



Evaluation of the Efficacy of Inactivated Oil-Emulsion Newcastle Disease Komarov Vaccine against Clinical Disease, Lesions and Immune Response, Following Challenge with Velogenic Newcastle Disease Virus in Laying Chickens

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SUMMARY

Since the first recognition of Newcastle disease (ND) in Nigeria, it has been observed to be enzootic despite the intensive vaccination policy, leading to significant economic losses in the poultry industry. This study evaluated the ability of inactivated oil-emulsion ND Komarov vaccine to protect laying chickens from challenge with a velogenic ND virus (VNDV). Two hundred and forty pullets were randomly divided into two groups of 120 each viz: vaccinated and unvaccinated groups. The vaccinated group was given ND vaccines. At peak production, 32-weeks of age, vaccinated and unvaccinated laying chickens were sub-divided into four groups of sixty birds each designated; Vaccinated and challenged (VAC); Vaccinated and unchallenged (VAU); Unvaccinated and challenged (UNC); and Unvaccinated and unchallenged (UNU). Groups VAC and UNC were each inoculated intramuscularly with 0.2ml of a VNDV with a median embryo infective dose (EID₅₀) of 10^{6.46} per ml. Groups VAU and UNU were each inoculated with 0.2ml of phosphate buffered saline. Group VAC showed no clinical signs, no clear lesions grossly and mild histopathologic changes. Group UNC showed severe depression, anorexia, whitish-greenish diarrhoea, nervous signs and necrosis of the organs. All infected groups (VAC and UNC) showed significantly higher ($P < 0.05$) sero-conversion determined by enzyme-linked immunosorbent assay (ELISA) 10 days post-challenge. The inactivated oil-emulsion ND Komarov vaccine not only induced higher immunity, but also conferred long-lasting protection against morbidity, mortality, and severe organ damage in VAC group. This immunization procedure can be recommended for prevention of ND in laying chickens in an endemic environment.

Key words: Newcastle disease, vaccine, laying chickens, immunity.

INTRODUCTION

Newcastle disease (ND) is a highly contagious viral disease of poultry that causes significant economic losses to the poultry industry worldwide since 1926 (Alexander, 2001). The causative agent, ND virus (NDV), is an avian paramyxovirus serotype 1 group, classified in the genus *Avulavirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, order *Mononegavirales* (Mayo, 2002; Lamb *et al.*, 2005). Strains of NDV can be classified into three pathotypes (lentogenic, mesogenic and velogenic) on the basis of the severity of disease they cause in chickens (Miller and Koch, 2013). Strains are defined as virulent if they (1) have three or more basic amino acids at position 113–116 of the un-cleaved fusion protein cleavage site (F0) with a phenylalanine at position 117, or (2) obtain a intracerebral pathogenicity index (ICPI) value of ≥ 0.7 in day-old chickens (*Gallus gallus*) (Alexander and Senne, 2008; OIE, 2012). Because of the severe economic consequences of an outbreak of velogenic NDV (VNDV) in commercial poultry, ND belongs on the List of Notifiable Diseases to the World Organisation for Animal Health (OIE), (OIE, 2013). Infection with VNDV produces severe diarrhoea, extensive haemorrhage and ulceration in the digestive tract, necrosis in the spleen and lymphoid tissues, and high morbidity and mortality in infected susceptible flocks (Okoye *et al.*, 2000; Sa'idu *et al.*, 2006; Igwe *et al.*, 2014). ND is also associated with neurological signs and a severe drop in the egg productivity of laying hens (Bwala *et al.*, 2012; Igwe, 2015; Silva *et al.*, 2016). Chickens that survive infection with virulent Newcastle disease virus develop a long lasting immunity to further infection with Newcastle disease virus (FAO, 2002). Reducing losses of large numbers of laying flocks to virulent Newcastle disease is an essential first step to improving their productivity and decreasing economic losses

