

CHAPTER ONE

INTRODUCTION

Newcastle disease (ND) is one of the most important viral diseases (Orsi, *et al.*, 2010). It is an acute infectious viral disease of domestic poultry and other species of birds regardless of variation in sex and age (Alexander, 2003). ND causes huge economic losses to the commercial poultry farmers round the world (Diel, *et al.*, 2012). Etiological agents of ND are virulent strains of avian paramyxovirus-1 (Choi *et al.*, 2010). The disease is characterized by respiratory, nervous system impairment, gastrointestinal and reproductive problems (Nanthakumar, *et al.*, 2000). Newcastle Disease virus (NDV) was first reported in the Sudan in 1951 (Anon, 1951). The ND virus was isolated and identified for the first time in the Sudan in 1962 from a natural outbreak (Sana *et al.*, 2004). The disease represents a permanent threat to both farm and industrial rearing of poultry (Vincent, 2009). Moreover, The major constraint to production of village chickens in many developing countries is Newcastle disease (ND) (Alexander, 1991). Throughout Africa these village chickens are the chief source of animal protein in rural area (Musiime, 1992). Most epidemics of ND in these countries result in great mortality rates from 75% to 100% in unvaccinated flocks and the disease has great socioeconomic impact in these rural communities (Martin, 1992).

In the Sudan the conventional production sector comprise about 30 million chickens from which the annual meat and egg production is 20.1 million birds and 900 million eggs, respectively (Suleiman,1996).The Major problem of the existing village production system in the Sudan is the high incidence of Newcastle disease, about 77% of surveyed households experienced disease problem. In a recent serological survey for ND in Sinar State of central Sudan (in village chickens with no history of previous vaccination and showed no clinical signs) the result showed that 92% of the tested birds had antibodies against ND virus (Selma and Ballal, 2013).

Vaccination is the most important way to control NDV. The available heat-labile vaccines like La Sota, which are used in the commercial poultry branch, are very unpractical for use in village chickens. These vaccines are not available in small amounts, which would cause large expenses for the rural poultry keeper. Another major problem is that the vaccines need a 'cold chain': they have to remain cold from manufacturing process to administration. The bad infrastructure and lack of refrigerators make this impossible for the rural poultry keepers. To avoid the problem with the cold chain, much research is being done to design 'thermostable' vaccines. These vaccines could offer a solution to the problems. The first thermostable vaccine produced was the NDV4-HR vaccine. This was proven to be effective in Cameroon, Ghana, South Africa, Tanzania, Zambia and many countries in Southeast Asia (Alders and Spradbrow, 2001). The virus, strain I-2 was chosen from a group of recent Australian isolates of avirulent

Newcastle disease virus (NDV). Strain I₂ was thermostable, readily spread among chickens and produced an adequate serological response (Spradbrow, 1995). It is cheap, it does not require strict cold chain facilities, and easy to administer by farmers and has been used successfully in some African countries. (Khalafalla, *et al*, 2000).

The first attempt to produce (I -2) thermostable vaccine in Sudan was made by (Khalafalla, *et al.*, 2000).

Inactivated vaccine is more capable of eliciting an immune response in the face of existing maternal immunity (Marangons and Busani, 2006), It can be used in day-old chicks because the maternal antibodies do not affect the vaccine efficiency (Nichol, *et al.*, 2012). There is no subsequent spread of virus or adverse respiratory reactions, no virus multiplication takes place after administration, (OIE, 2012).

Objective In this study an experimental batch of I₂ inactivated thermostable ND vaccine will be produced and tested at the CVRL-Sudan, for safety and efficacy.

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical background

Newcastle disease is a contagious viral bird disease affecting many domestic and wild avian species; it is transmissible to humans (Nelson, 1952). It was first identified in Java, Indonesia, in 1926, and in 1927, in Newcastle-upon-Tyne, England (whence it got its name). However, it may have been prevalent as early as 1898, when a disease wiped out all the domestic fowl in northwest Scotland (Macpherson, 1956). Its effects are most notable in domestic poultry due to their high susceptibility and the potential for severe impacts of an epizootic on the poultry industries. It is endemic to many countries. No treatment for NDV exists, but the use of prophylactic vaccines and sanitary measures reduces the likelihood of outbreaks, (FAO, 2017).

2.2. Newcastle disease in Sudan

In the Sudan, NDV was first reported in Khartoum in 1951 (Anon, 1951). Since then the disease has been regularly mentioned in all reports of the Sudan veterinary services. Diagnosis was based on the picture of disease, but the virus was isolated and identified for the first time in 1962 (Karrar and Mustafa, 1964 and Eisa, 1979).

According to (Ballouh, *et al* 1983), twelve NDV isolates obtained during 1963-1979 in the Sudan were mesogenic (n=4) and

velogenic(n=8). During the year1984-1985, four virus isolates were found to be velogenic,(Haroun, *et al.*, 1992). In another study, six isolates were obtained from outbreaks in the country between1988 and 1991 and found to possess the characteristics of the viscerotropic velogenic strains of NDV [VVNDV], (Khallafalla, *et al.*, 1992), what suggested that the VVNDV was the most prevalent pathotype in the Sudan.

2.3. Economical impact:

In many developing countries, chickens are the livestock most commonly owned by rural families. Many of these families have scarce resources and many may be headed by women. Increasing the productivity of their chickens would make a significant contribution towards increasing their food security and their ability to have secure livelihoods. Village chickens provide meat and eggs, food for special festivals, offerings for traditional ceremonies, pest control and petty cash to, for instance, purchase medicines or pay school fees (Alders and Spradbrow, 2001). ND is the single greatest constraint on the production of village poultry (Alders and Spradbrow, 2001). ND can cause up to 100 percent mortality in susceptible populations during devastating outbreaks and sporadic losses throughout the year where the disease is endemic. In areas where ND is endemic, the disease is generally well recognized by farmers and it discourages them from investing time and money in improving the standard of their poultry husbandry (Spradbrow, 1996). In such areas, control of ND will result

in substantial increases in village chicken numbers (Alders and Spradbrow, 2001).

2.4. Causal agent

2.4.1. Description

NDV, or avian paramyxovirus type 1 (APMV-1), is classified in the genus Avulavirus of the subfamily Paramyxovirinae (Mayo, 2002), NDV viruses belong to one serotype and there are two classes (Czeglédi, 2006). The genome of class I viruses consists of 15- 198 nucleotides (nt) and the genome of class II viruses consists of 15 -186 or 15- 192 nt. (Czeglédi, 2006). The genome contains six open reading frames (ORF) which encode the nucleoprotein (NP), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the haemagglutinin-neuraminidase (HN) and the large protein (L). At least one additional, non-structural protein (V) and possibly a second one (W), are generated by RNA editing during P gene transcription (Steward, 1993) .ND virus (NDV) has been designated APMV-1(Alexander and Senne, 2008).

