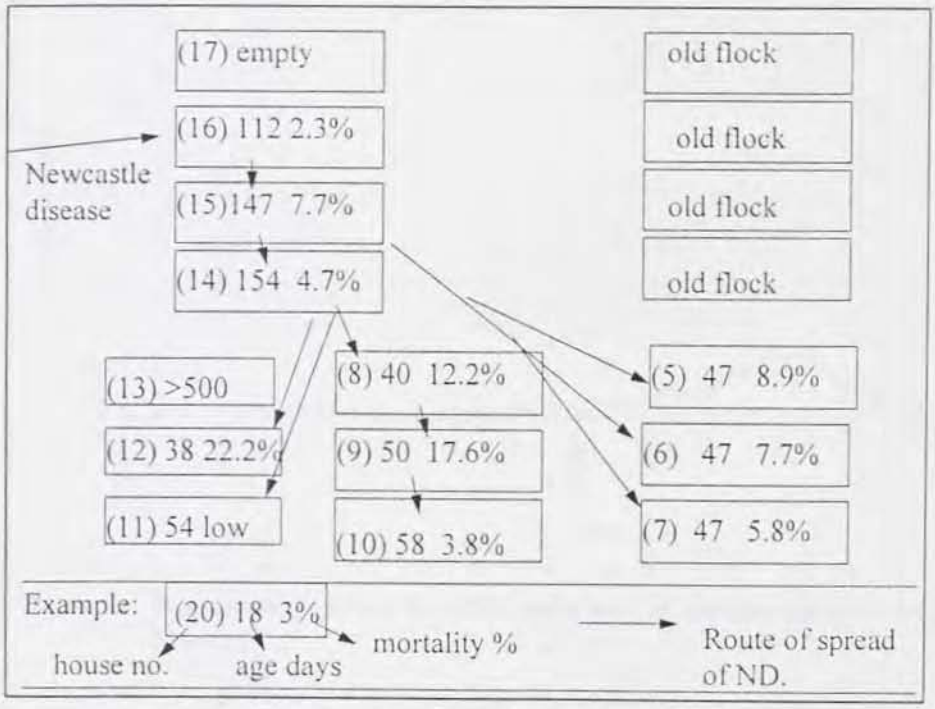

Figure 7. Route of spread of the ND outbreak at Dembi farm in 1989 in Ethiopia.

Fig 7 shows the route of spread of ND during the 1989 outbreak in Dembi farm. In this outbreak, 11 out of 14 functional houses were affected by the disease, younger birds were

highly affected, and their mortality rates was four times higher than those of older birds (Figs 8, 9 and 10).