at harvest. Newcastle disease is mostly controlled by the use of vaccines in Nigeria (Adu *et al.*, 1990). The goal of current vaccination procedures is to induce protective immunity while producing a minimal antagonistic response / immune responses without causing severe disease in the bird (FAO, 2002). The traditional vaccination programme in Nigeria typically involves the use of 3 types of live ND vaccines, and the regime of four successive vaccinations (Hichner B1 at day old intraocularly, ND Lasota (orally) at day 21, at 6 and 16 weeks ND Komarov (intramuscularly) (Adu *et al.*, 1990) designed to build sufficient immunity to withstand/control the endemic velogenic ND strains. Although the efficacy of currently available NDV vaccines against velogenic NDV is widely accepted (Okeke and Lamorde, 1988; Kapczynski and King, 2005), ND outbreaks in Nigeria have been frequent and widespread in both vaccinated and unvaccinated flocks (Adu *et al.*, 1990; Shoyinka, 1983). Furthermore, the characterization of NDV strains isolated from outbreaks in several regions in Nigeria (Solomon *et al.*, 2012; Shittu *et al.*, 2016) underscores the need for continued evaluation of NDV vaccines and vaccination programs to controlling spread of disease. In Nigeria farmers have been using combinations of live lentogenic vaccines and subsequent revaccination with imported oil-emulsion inactivated ND-Komarov vaccines following different schedules of vaccination. These combinations of live and inactivated oil-emulsion vaccines are assumed to be highly effective against ND although there is paucity of information on the immune performances of combination of these vaccines. The objectives of the present study were to extend the knowledge of protection by inactivated oil-emulsion NDV vaccine, against clinical disease, lesions and

determine the immune response, following

MATERIALS AND METHODS

This study was scrutinized and approved by the University Committee on Medical and Scientific Research Ethics, University of Nigeria, Nsukka.

Experimental Chickens

Two hundred and forty Isa-Brown pullets obtained from Zartech Farms, Nigeria were used for the study. They were randomly assigned into four groups of 60 each. The groupings and their treatments were Vaccinated against ND and challenged with VNDV group (VAC); Vaccinated against ND and unchallenged group (VAU); Unvaccinated and challenged with VNDV group (UNC); and Unvaccinated and unchallenged group (UNU). Brooding of all the pullets was done on deep litter. Each of the groups was brooded separately under the same environmental conditions at the animal facilities. Feed and water were provided *ad libitum*. General care of the birds was provided in accordance with the Institutional Animal Care and Use Committee, as outlined in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching and Quarantine Service*.

Velogenic NDV Inoculum

The virus used was the VNDV strain, duck/Nigeria/903/KUDU-113/1992 (Shittu *et al.*, 2016). The virus was isolated in Kuru, Plateau State of Nigeria from an apparently healthy duck and characterized biologically by Echeonwu *et al.* (1993). The inoculum had a median embryo infective dose (EID₅₀) of 10^{6.46} per ml.

Experimental Design

The experiment was designed to determine if current industry routine NDV vaccine strategies would protect against the challenge of VNDV to 32 weeks old laying chickens at the peak of egg production (that

challenge with VNDV in laying chickens. is, to extend the knowledge of protection by live, and inactivated oil-emulsion NDV vaccines, against clinical disease and lesions). Also to determine the immune response in laying chickens following challenged with VNDV.