2.4.2. Classification of the causative agent

Newcastle disease (ND) is a member of the family Paramyxoviridae in the genus Avulavirus. NDV strains have been categorized into three main pathotypes:lentogenic, mesogenic and velogenic strains.

Lentogenic strains are avirulent and may cause mild or inapparent respiratory infection. Mesogenic strains are of intermediate virulence

and cause respiratory symptoms with low mortality, whereas velogenic strains are highly virulent and cause high mortality (Alexander, 1996).

2.5. Susceptibility to physical and chemical agents

The virus is inactivated by acidic pH ≤ 2 , and survives for longer periods of time at ambient temperature, especially in the feces (Nichole, *et al.*, Miller, 2012). The infectivity of ND virus and other avian paramyxoviruses may be destroyed by physical and chemical treatments such as heat, irradiation (including light and ultraviolet rays), oxidation processes, pH effects, and various chemical compounds. The rate at which infectivity is destroyed depends on the strain of virus, the length of time of exposure, the quantity of virus, the nature of the suspending medium and the interactions between treatments. No single treatment can guarantee destruction of all viruses but may result in low probability of infective virus remaining. (Alexander, 1997).

2.6. Biological properties

Several biological properties are associated with paramyxoviruses, which characterized the group such as:

2.6.1 Haemagglutination activity

All strains of Newcastle disease virus will agglutinate chicken red blood cells. This is the result of the haemagglutinin part of the haemagglutinin/neuraminidase viral protein binding to receptors on the

membrane of red blood cells. The linking together of the red blood cells by the viral particles results in clumping. This clumping is known as haemagglutination. Haemagglutination is visible macroscopically and is the basis of haemagglutination tests to detect the presence of viral particles. The test does not discriminate between viral particles that are infectious and particles that are degraded and no longer able to infect cells. Both can cause the agglutination of red blood cells (Nichol *et al.*, 2012).

2.6.2. Neuraminidase activity

The enzyme neuraminidase is also part of the HN molecule and present in all members of the rubulavirus genus Avulavirus of the subfamily Paramyxovirinae. An obvious consequence of the possession of this enzyme is the gradual elution of agglutinated RBCs it seems likely that, neuraminidase removes virus receptors from the host cell which prevents the reattachment of released virus particles and virus clumping (Alexander, 1997).

2.6.3 Cell fusion and Haemolysis

ND virus and other paramyxoviruses may cause haemolysis of RBCs or fusion of other cells by essentially the same mechanism. Attachment at the receptor site during replication is followed by fusion of the virus membrane with cell membrane, which may result in the fusion of two or more cells (Similar to syncytial formation that occurred when virus particles are budded from cells). The rigid membrane of the RBCs

usually results in lyses from the virus membrane fusion (Alexander *et al.*, 1997).

2.7. Thermostability

The thermostability of the HA activity of ND virus isolates varies and has been used as a characterization test. This property has been proven to be a useful tool in epizootiologic studies and a rapid method for distinguishing between some avirulent and virulent viruses. (Alexander, 1997).

2.8. Epidemiology

After the outbreaks of ND in the United Kingdom in the 1960s and early 1970s, the spread of the virus was reported by the British to be significantly by wind (Dawson, 1973). This was reinforced by comments by(McFerran jB , 1988) on the outbreaks in Northern Ireland. However, in other outbreaks in the 1970s, 1980s and 1990s, airborne spread of velogenic ND has been ascribed a low importance compared to the movement of birds, humans, equipment, vehicles and other fomites. The importance of these latter routes of transmission relates to the ready demonstration of ND transmission by faeces as opposed to relatively little experimental evidence for the spread of infection by aerosol (Alexander, 2000). Nonetheless, where poultry farms are concentrated in a region and climatic conditions are favorable, it is difficult to conclude that airborne spread will not play a role. The stability and persistence of ND virus in faeces are well

established. Transmission studies with Australian-origin ND viruses have demonstrated low transmissibility in the laboratory compared with exotic strains of ND viruses, suggesting that bird, human and fomite movements and windborne spread of contaminated chicken debris and litter from infected flocks are likely to be the major reasons for the spread of Peats Ridge family viruses and Australian-origin ND viruses.

2.8.1 Hosts

a- Many species of birds both domestic and wild

b- Chickens are highly susceptible to disease; turkeys do not tend to develop severe signs

c- Game birds (pheasants, partridges, quail and guinea fowl) and parrots (order Psittaciformes) vary in susceptibility; cockatiels are susceptible

d- Wild birds and waterfowl (order Anseriformes) may harbour virus subclinically; some isolates within certain genotypes have caused epizootics within these species

e- Young cormorants (*Phalacrocorax*spp.) have demonstrated disease associated with APMV-1

f- Disease has been recorded in ostriches (order Struthioniformes) and pigeons (order Columbiformes) are known to be susceptible

g- Raptors are usually resistant to ND; except reports of acute disease in bearded vulture (*Gypaetus barbatus*), white-tailed sea eagle

(*Haliaeetus albicilla*), a wild osprey (*Pandion haliaetus*) and some species of falcons

h- Other birds known to have been affected by NDV include: gulls (order Charadriiformes), owls (order Strigiformes), and pelicans (order Pelecaniformes).

i- Passerine birds (order Passeriformes) are variable in their susceptibility; some species show no signs of disease but excrete NDV while others may develop severe disease

j- Reports of deaths in crows and ravens (genus *Corvus*) have been recorded

k- Acute ND has been recorded in penguins (order Sphenisciformes)

l- The morbidity and mortality rates vary among species, and with the strain of virus

m- Humans may become infected; manifested by unilateral or bilateral reddening, excessive lachrymation, oedema of the eyelids, conjunctivitis and sub-conjunctival haemorrhage, (OIE, 2012).

2.8.2. Transmission

Dissemination of virulent ND virus between flocks has been attributed to the following (in descending importance):

a- movement of infected birds (including vaccinated birds);

B-movement of feedstuffs, personnel and equipment into and out of premises;

C-movement of infected poultry products and byproducts; and

d- faecal virus contamination of clothing/footwear, equipment, litter, manure and feed (Utterbuck, 1972; Alexander 1988, 1997, 2000). The movement of infected and contaminated live birds is the single most important means of spreading ND. Within a flock, the main method of transmission is by inhalation of virus-laden expired air or by ingestion of drinking water or feed contaminated with nasal secretions or faeces containing virus. Air sampling in hen houses during outbreaks showed high levels of virus in houses, detectable levels 64 metres away at night, and undetectable virus at 165 meters from infected flocks. (Hugh-Jones, et al 1973) .Transmission of ND virus from aquatic birds to nonaquatic birds has not been investigated; migratory birds are believed to have spread virulent ND virus infection in Europe in the 1990s (Alexander, 2000). Day-old chickens transported in contaminated carrier boxes caused significant spread of infection in California in 1972 (Utterbuck and Schwartz, 1973). Pigeons can spread ND virus by contaminating poultry feed. Cage and aviary birds could become infected by contact with infected pigeons.