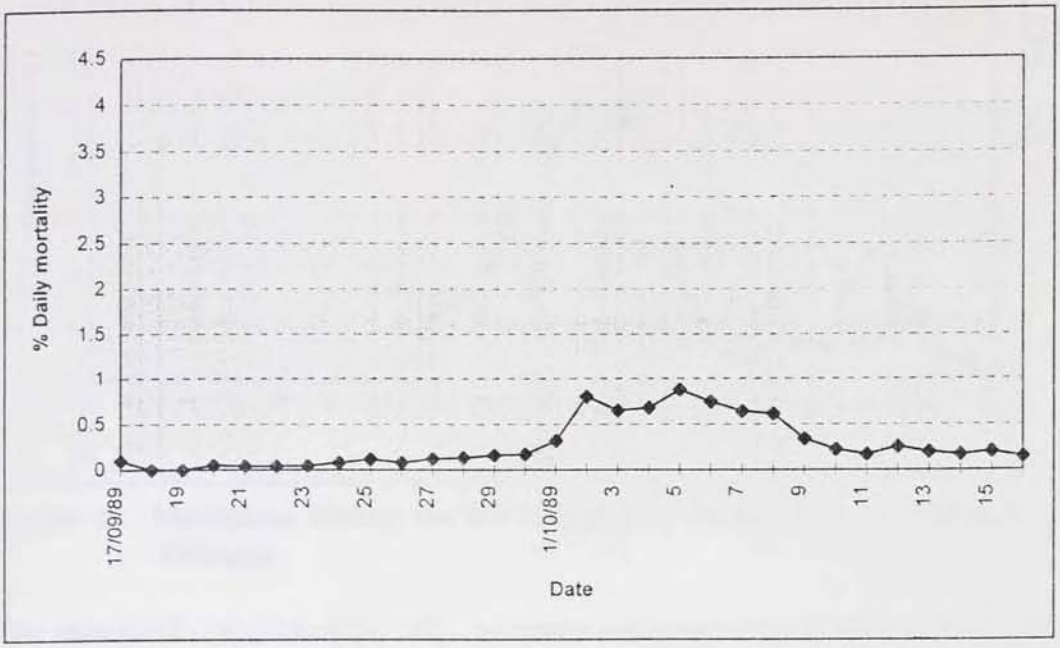

Figure 8. Mortalities during the ND outbreak at Dembi farm in 1989 in house 15 in Ethiopia.

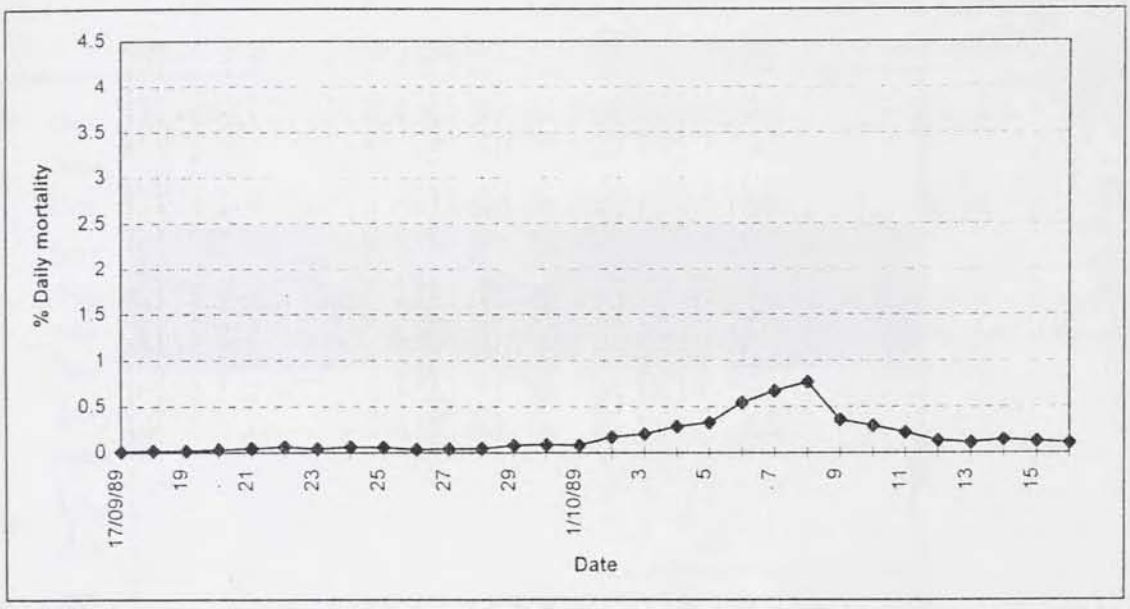

Figure 9. Mortalities during the ND outbreak at Dembi farm in house 14 in 1989 in Ethiopia.