Experiment I

Comparison of protective immunity to VNDV before and following challenge of vaccinated and unvaccinated laying chickens: Pullets in groups VAC and VAU were given ND vaccines intraocularly at day old- Hitchner B1, four weeks ND Lasota vaccine orally in drinking water, and at nine and sixteen weeks of age, Komarov, an oil-emulsion inactivated vaccine, intramuscularly (IM) according to National Veterinary Research Institute (NVRI), Vom, Nigeria and Biovac®, Israel respectively. Each bird in group VAC and VAU was inoculated intramuscularly (IM) with 0.2 ml of the inoculum at the beginning of the experiment (day 0). Unchallenged birds were each inoculated intramuscularly with 0.2 ml of phosphate buffered saline (PBS) (groups VAU & UNU) as placebo. The birds were observed twice daily for clinical signs of ND from day 0 through to day 21 PC. Three birds in each group were humanely euthanized and along with the mortalities examined for pathological changes on days 3, 5, 6, 7, 8, 10, 15 and 21 PC. In the challenged groups the euthanized birds were those showing clinical signs. The ovary, infundibulum, magnum, isthmus, uterus and vagina were carefully excised and fixed in Bouin's fluid for 12 hours and thereafter transferred to 70% alcohol. The samples of the spleen were fixed in 10% formalin for not less than 24 hours. The fixed tissues were trimmed, and processed to paraffin using routine procedures. Five µm sections were cut and used for haematoxylin and eosin (H&E) staining.

Experiment 2

Serological/Immune response in vaccinated and unvaccinated laying chickens before and following challenged with VNDV: At 32 weeks-of age, each bird in groups VAC and UNC was challenged by inoculating intramuscularly (IM) with 0.2 ml of the inoculum at the beginning of the experiment (day 0). Birds in group VAU and UNU were each inoculated intramuscularly with 0.2 ml of phosphate buffered saline (PBS) as placebo and kept as unchallenged controls. Blood samples were collected from ten (10) birds in each group on days 0, 10, 15 and 21 post challenge (PC). The separated serum was stored at -20°C until used for NDV enzyme-linked immunosorbent assay (ELISA) test. A commercial ELISA test kit (Flockcheck™ IDEXX Laboratories Inc., Westbrook, ME) was used to test serum for antibodies against NDV. Chicken serum samples were diluted 1:500 and incubated in 96-well microtiter plates containing NDV antigen. The ELISA was performed according to the manufacturers' recommendations. Duplicate titres were obtained and calculated using XCHEK software (IDEXX Laboratories Inc) at Virology Department, NVRI, Vom, Plateau State Nigeria. An optical density of 650 nm wavelength was used to detect the colour change using an Emax reader (Molecular Devices, Sunnyvale, CA).

Statistical Analysis

Data generated from immune responses were subjected to one way analysis of variance (ANOVA). Variant means were separated post hoc using the least significant difference (LSD) method (Okafor 1992). Probabilities less or each to 0.05 were accepted as significant.

RESULTS

Experiment 1

Protection of vaccinated commercial laying chickens against challenge, compared with unvaccinated infected group: No clinical signs of Newcastle disease were observed in any laying chicken prior to challenge. Protection from VNDV challenge was determined by absence of clinical signs during the 21 days PC observation period and protection from severe damage in the organs (Table 1). Laying chickens in the vaccinated challenged group had no clinical signs throughout the course of the study. Eighty two percent of the unvaccinated challenged group displayed severe depression, ruffled feathers, reduction in feed and water consumption at day 2 to 3 PC. At day 4 PC, morbidity was 100%, and all laying chickens in this group displayed whitish-greenish diarrhoea which soiled the vent. Mortality was first observed on day 5 PC. Peak mortality occurred on day 6 PC and involved 21 laying chickens. Some of the remaining laying chickens showed by days 6 to 9 PC, nervous signs such as head tremors, torticollis, paralysis of the legs and wings were evident in 6 hens by days 6 to 9 PC. Total mortality was 89.58% excluding the euthanized sacrificed layers (Table 1).

In the VAC laying chickens the muscles of the breast, thigh and legs were mildly congested at days 6 to 10 PC. At days 6 to 10 PC the caecal tonsils were mildly ulcerated, haemorrhagic and contained cheesy necrotic materials. The female reproductive tract showed no clear lesions grossly (Figures 1 and 2), however, there were mild histopathologic changes (Figure 3) throughout the experimental period. In the UNC laying chickens, gross lesions were congested breast, thigh and leg muscles by days 5 to 10 PC, petechiae haemorrhages at the tips of the proventricular glands and haemorrhagic band at oesophageal-proventricular junction at days 5 to 7 PC. Caecal tonsils showed severe swollen,