2.8.3. Incubation period

The incubation period is usually 2–6 days in domestic fowls, but can be up to 15 days. It is generally shorter for younger birds, (Aus-Vet Plan, 2010).. The OIE defines the maximum incubation period as 21 days. During the incubation period, the virus replicates at the site of introduction. Virulent and mesogenic viruses are then discharged into the bloodstream where they replicate in the visceral organs. Another

release into the bloodstream, about two days after infection, coincides with the excretion of virus via the respiratory tract and in the faeces. Clinical signs occur 24 hours later.

2.9. Diagnosis

Diagnosis of avian Paramyxovirus infections have usually been done using serological methods or by virus isolation. In common with ND, antibodies to APMVs may be detected by HI tests using the relevant antigens and controls. ND virus can be isolated from tracheal or faecal swabs or tissue samples from infected birds by inoculation of eight to ten-day-old embryonated chicken eggs via the allantoic cavity. Confirmation of the virus as belonging to the APMV serotype can be performed by HI tests with specific antiserum (Alexander, 2000).

2.9.1 Clinical signs

In chickens, symptoms indicative of ND included prostration, ruffling of feathers, depression, leg and wing paralysis, or other neurologic signs along with high mortality reaching 100% in the fully susceptible flocks. Clinical symptoms in the field may not be a reliable measure of the virulence of virus. Laboratory diagnosis is necessary for confirmation and pathotyping of ND virus to rule out other diseases which may cause similar symptoms including highly pathogenic avian influenza virus (Nichol, *et al.*, 2012).

2.9.2. Serological Test:

2.9.2.1. Haemagglutination Inhibition test (HI)

The HI test is also performed in a microtiter plate. The OIE standard HI method employs a v- bottom microtiter plate in which serum test specimens are serially diluted in two fold dilution using phosphate buffered saline (PBS). A known quantity of NDV antigen usually 4 haemagglutinating units is added to each well and incubated to allow antigen-antibody binding. A 1% suspension of RBCs is added to each well and incubated again. Some laboratories employ a slightly different version of the HI test method. U-bottom microtiter plates are used instead of V-bottom plates. The NDV antigen is added to the plate, and serum is diluted directly in the antigen leaving out the need for PBs in the test wells. The RBCs are prepared in 0.5% suspension instead of 1% suspension used in the standard method. The serum HI titer for both methods is determined by taking the reciprocal of the highest dilution of test serum which is able to completely inhibit hemagglutination of the RBCs (Nichol, *et al.*, 2012).

2.9.2.2 Enzyme Linked Immunosorbent Assay

The ELISA consists of a microtiter plate that has NDV antigen attached to the bottom of each well. Serial dilution of the anti-ND virus antibody in the test serum can be used to determine the titer (Nichole, *et al.*, 2012). There are varieties of commercial ELISA kits available and these are based on different strategies for the detection of

ND virus antibodies, including indirect, sandwich and blocking or competitive ELISA using MAbs. Competitive ELISA may not recognize all strains of APMV-1 if they use MAb known for their specificity for a single epitopes (OIE, 2012).

2.9.3 Identification of the agent

Suspensions in an antibiotic solution prepared from tracheal or oropharyngeal and cloacal swabs (or faeces) obtained from live birds, or of faeces and pooled organ samples taken from dead birds, are inoculated into the allantoic cavity of 9–11-day-old embryonating fowl eggs. The eggs are incubated at 37°C for 4–7 days. The allantoic fluid of any egg containing dead or dying embryos, as they arise, and all eggs at the end of the incubation period are tested for haemagglutinating activity and/or by use of validated specific molecular methods. Any haemagglutinating agents should be tested for specific inhibition with a mono specific anti serum to APMV-1. APMV-1 may show some antigenic cross-relationship with some of the other avian paramyxovirus serotypes; particularly APMV-3 and APMV-7. The intracerebral pathogenicity index (ICPI) can be used to determine the virulence of any newly isolated APMV-1. Alternatively, virulence can also be evaluated using molecular techniques, i.e. reverse-transcription polymerase chain reaction and sequencing. (OIE, 2012).

2.10. Virus isolation

Virus can usually be isolated from tracheal/ oropharyngeal swabs, fecal or cloacal swabs from live birds, tissues collected from affected organs of dead birds. Intestinal tissue and trachea are the most likely organs to contain virus, but other organ demonstrating clinical signs could be used for virus isolation. Swabs are collected in viral transport media such as brain heart infusion (BHI) broth. Upon arrival in the laboratory, tissues are homogenized to a 20% weight /volume suspension in antibiotic media such as BHI broth. Swab media and tissue homogenates are centrifuged. Supernatant is then used to inoculate cultured system such as chicken embryo kidney (CEK) cells, chicken embryo fibroblast (CEF) cells, or specific-pathogen-free (SPF) embryonating chicken eggs. The SPF chicken egg is the most commonly used culture system. Inoculated eggs are examined daily for embryo mortality. The allantoic /amniotic fluid (AAF) is harvested from dead embryos on the same day they die to reduce hemolysis of RBC's within the eggs. At the end of the incubation period live embryos are chilled at 4°C to kill the embryo and the AAF is harvested. Presence of live virus in the AAF is determined by the haemagglutination (HA) test,(OIE, 2012).

2.11. Molecular Diagnosis

2.11.1. Polymerase chain reaction(PCR)

Rapid diagnostic tests such as RT-PCR and sequencing to determine pathogenicity greatly reduced the time required for implementing control measures. Molecular diagnostic assays have come a long way from conventional polymerase chain reaction (PCR) followed by gel electrophoresis for amplicon analysis to the current methods of real-time reverse transcription (rRT) PCR assays that provide quick amplification making them essential diagnostic tools for viral detection (Nichol, *et al.*, 2012).

2.11.2. Sequencing

Nucleic acid sequencing is used in the diagnostic laboratory to analyze the virulence potential of APMV-1 isolates. Sequencing techniques originated with (Sanger, *et al.*1977) using the dideoxynucleotide triphosphate (ddNTP) mediated chain termination, and(Maxam, and Gilbert.,1977) using the chemical degradation methods .Sequencing techniques have been rapidly improved and next-generation of automated sequencing techniques has become standard for laboratory analysis of gene sequences (Nichol, *et al.*, 2012).