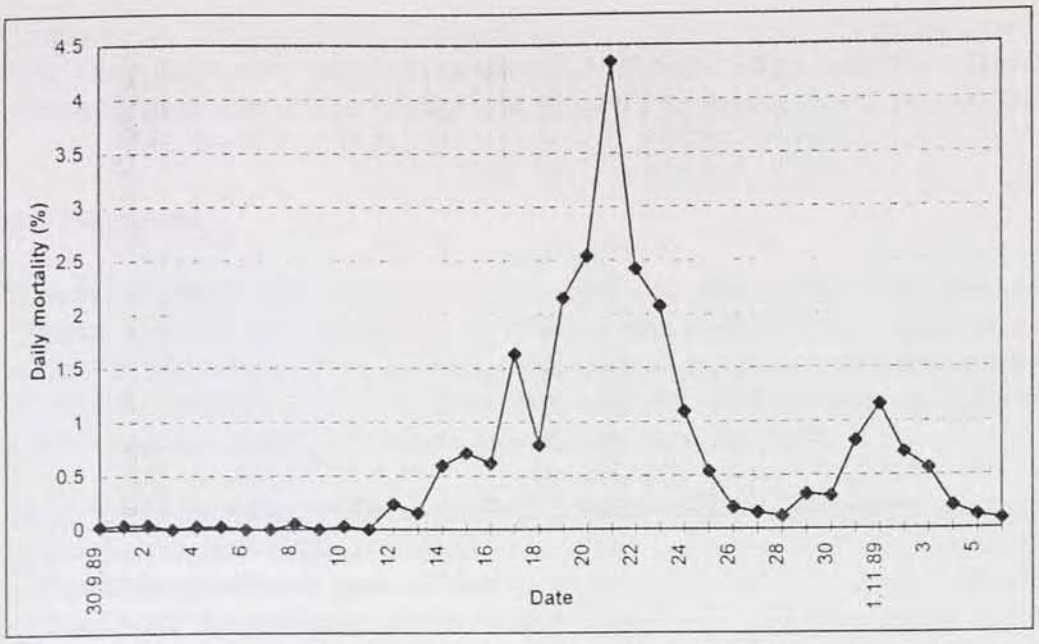

Figure 10. Mortalities during the ND outbreak at Dembi farm in 1989 in house 12 in Ethiopia.

The seasonal distribution of the NDV outbreaks is shown in Fig 11. Out of the nine outbreaks only two occurred in the same month (March), while the others occurred during different months. The 1995 and 1989 outbreaks persisted for 5 months at Dembi and Lemlem farms, the 1992 outbreak persisted for 3 months and the other outbreaks only for 2 months.

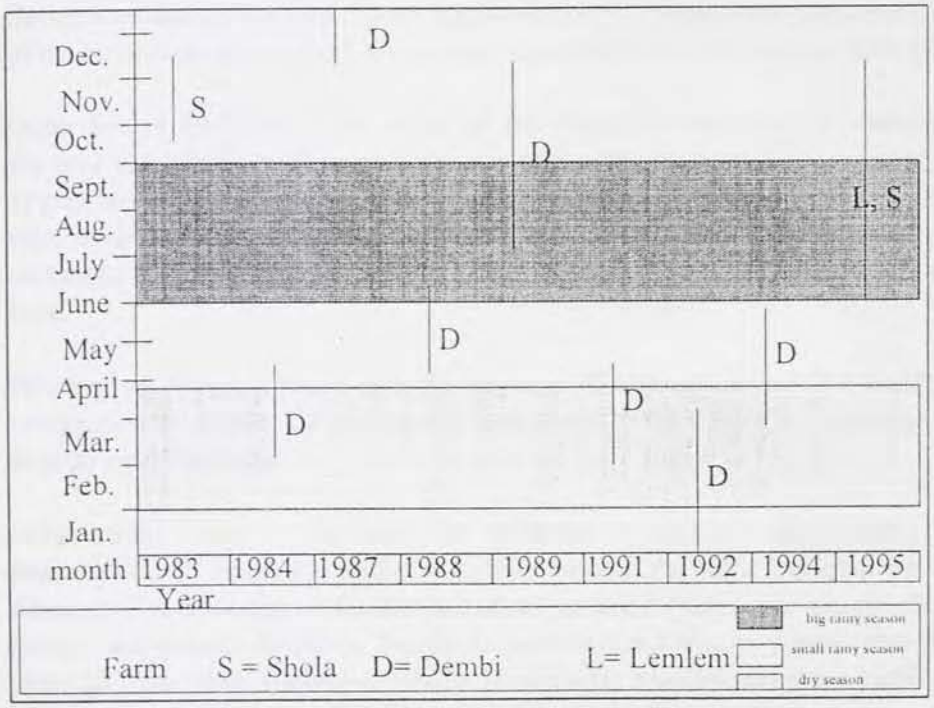

Figure 11. Persistency and months of ND outbreaks between 1983 to 1995 in state poultry farms in Ethiopia.

If seasons are considered two, three and four outbreaks respectively had occurred during the long rainy, short rainy and dry seasons, i.e. long rainy season lasts from June-September, short rainy season lasts from February-April and the dry season during the other months.