TABLE 1: Protection of vaccinated commercial laying chickens with inactivated oil-emulsion vaccines against challenge with virulent NDV, compared with the unvaccinated challenge group. Titers >396 are considered positive

Groups	ELISA antibody titer at day 0	Protection														
		Days post challenge														
		1	2	3	4	5	6	7	8	9	10	11	12-13	14-15	16-21	
VAC	1473 ± 187.05 ^a (n=10)	0 ^A /60 ^B		0/60	0/60	0/57	0/57	0/54	0/54	0/54	0/5	0/5	0/51	0/5	0/5	0/48
VAU	1484.2 ± 211.98 ^a (n=10)	0/60	0/60	0/60	0/57	0/57	0/54	0/54	0/54	0/5	0/5	0/51	0/5	0/5	0/48	
UNC	0 ± 0.00 ^b (n=10)	0/6	12/6	49/6	57/57	55/5	42/4	20/2	11/1	8/1	5/1	2/11	1/1	0/1	0/8	
UNU	0 ± 0.00 ^b (n=10)	0/6	0	0	0	7	4	3	6	4	4	0/51	1	1	0	
VAC	1473 ± 187.05 ^a (n=10)	0	0/60	0/57	0/57	0/54	0/54	0/54	0/54	0/5	0/5	0/5	0/5	0/4	0/60	
VAU	1484.2 ± 211.98 ^a (n=10)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
UNC	0 ± 0.00 ^b (n=10)	0	0	0	0	13 (22.8 1)	21 (48)	7 (30.4 4)	2 (12.5 0)	0	0	0	0	0	0	
UNU	0 ± 0.00 ^b (n=10)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

A= Number positive for clinical signs.

B= Total number of layers per group. It is also the number of layers remaining in a group when first and subsequent mortalities were observed

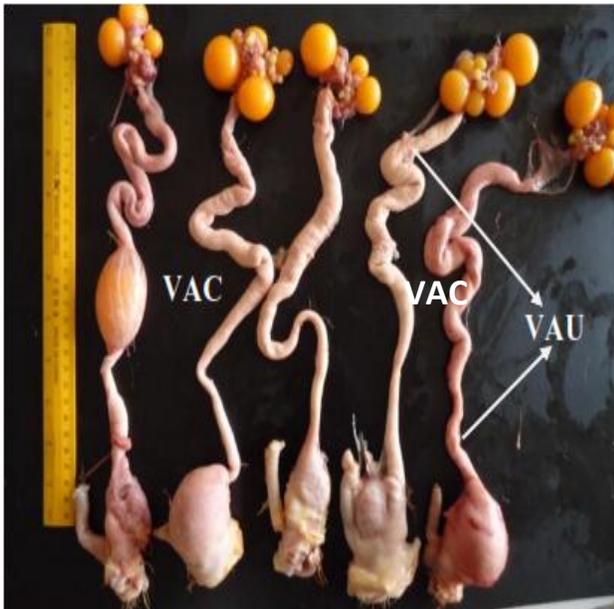


Plate 1: The oviduct of group VAC laying chickens compared with group VAU on day 3 PI showed no lesion



Plate 2: No appreciable change of the ovarian follicles in group VAC laying chickens compared with group VAU on day 6 PI