2.12. Immunity

2.12.1. Innate and passive immunity

Different strains of chickens vary in their response to ND infection. Younger birds develop clinical signs more quickly and are more severely affected, although chicks from immune hens may be protected by antibody derived from the yolk (Arzey and Pearce, 2001).

2.12.2. Active immunity

Cell-mediated immunity can be demonstrated two days after infection. All ND virus strains cause an antibody response in chickens and other avian species. However, titres in cage and aviary birds following natural infection with lentogenic strains are not known. Serum antibody can be detected in chickens 6–10 days after infection. Titres peak after 3–4 weeks and decline to undetectable levels in 8–12 months. Neutralising antibody protects chickens, chicken embryos and cell cultures from infection. Birds resistant to infection have high levels of circulating antibody. Low levels of antibody may not prevent infection but can protect chickens from severe disease and mortality. It has been demonstrated that vaccinated birds without detectable antibody may survive challenge with virulent virus. This may be due to low levels of humoral antibody, interference between vaccine and challenge virus competing for cell attachment sites, cell-mediated immunity, and/or local immunity,(Arzey and Pearce, 2001).

2.13. Control of ND

2.13.1. Biosecurity and hygiene

Biosecurity aimed at preventing disease should begin at the planning stage of commercial poultry farms. Farms and flocks should be well separated; hatcheries should be isolated from poultry farms, and there should be an adequate fresh water supply (Alexander, 2000). The greatest aid in ND control might be the extension and education of farmers and workers.

2.13.2 Vaccination

Vaccination is the most successful tool for prevention of ND. Non usage of ND vaccine in rural areas is one of the factors for outbreak of ND. For the prevention of ND in chicks, the birds should be vaccinated against ND. The vaccine against the local strain of ND virus prevalent in the respective areas may be used for vaccination against ND. The improper vaccination may result in the outbreak of ND (Khan, *et al.*, 2000, Mustafa and Ali, 2005).

2.13.2.1 Inactivated vaccines

Where inactivated vaccines have been used extensively in commercial farms. Although inactivated vaccine gives good protection, it is relatively expensive to produce. It also carries a slight risk to the user of accidental self injection (Alexander, *et al.*, 2004). Oil emulsion inactivated vaccines can be used in day-old chicks because the

maternal antibodies do not affect the vaccine efficiency (Nichol, *et al.*, 2012).

2.13.2.2. Live Vaccines

Live virus vaccines are usually lyophilized allantoic fluid produced by infecting embryonated chicken eggs. The majority of live ND vaccines are derived from a symptomatic enteric or lentogenic strains, although some vaccines were derived from mesogenic strains (Alexander, *et al.*, 2004). To provide the best protection, vaccine programs have adopted method of progressive vaccinations which involves successive booster vaccines with increasingly virulent strains. Another method entails combining inactivated and live virus vaccines that lead to stimulation of the cell-mediated, innate, and humoral immune responses to improve protection.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Pre-Clinical Stage

A new batch of Newcastle disease vaccine (I-2) strain was reproduced using chicken embryonated eggs derived from a healthy flocks vaccinated against the major poultry disease.

3.1.1. Source of the I-2 master seed virus

The virus was originally isolated in Australia with funding from the Australian Centre for International Agricultural Research (ACIAR) (Alders *et al*, 2005) the master seed I-2 virus was first supplied by the Department of Veterinary Pathology of the University of Queensland, Australia, which then kindly handed over by the Department of Veterinary Virology of the Veterinary Research Institute.

3.2. Inoculation of vaccine strain

Three vials of each lyophilized I₂ vaccine strain were obtained from their working seed vaccine lots and diluted in sterile normal saline and inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs previously disinfected by wiping the inoculation site using 70% alcohol. The inoculated eggs were sealed using melting paraffin wax, and then incubated at 37c° for 120 hours. Dead embryos within 24 hours were discarded. (Alexander DJ, 2004).

3.2.1. Harvesting

At the end of the incubation period the infected eggs was chilled at 4C° over night before being harvested. The tops of the eggs was removed, and the allantoic amniotic fluid (AAF) was aspirated using 10ml pipette after the depressing the embryos aside. The invasion of the yolk and albumin was avoided, and then the harvested AAF was centrifuged using cold centrifuge at 1000 RPM for 7 minutes, before the vaccine to be pooled and stored at 4 C°.

3.2. 2 Test of virus content using Haemagglutination

Assay (HA)

The HA test was used to determine the titer of Newcastle virus. Briefly, samples (25 µl) were serially diluted 2-fold in PBS in a U-bottom micro titer plate. An extra 25 µl of PBS was added to all wells. Each well was mixed with 50 µl of 1% chicken red blood cells and incubated at room temperature for 30 minutes. The HA titer was expressed as the reciprocal of the highest virus dilution exhibiting complete haemagglutination (OIE, 2012).

3.2.3. Strain of vaccine production

For phase III clinical trial, The vaccine was prepared by using a virulent ND virus of I₂ strain, concentration of 10^{9.1} EID₅₀ /ml was used as stock virus for the inactivated vaccine formulation i.e. a high

amount of antigen was used .The virus concentration was identified by HA test (OIE, 2016).

3.2.4. Inactivation of the viruses

The vaccines strain of I₂ was inactivated by treatment with 0.05% laboratory grade formaldehyde, this was according to (Wanasawaeng, et al., 2009) and for the product escalation larger amount of 1000 ml AF of I₂ strain was inactivated by adding 500ul of the concentrated formaldehyde. and then the mix were shaken well, and incubated at 37 C° for 16 hours ,after incubation the bottles were stored at 4 C°.

3.2.5. Test for complete inactivation

The test has been performed on the formaldehyde treated allantoic fluid, after the incubation for inactivation has been completed, the allantoic fluids of the strain of I-2 and was inoculated into 10-day-old embryonated chickens eggs and incubated at 37C° for 120 h. (Palya.1991).

3.2.6. Interference by maternal antibodies

Oil-emulsion inactivated vaccines have been successfully used in day-old chicks with maternal immunity in the prevention of ND (Alexander and Jones, 2001). The major advantages of those inactivated vaccines are the very low level of adverse reactions in vaccinated birds and extremely high levels of protective antibodies of long duration that can be achieved (Alexander and Jones, 2003).

Moreover, these inactivated oil-emulsion vaccines are not as adversely affected by maternal immunity as live vaccines (Box et al., 1976) because the oil adjuvant acts as stimulus of defense mechanism and disperse antigen slowly. In these circumstances, there is a progressive stimulation of the active immunity while the passive immunity declines and the immune system reaches full competence (Bennejean et al., 1978; Box et al., 1976; Warden et al., 1975.)