6.9 Discussion

Nine ND outbreaks had occurred between 1983 and 1995 in the three farms belonging to "Poultry Development Enterprise" in Ethiopia and caused 14.9% mortality during these outbreaks. The source of the outbreaks was difficult to identify. Management and hygienic conditions, nutrition, type of vaccines used and concurrent diseases all contributed to the introduction, spread and persistence of the disease and to mortality.

Multiple age-groups in the farms, minimal distances and lack of physical separation between different units, poor disposal of dead birds, absence of an all-in all-out system on the farms and maintaining different types of birds in the same farm created favorable conditions for the outbreaks and for persistence of the disease in the farms. A good example is the 1995 ND outbreak at Lemlem poultry farm. Regardless of the acute outbreak on the farm, new batches of day-old broilers were introduced. This extended the persistence of the disease on the farm over five months (Fig 11). The present study shows that it was always the young chicks which were highly affected by the disease (Table 19).

Lack of strict vigilance of the movement of staff, visitors' vehicles and objects in and between farms, repeated use of feed sacs without disinfecting or circling them between farms and other private farms facilitated the introduction of the disease into the farms. Without changing clothes or boots between houses a farm manager or supervisor made visits (after entering the diseased house) to all functional houses, carrying the disease from one house to the next. Sometimes attendants entered the poultry houses without changing overalls. More than 75% of the attendants also keep 2 or more unvaccinated local chickens at their homes.

Other factors facilitating the spread of the disease are absence of sanitary rooms for taking showers and for the change of clothes before entering the poultry houses, the free movement of pets and wild animals freely moving in and around the farms, and lack of protection against wild birds entering the chicken houses and gaining access to feed. Out of the seven ND outbreaks at Dembi farm, five were reported a few days after contract workers had entered the farm.

Shortage of overalls, boots and the absence of sanitary rooms for contract workers for the vaccination or transfer of chickens from one site to the other facilitated the introduction of the disease onto the farms.

Only healthy, vigorous and well fed chickens can respond satisfactorily to vaccination and attain sufficient protective antibodies. Nutritionally deficient chickens are more susceptible to diseases. The chickens on the farms had aminoacid-mineral-and vitamin-deficiencies, and low energy and protein levels in the feed were not uncommon. Apart from the poor feed, feed shortage was also common. These conditions resulted in poor antibody response after vaccination and to susceptibility to other diseases. The serological test results in most of the cases showed low antibody titres ($< \log 2^5$) (Nasser, unpublished).

This retrospective study revealed that mortality in those nutritionally deficient chickens was 9 times higher than in healthy ones. Considering individual outbreaks, in the 1992 outbreak, the pullets were highly affected by nutritional deficiencies and coccidiosis, the average mortality for the four houses was more than 60.7% (table 11), while individual house mortality approached 100% (even where the pullets were vaccinated for the third time).

Other diseases such as aflatoxicosis, salmonellosis, coccidiosis and mycoplasmosis were prevalent in the farms. These diseases, apart from their specific effects, could inhibit a good response to vaccination and render the birds more susceptible to other diseases, such as Newcastle disease. The study shows that chickens with concurrent diseases had a 2.6 to 9.3 times higher mortality than birds free of the disease. Hanson (1978) also pointed out that a reduction in the degree and duration of immunity is associated with intercurrent infectious diseases and internal parasites. Also Allan *et al.* (1978) pointed out that the variation in the immune response to vaccination may be due to faulty administration of the vaccine, the presence of intercurrrent diseases or varying to susceptibility.

In most cases live vaccines were used, first HB1 and later the LaSota strains, occasionally also an inactivated Newcastle disease vaccine. HB1 was given via the ocular or the drinking water route, while the LaSota strain was only given via the drinking water. Allan *et al.* (1978) stated that the drinking water method is the easiest way but gives the lowest results. Lancaster (1964) also emphasized that this method results in the greatest variation of immunity in chickens within the same flock some birds thus may be fully protected while others still remain susceptible to the disease. It is for this reason why the results of ND serology in this study are not only given as a mean titres but by showing the distribution of the titres in the flocks.