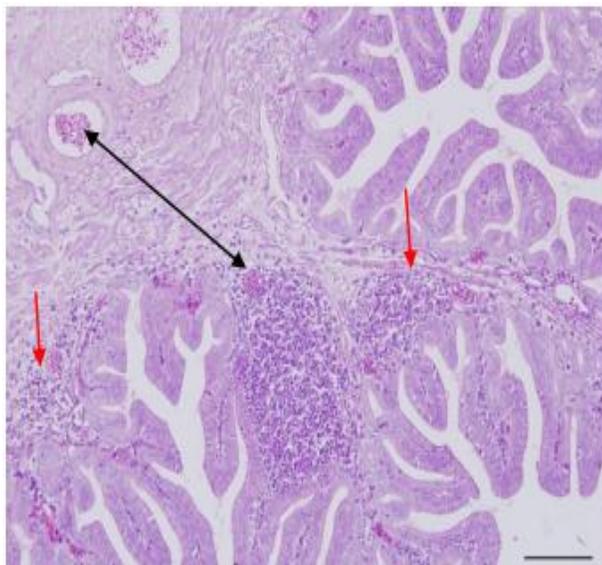


Plate 3. Section of infundibulum of a sacrificed laying chicken from group VAC, showing hyperaemia (double arrow), inflammatory cells (arrows) in submucosa and lamina propria on day 6 PI. H & E, X200

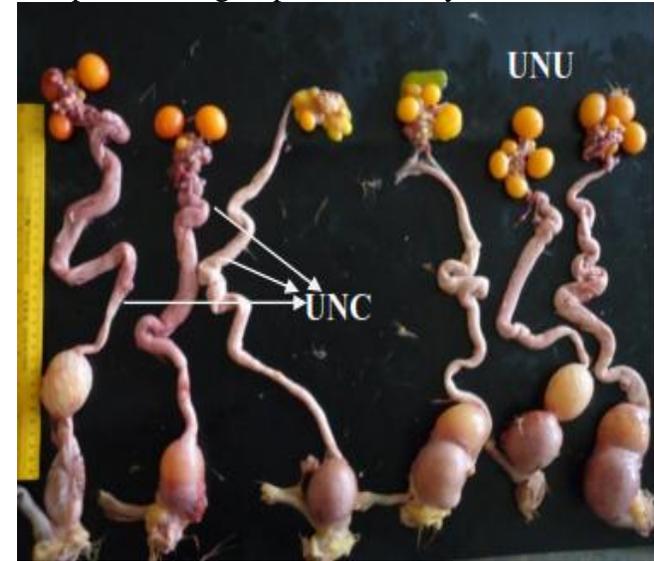


Plate 4: Swollen and congested oviduct with hyperaemic ovarian follicles (arrows) in sacrificed group UNC compared with group UNU laying chickens on day 3 PI

ulcerated and haemorrhagic lesions by day 5 PC which persisted to day 21 PC in less severe form. Spleens were enlarged and mottled 3 to 6 days PC. At days 3 to 6 PC the kidneys were swollen, congested or haemorrhagic.

The reproductive tract showed marked swelling, hyperaemia and haemorrhages in the

ovarian follicles and all through the oviduct at day 3 PC (Figure 4). The infundibulum at day 3 PC had extensive areas of oedema, heterophil accumulation and scattered lymphocytes which expanded the mucosal folds and submucosa. There were multifocal large scattered lymphocytic aggregates

in the submucosa. There was deciliation of superficial epithelial lining. At days 5 to 6 PC, there was generalized extensive, marked necrosis of the epithelial cells and ulceration (Figures 5a and b). The cells of the lymphoid follicles were also necrotic. Similar histopathologic changes were observed in the magnum and isthmus. In the uterus (shell gland) the most common change was acute inflammation of the uterus at day 3 PC. The oedema led to swelling of the organ with separation of the necrotic tubular glands and multifocal accumulations of inflammatory cells (Figure 6). By days 5 and 6 PC there was generalized and extensive oedema, severe necrosis of the superficial mucosal epithelium and tubular glands, ulceration of luminal epithelia with exocytosis and scattering of necrotic debris and inflammatory cells and macrophages in the lumen (Figure 7). More inflammatory cells were present in the submucosa.

The spleen of VAC chickens was histologically normal whereas the spleen of laying chickens in the UNC group had severe

necrosis of the lymphocytes around the sheathed arteriole (Figures 8 and 9). Regeneration of the lost lymphocytes was observed by day 10 PC. Neither clinical signs of disease, severe lesions nor mortality were observed in the non-challenged control groups during the course of the experiment.