3.2.7. Correlation between HI antibodies titer and protection

Humoral immunity plays an essential role in the protection against NDV infection. Chickens with high antibody titers are usually protected. For example, young chicks with high maternal antibody titers are protected against a challenge with a virulent strain during the first few days (Umino Y, *et al* .1987). Recently, higher and specific levels of antibodies were not only related with protection against mortality, but also with reduction of viral replication and secretion. (Miller PJ, *et al*, 2013). Hence, measuring the neutralizing antibodies (nAbs) against NDV is highly essential to evaluate the efficacy of a vaccine.

3.3. Formulation of the water (W/O) in oil emulsion

vaccines

For phase III clinical trials about 2 liters of W/O emulsion was prepared from a pharmaceutical grade white mineral (paraffin oil) as follow: the aqueous phase was made up by adding 40 ml of tween 80 (add 10 ml each time while stirring) to the 960 ml allantoinic fluid then was gently stirred for 30minutes for a proper mixing. Equal volume of the oil phase was prepared by adding 100 ml manidmonoleate (span 80) (also add 10 ml each time) to the 900 ml of the purified paraffin oil, then was gently mixed for 45 minutes using magnetic stirrer. Using the same procedure (100 ml each time) 1000 ml aqueous phase was added drop wise to the 1000 ml oil phase i.e. 1:1 ratio and then was thoroughly mixed by stirring for 20minutes then using the rotary machine method product was emulsified by using machine of Silverson type with head suitable for larger volumes, this process lasted for 20 minutes then a very homogenous product was obtained (Palya, 1991).

3.3.1 Randomized-controlled trial for the prepared inactivated Newcastle disease vaccines in day-old-broiler chicks

3.3.2. Study design

This is phase III single blinded randomized controlled trial which was conducted in 120 healthy day-old commercial broiler chicks purchased

from Arab Poultry Breeders CO (OMMAT). at the Central Veterinary Research Laboratory, Department of ViralVaccine Production. One hundred twenty day old broiler chicks were randomly assigned to three groups of 40 as a safety, 40 as efficacy, and 40 non inoculated control named group S, E, and C respectively.

Using this preparation of I₂ inactivated vaccine the S group received 0.4ml subcutaneously in the nap of the neck, group E received 0.2ml using similar route as group S (field dose) , and chicks in group C was kept as un-inoculated control.

3.3.3. Safety

Occurrence of local or systematic adverse events or tissue reaction was observed for 21 days post inoculation in Chicks inoculated with double recommended dose for any local or general adverse events, this test was done according to the OIE terrestrial manual (OIE, 2016).

3.3.4. Efficacy

Chicks were bled 4 times, on day-old to evaluate the maternal antibodies level, and after 14 days, 21 days and 30 days post vaccination to evaluate the vaccines derived antibodies. Accordingly the antibodies titers was measured on day, 1, 14, 21, and 30 days post vaccinations i.e. the humoral immunity derived by the inactivated vaccine was assessed along the broiler chickens life span. Chicks were inoculated with recommended dose of 0.2 ml then the seroconversion

levels were evaluated at ND risk time of 21, and 30 days post vaccination using objective measurements of HI tests (OIE, 2016).

3.3.5. Haemagglutination inhibition test

Haemagglutination inhibition (HI) test was performed on the serum samples using the protocol recommended by (OIE, 2012). The representative samples were counter tested using live Newcastle vaccine I₂ strain.

Test procedure :

The sera were tested for the presence of antibodies against ND as described by (OIE, 2012), using 4 haemagglutination (HA) units of virus. Serial 2-fold dilutions of sera (1:2 to 1:2048) were prepared with phosphate- buffered saline (0.01 M, pH 7.2) in micro plates with U-bottoms. Phosphate- buffered saline (25 µl) was dispensed in all wells. An equal amount of serum was added to the first well of a row of 12 and was titrated. The last well was left as a control. The antigen was diluted to give a dose of 4 HA units. To each serum dilution 25 µl of 4HA units of virus were added. All plates were incubated at room temperature for 30 min. To all wells 50 µl of 1% chicken erythrocytes (RBCs) was added and all the plates were incubated at room temperature for 30 min. The results were read as reciprocals of the highest dilution of serum that completely inhibited haemagglutination.

3.4. Statistical analysis

The obtained results were subjected to statistical analysis using the computer program (SPSS 20) software, comparison between control group and efficacy group was performed by independent sample T test. In all analysis *P* value less than 0.05 were considered as statistically significant.

CHAPTER FOUR

RESULTS

4.1. Production of the I₂ inactivated thermostable Newcastle disease vaccine

Preliminary and final product sterility test showed no bacterial and fungal growth in thioglycolcate broth media.

4.2. Confirmation of virus inactivation

The formalin treated virus was confirmed to be completely inactivated by absence of HA evidences in the tested allantoic fluid. The positive, and negative HA reactions for positive and negative control groups were recorded.

4.3. Vaccine safety test

There were neither deaths in vaccinated chickens, nor any ND clinical signs or any other intercurrent infection. The vaccine was safe.

4.4. Vaccine efficacy test:

The mean HI Abs titers for group E after 14 , 21 and 30 days post vaccination were 62.9 , 59.2 , and 35.7 HIU respectively, the mean Abs titers for control group after 14 , 21 ,and 30 days were 25.2 , 5.6 and 5.4 HIU respectively, while the maternal immunity level was

605.8 HIU. The results showed that Levels of HI Abs were high in vaccinated group even at 30th day and decreased in unvaccinated chickens at the end of the trial.

This study has shown that the locally produced I₂ ND vaccine induces high antibody levels in vaccinated chickens.