Different workers (e.g. Bennejean, 1981; Sagild and Haresnape, 1987) showed that live vaccines do not give 100% protection, especially not against a very pathogenic strain of NDV. Allan *et al.* (1978) emphasized that the efficacy of a live vaccine depends on its invasiveness and its power to multiply sufficiently within the chickens in order to provoke an adequate immune response.

According to Papparella (1973), Rossi and Di Giuseppe (1968), Cessi and Nardelli (1974), Edison *et al.* (1981) and Nonnewitz (1986) the oil-based vaccines seem to offer substantial advantages over the currently available live vaccines. In the present work, where broilers and layers were vaccinated with an inactivated vaccine and birds were well protected when compared to the group vaccinated with the live vaccines.

This retrospective study also indicates that mortality rates were negatively correlated with frequencies of vaccination and with age, unless the mortality was influenced by concurrent diseases and nutritional deficiencies. Booster vaccinations and increasing age decreased mortality.

In most East African countries ND in scavenging chickens flares up during the dry season when it is also very windy (Musiime, 1992). However, he also added that in other countries there was no indication of a seasonal pattern. Mukibi-Muka (1992) reported that in Uganda the seasonal occurrence of ND is a well-known phenomenon among rural farmers, occuring just before the dry season, when farmers start selling their chickens. In contrast to this reports the present study did not reveal any seasonality in disease occurrence. Only in the month of March two outbreaks of ND were recorded, while the other seven outbreaks occurred at

different months of the year, and the incidence during the dry and the rainy seasons was almost the same. Tadelle (1996) also confirmed that after the villigization program, ND remains a problem throughout a year, even though it is most serious at the beginning of the rainy season. (Mabratu, unpublished) also suggested that ND is more serious at the beginning of the rainy season than during the rest of the year.

PART 7. CONCLUSIONS

In Ethiopia, Newcastle disease is the most important cause of loss in village and commercial chickens. The present study showed no indications of any ND seasonality. The disease. rather occurs almost any time of the year, inflicting heavy losses. Velogenic strains of NDV are widely distributed throughout the country and strain characterization tests performed on field isolates from different parts of the country, in Ethiopia and Germany, confirmed the same.

The retrospective part of this study revealed that Newcastle disease mortality rates were strongly aggravated by concurrent diseases and nutritional deficiencies. Most outbreaks were associated with poor management and poor hygienic conditions. Inactivated Newcastle disease vaccines effectively decreased mortality and production losses. Effective ND control on the study farms requires attention to hygienic measures, maintenance of a high level of management, good nutrition, better disease control programs as well as appropriate vaccination programs in combination with monitoring of the HI antibody levels after vaccination.

Previous laboratory and field trials by many researchers had demonstrated, that the V4 vaccine was highly protective when given by conventional routes. Our trials under laboratory conditions confirm this. Without doubt, the conventional way of application can be applied also in village chickens. In Ethiopia, chickens can usually be caught. Untreated barley and sorghum proved to be unsuitable as vaccine carriers, eliciting neither a measurable antibody responses nor substantial immunity. Short boiling (parboiling) barley as a vaccine carrier gave good antibody response and protection, parboiling of sorghum gave negative results. However, further field studies with other types of feed as carriers for the thermostable NDV vaccine are required and the practicability of this method with parboiled feed under local conditions needs to be evaluated.

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PART 9: ANNEX

9.1 Preparation of PBS

Chemicals used for preparation PBS:

	NaCl	8 g
	K Cl	0.2g
	$K H_2 PO_4$	0.2g
	$Na_2 HPO_4 . 7H_2O$	2.16g
or	$Na H Po_4 . 2H_2O$	1.44g
	made up with distilled water	1000 ml.

This solution was sterilized by autoclaving at 121°C for 15 minutes. The liquid level was marked before autoclaving then stored at room temperature. If evaporation had occurred, it was made up to the mark with sterile deionized distilled water before use. The pH was adjusted to 7.2 . 7.4 at 20°C - 36.5°C with sterile 1 m. NaOH.