Experiment 2

Serological response of laying chickens, compared with uninfected groups before and following challenge with VNDV: No clinical signs of Newcastle disease were observed in any vaccinated laying chicken prior to challenge. Protection from VNDV challenge was determined by absence of clinical signs during the 21-days PC observation period. Birds in the unchallenged control group had no clinical signs during the course of the study. All pre-challenge sera at 32 weeks-of age (day 0) tested positive to NDV with significantly high ($P < 0.05$) antibody titers of between 1473 and 1484 in VAC and VAU groups (Table 2). After challenge, the NDV antibody titers of VAC dropped slightly but

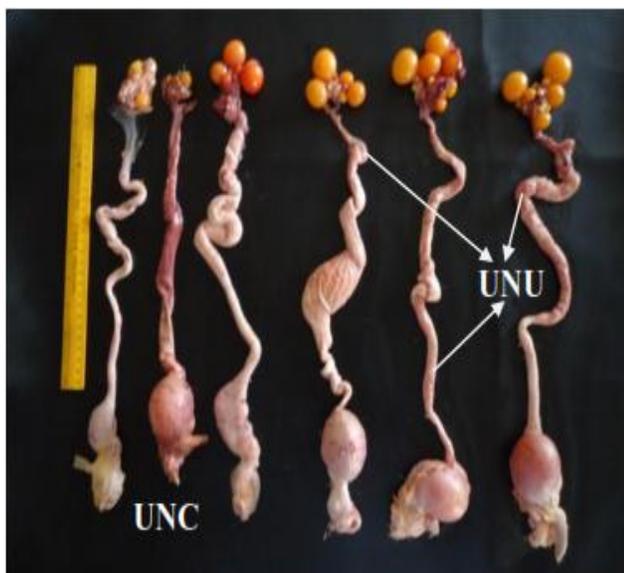


Plate 5a: The reproductive tract of dead UNC laying chickens on day 6 PI. The oviduct is thin-walled and flacid, atrophic with pale (in middle), haemorrhagic, congested and atretic ovarian follicles, compared with the control group, UNU on the right

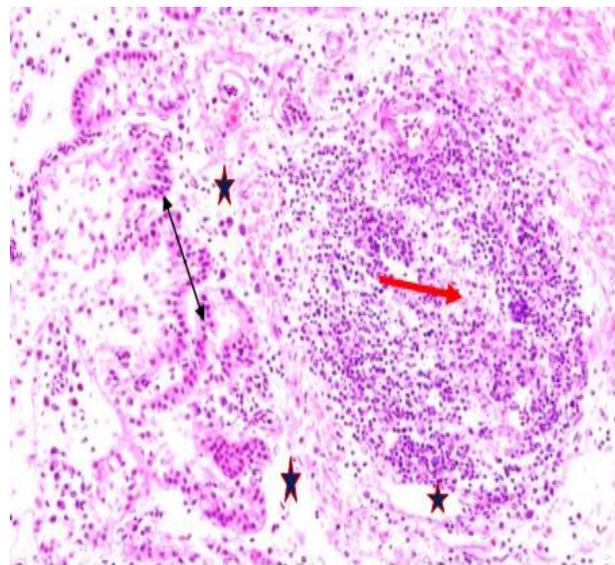


Plate 5b: Section of infundibulum: Severe oedema (asteriks), necrosis and ulceration in mucosa and submucosa (double arrow), necrosis and depletion (red arrow) in the submucosal lymphoid follicle of a dead UNC laying chicken on day 6 PI. H & E, X400