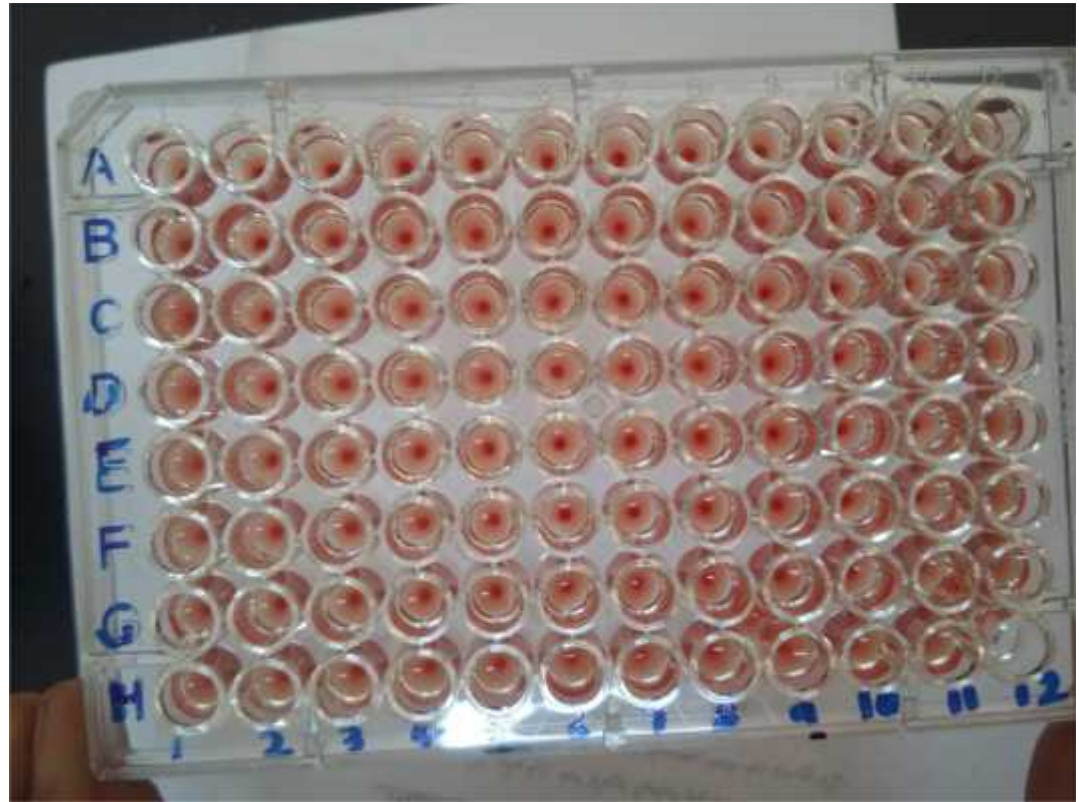


Figure 1: show Haemagglutination Inhibition (HI) test of sera from vaccinated group using 4 (HAU) , The row A show the result of control RBCs , the rows (B - H) show the result of the tested sera.



Figure 2: show Haemagglutination (HA) test of the I₂ virus working seed as antigen in (HI) test using 4 (HAU) , the rows (A.B)on the top show the result of the tested I-2 working seed bank (WSB).the HI titer(6 log₂) HIU, while the row(C) is the control RBCs result of the test .

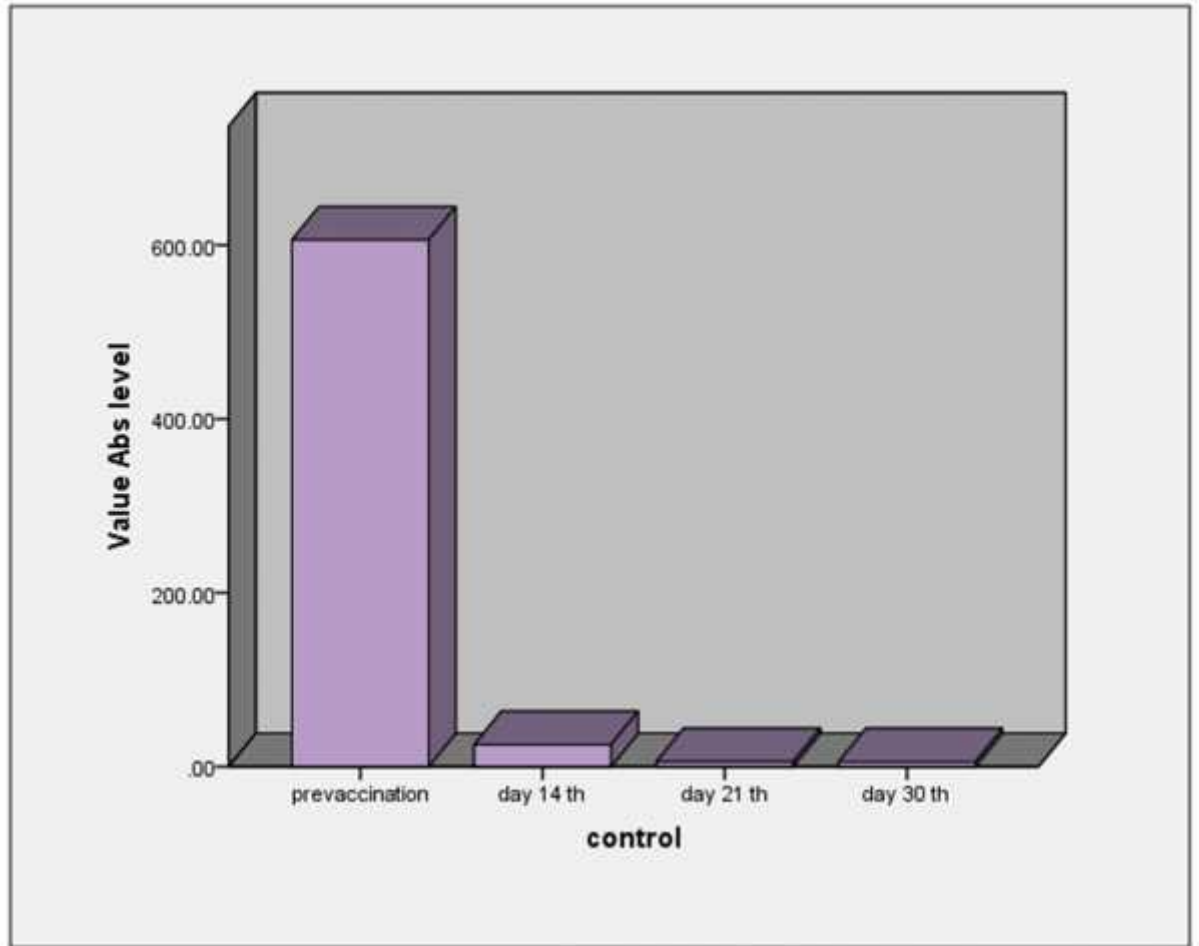


Figure 3: show the levels of maternal Abs in unvaccinated group at different times.