9.2 Preparation of Antibiotic Stock

Stock solutions. Penicillin 5x10 million IU concentrate solution
Streptomycin sulfate 5x1gm
PBSA = 100 ml

Aseptically 5ml PBS was added to each vial of sodium penicillin (5x10 million I.U) and Streptomycin sulfate (5g) and was left for a few minutes to dissolve. The PBSA was removed from the vials back to PBS bottle. Then another 5ml PBSA was added to the vials to rinse and returned back to the PBS bottle. 5ml was dispensed into a sterile container and stored at -20°C.

1ml of the solution was used for each 5 ml of solution in order to give the final antibiotic concentration of

Penicillin	100 units/ml
Streptomycin	100 mg/ml

Neomycin and Polymycin B stock solution

Neomycin sulfate	2500 mg
Polymyxin B sulfate	2500 mg
PBSA	100 ml

This was made by the same method as the penicillin and streptomycin stock solution. 1ml of this solution was used for each 500 ml solution, giving a final concentration of 50 mg/ml.

9.3. Preparation of Alsevers Solution

The solution was made by the following formula

d-Glucose (dextrose)	20.5g
Sodium citrate	8.0g
Sodium chloride	4.2g
Distilled water	1000 ml

The pH was adjusted to approximately 6.1 by addition of 0.55g of citric acid.

9.4 Calculation of End point (Spearman-Karber)

After performing titration and determining the number of embryonated eggs that responded to each dilution, the end point dilution was determined mathematically using Spearman-Kerber method as follows.

To calculate EID_{50} by this method I, used the following formula.

$$EID^{50} = \chi + 1/2\partial - \frac{\partial \Sigma \Gamma_1}{\eta}$$

Where
- χ = The highest dilution level tested
- α = The interval between successive logarithmic doses (dilution factor)
- $\Sigma\Gamma_1$ = The total number of uninfected embryos
- η = The number of hosts used at each dilution level (constant)

CURRICULUM VITAE

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Education background

DVM	1979-1985 Addis Ababa University Faculty of Veterinary Medicine, Debre Zeit, Ethiopia.
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High schools	1976-1979 Winget and Addis Ketema comprehensive high schools
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Language Proficiency

Amharic	Fluent written and spoken
English	Fluent written and spoken

Current Duties and Responsibilities

Head of Vet Dept. Poultry Development Enterprise since 1994.

Employer	Poultry Development Enterprise.
Main function	Monitoring the health and production activities of the farms.

Offering lecture and practical sessions on Avian diseases for the 5th year students Addis Ababa University, Faculty of Veterinary Medicine since 1991.

Consultancy service to poultry farmers.

Previous Post

Post	Head of Vet. Dept. of Dembi Poultry farm since 1985-1993.
Employer	Ministry of State Farms, Poultry Development Enterprise.
Main function	Monitoring the health and production activities of the Dembi Poultry Farm.

Other Experiences

Children Village	1987-1991
Post	Head of the Vet Service Section
Main function	Monitoring the health and management activities of poultry , Dairy, Beef, and Swine Farms.

Hamid and Family Center for Agriculture 1992

Post	Manager of the farm
Main function	Monitoring the health and management activities of beef, sheep fattening and also the dairy farm.

Publication

Published	Assisted and collaborated with research activities of Experimental Oil emulsion Newcastle disease vaccine in 1987 EMVT 1988. Collaborate author of Newcastle disease in Ethiopia. In: Rweyemamu *et al.* (ed.): Newcastle disease vaccines for rural Africa.
Unpublished	Survey of common ticks in Wolita Awraja Southern Ethiopia DVM thesis FVM/AAU, 1985. Newcastle disease at Dembi Poultry Farm. Salmonellosis in State Poultry Farms. Nutritional Deficiency Diseases in State Poultry farms.

Leisure time and activities Reading, cinema, indoorgames and music.

I the under signed, declare that the thesis is my original work and has not been presented for a degree in any University.

Name	Mohammed Nasser Hassen
Signature	
Date of submission	January, 7, 1998.

This thesis has been submitted for examination with our approval as University advisors.

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1998/MOH/1412 C-1

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TITLE ~~Oral newcastle disease~~ vaccination trials & studies of....

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1998
MOH/1412

Oral newcastle disease vaccination trials & studies of newcastle disease in Eth.

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