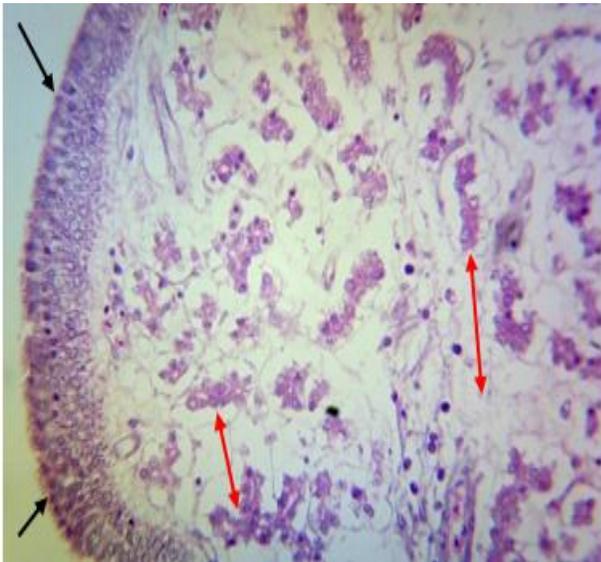


Plate 6: Section of uterus of a sacrificed UNC laying chicken showing severe oedema separating the necrotic shell glands (double arrows) on day 3 PI. Note non/de-ciliation of mucosa epithelium (arrows). H & E, X400

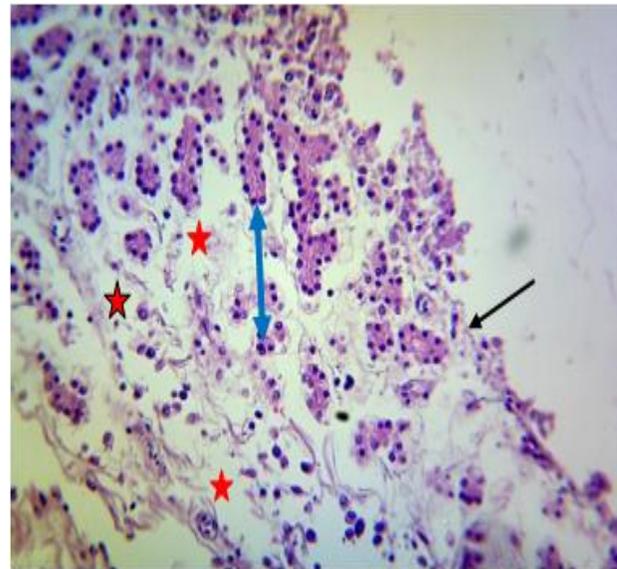


Plate 7: Section of uterus of a dead UNC laying chicken showing severe oedema (asterisks) and necrosis of shell glands (double arrows), and ulceration of mucosa epithelia (arrow) on day 6 PI. H & E, X400

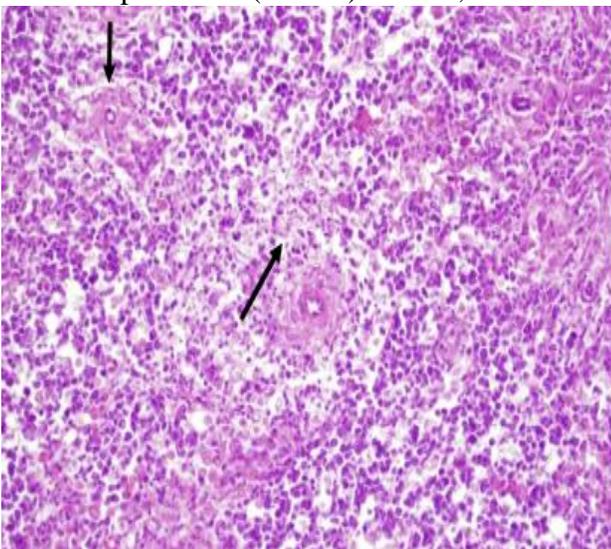


Plate 8: Section of spleen of a dead UNC laying chicken, showing severe necrosis and depletion of lymphoid follicles around the sheathed arterioles (arrow) on day 6 PI. H & E, X400