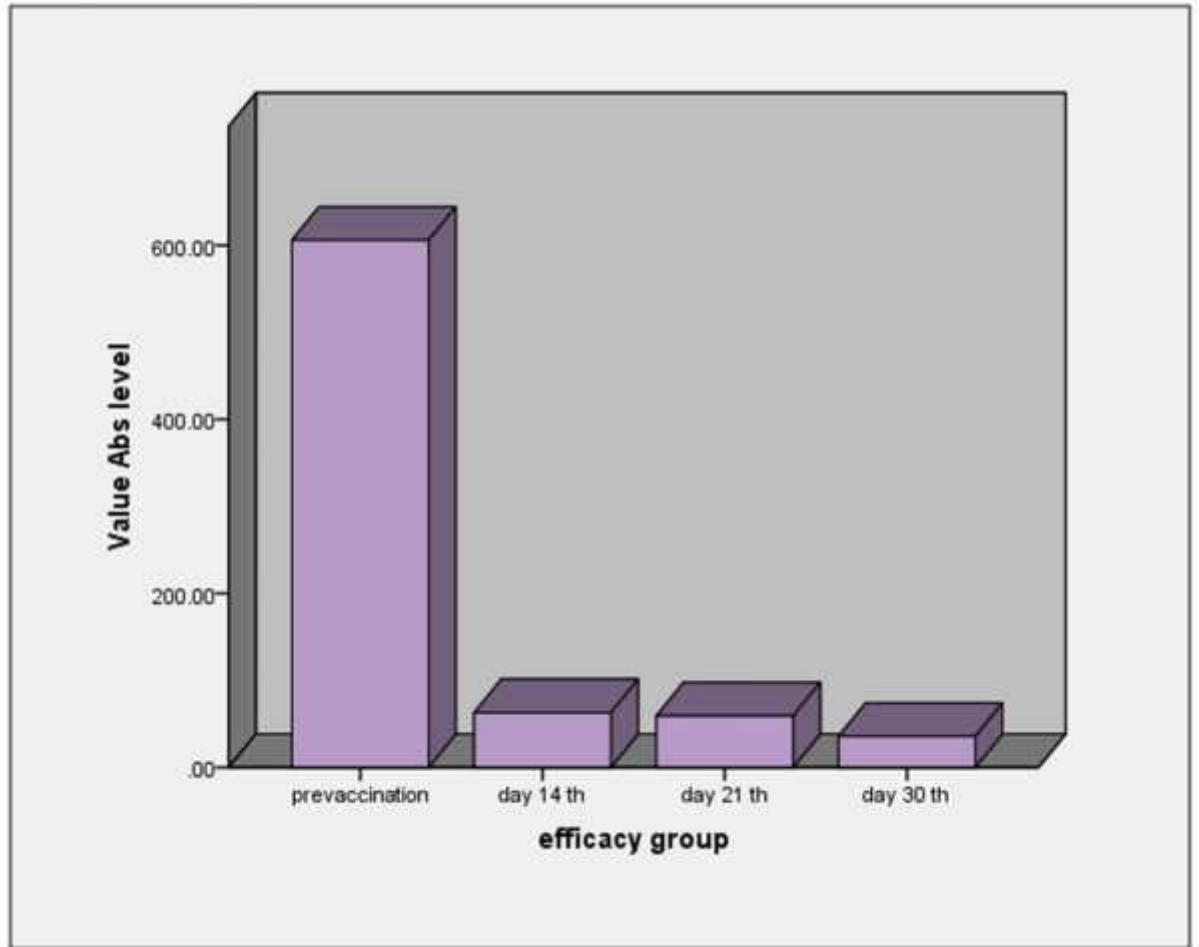


Figure 4: show the Abs level of the pre vaccination and post vaccination sera against one field dose of inactivated I₂ ND vaccine as measured by (HI) test .

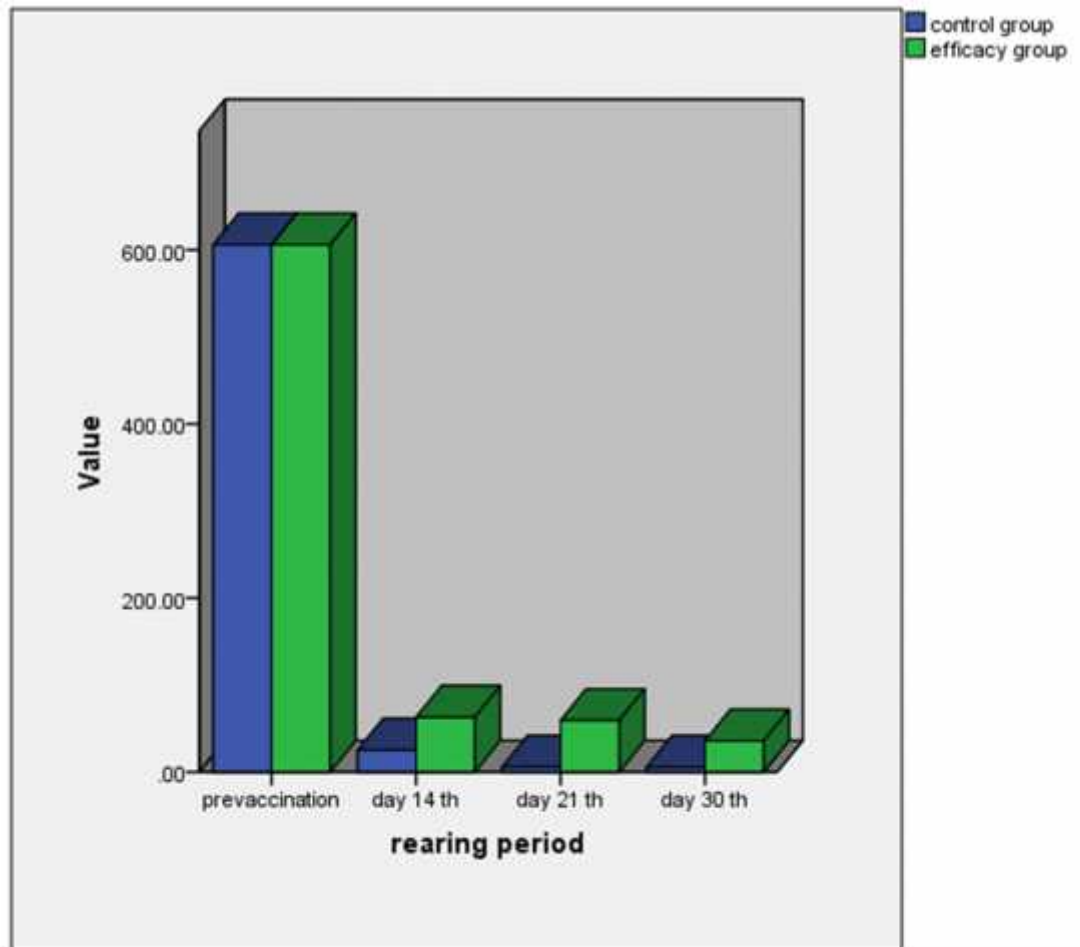


Figure 5: show the Abs level in unvaccinated and vaccinated group at the same times.

Table (1): Descriptive statistics results of independent sample *t*-test for Abs level between unvaccinated and vaccinated group used inactivated I₂ND vaccine .

Immunity	Mean	SD	Sig
Control After 14 days	25.2 ± 23.2		**
Vaccinated after 14 days	62.9 ± 31.11		
Control after 21 days	5.6 ± 3.56		**
Vaccinated after 21 days	59.2 ± 27		
Control After 30 days	5.4 ± 3.4		**
Vaccinated after 30 days	35.7 ± 24.9		

** means highly significant

CHAPTER FIVE

DISCUSSION

Vaccines are biological products that are sensitive to excessive heat, cold or light. Many vaccines lose potency if stored above 22–25 °C (Nayda, *et al.* 2001) or below 0 °C (Hedenström and Kahler, 1992). The degree of loss of potency is specific to the type of the antigen (virus or bacterium, live or inactivated), and depends also on the solvent, the types of preservatives, stabilizers and protectants used, and whether it is lyophilized or in liquid form (Hedenström and Kahler, 1992). In general, the ideal temperature range for storage and transport of vaccine is 2–8 °C (Allan, *et al.* 1978). During the preparation for vaccination campaigns, the slogan should be ‘Maintain the vaccine refrigerated and maintain the vaccine’ (Nawathe, 1988). Many factors can contribute to poor cold-chain maintenance: inappropriate refrigerators for vaccine conservation, poor monitoring, over filling the refrigerator, deficiencies in electrical supply and problems with the capacity and knowledge of the personnel (Allan, *et al.* 1978; Hunter, 1997; Nayda, *et al.* 2001; Young, *et al.* 2002). Factors that can affect the transport of vaccine in insulated containers include the number, distribution and the physical state of the ice packs, the volume and properties of the insulated container, the number of times it is opened and the duration of opening, and the ambient temperature (Young, *et al.* 2002).). Under these conditions, heat-tolerant vaccines are recommended, such as liquid or lyophilized I₂ ND vaccine (Young, *et*

al. 2002), Inactivated oil emulsion ND vaccines are less heat sensitive than the conventional live ND vaccines, making their transport to villages more feasible (Bell, 2001). These vaccines must not be frozen. Prior to use, the vaccine must be slowly brought to room temperature and shaken well to ensure that the emulsion is fluid and the contents are evenly distributed. Although inactivated vaccine gives good protection (the standard re-vaccination interval is six months), it is relatively expensive to produce (Alexander, 2000; Bell, 2001). Quality control of inactivated vaccine is often difficult, and mineral oils may cause serious problems to the vaccinator if accidentally injected (Alexander, 1997). Adverse reactions to inactivated vaccine post-vaccination are rare (Alexander, 1997). ND I₂ vaccine was produced in embryonated chicken eggs. Observation throughout the period of safety test proved that the experimental batch of the vaccine is safe, there were no clinical signs attributed to the vaccine or any other viral infection. This strongly fosters the freedom of the vaccine from any contaminant or pathogenic microorganisms, and indicates that the experimental batch complied with the main quality control tests. When paraffin oil adjuvant was used to stimulate better ND immune-responses; the results obtained confirmed that the vaccinated birds were apparently healthy, no allergic reactions or clinical signs attributed to either ND virus or any other avian pathogens, indicating that the locally formulated vaccines were safe and sterile.

The study shows conclusively that vaccination using locally produced I₂ ND vaccine can benefit the chicken industry in Sudan.

The value of serology in diagnosis is clearly related to the expected immune status of the affected birds. HI titers may be regarded as being positive if there is inhibition at a serum dilution of 1/16 (2^4 or $4 \log_2$ when expressed as the reciprocal) or more against 4 HAU of antigen, (OIE. 2012).

High levels of humoral immune response were detected when liquid paraffin oil was used as an adjuvant with the ND I₂ inactivated vaccine. This result is consistent with nature of the inactivated vaccines which produce better antibody derived immunity than cellular immunity. (Murphy, *et al*, 1999).

The results obtained from this trial confirm that inactivated vaccine in oil emulsion evokes very high antibody levels ($5 \log_2$ in day 30th), and that agree with (Young, *et al*, 2002) who stated that, titers greater than $3 \log_2$ are considered protective.

In this clinical trial there was a dramatic decrease in the maternal Abs of the control group. This indicated that the passive immunity always waned over time while the active immunity increased at the same time in levels of the protective Abs along the broiler life span ($P < .05$).

Finally, the candidate vaccine complied with the main quality control tests of sterility, safety and efficacy.

CHAPTER SIX

CONCLUSION

The inactivated ND I₂ vaccine produced locally in this study has the potential to replace the imported ND oil vaccines.

The study shows conclusively that vaccination using locally produced inactivated I₂ ND vaccine can benefit the chicken industry in Sudan.

HI titer was significantly different, ($P < 0.05$), on 14th, 21th, 30th day of vaccinated compared to unvaccinated group. It indicates that inactivated vaccine was sufficient to induce high protective level even after 30 days post vaccination.

To produce inactivated ND vaccine, a much larger amount of antigen is required for immunization than for live virus vaccination, (OIE, 2012).

RECOMMENDATIONS

1-Education of local community about the importance of I₂ vaccine and vaccination because most of them remain less interested vaccination campaign.

2- Application of I₂ inactivated vaccine against the deadly VVNDV.

3- Insurance the I-2 vaccine availability and accessibility to all Sudanese poultry back yard chickens.

4- More studies of the effect of inactivated I₂ ND vaccine on the broiler chick's performance

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APPENDIX

1. Preparation of Reagents

Following reagents have been used during the production and testing of the 1-2 ND vaccines.

1.1. Normal saline (N.S.) "Buffer solution"

To prepare 1 liter of normal saline 8.5 gram of purified NaCl was dissolved into 1000 ml distilled deionizer water, then mixed well and autoclave at 121°C for 15 minutes, then, cooled and stored in the refrigerator.

1.2. 70% alcohol

Using 250 ml measuring cylinder, 140 ml of absolute ethanol was measured, and mixed with 60 ml of distilled water.

1.3. Preparation of 1% washed chicken red blood cells

Chicken used for the supply of blood were housed separately and at least not vaccinated with Newcastle disease vaccines.

A tiny amount of Ethylene diamine tetracetic acid "EDTA" has been placed in to the barrel of sterile 5 ml syringe. 4ml blood were collected from the wing vein, then immediately the blood gently shaken and the needle removed from the syringe, then gently the plunger pushed down to transfer the blood into the sterile disposable tube, and the lid was replaced and the tube rotated gently to be mixed.