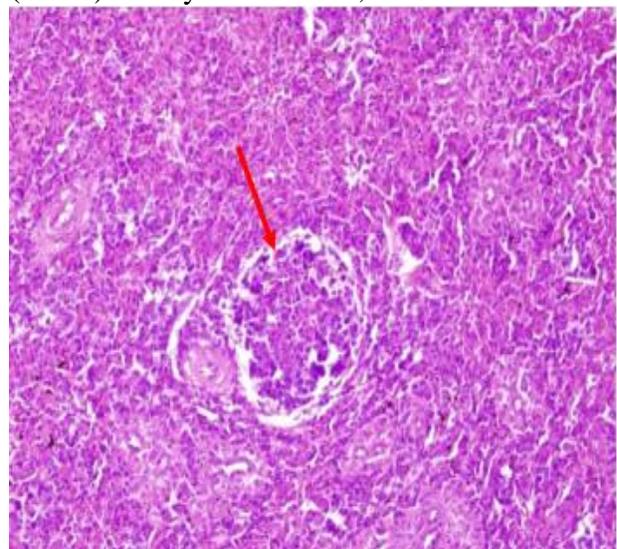


Plate 9: Section of spleen of a sacrificed VAC laying chicken showing lymphoid follicles around the sheathed arterioles (arrow) on day 6 PI. H & E, X400

was still positive and significantly ($p < 0.05$) high by day 10 and 15 PC (1158.4 and 1010.5), and rose gradually by day 21 PC. In the VAU group, the NDV antibody titer fluctuated but still remained positive from days 10 to 21 PC (707.5). Prior to VNDV challenge, the NDV antibody titer was

negative in UNC and UNU groups. On 10, 15 and 21 days PC the NDV antibody titers increased significantly ($P < 0.05$) in surviving UNC groups (Table 2). The non-vaccinated non-challenged laying chickens did not contain detectable antibody titers to NDV throughout the course of the study (Table 2).

Table 2: Serum antibody responses pre and post challenge with virulent NDV (Kudu 113) in commercial laying chickens vaccinated with live ND-Hitcher B1 and ND Lasota, and inactivated oil emulsion Komarov vaccines, at 9 and 16 weeks-of-age, compared with the unvaccinated group

Groups	ELISA antibody titer (\log_{10})			
	Days post challenge			
	0	10	15	21
VAC	1473 \pm 187.05 ^a (n=10)	1158.4 \pm 127.22 ^a (n=10)	1010.5 \pm 124.64 ^a (n=10)	1335.1 \pm 100.68 ^a (n=10)
VAU	1484.2 \pm 211.98 ^a (n=10)	821.1 \pm 105.34 ^a (n=10)	1070.2 \pm 233.25 ^a (n=10)	707.5 \pm 136.18 ^b (n=10)
UNC	0 \pm 0.00 ^b (n=10)	2335.3 \pm 183.71 ^b (n=10)	1856.4 \pm 168.78 ^b (n=10)	2132.8 \pm 104.84 ^c (n=8)
UNU	0 \pm 0.00 ^b (n=10)			

^{a, b, c} Different superscripts in a column indicate significant differences between the groups, $p < 0.05$. Titers >396 are considered positive

DISCUSSION

The continued outbreaks of velogenic NDV in domestic poultry in Nigeria emphasize the importance for continued research on vaccine efficacy against newly isolated strains. Information regarding vaccine efficacy against VNDV strains will provide valuable knowledge for the poultry industry when considering vaccine types and vaccination strategies. The present study demonstrated that priming with live ND B1 and ND Lasota vaccines and subsequent re-vaccination with inactivated oil-emulsion vaccines at 9 and 16 weeks of age induced high immunity levels that resulted in increased and persistent antibody titres at point of challenge at 32 weeks (16 weeks after the last vaccination), and 21 days PC. This finding is in agreement with Eidson *et al.* (1982) who reported that laying chickens vaccinated with live ND vaccines and subsequently re-vaccinated with an inactivated oil emulsion ND vaccine had higher and more persistent antibody titres than birds vaccinated with live ND vaccines. This increased immune response might be due to the higher number (quantity) of virus titres and primary antigenic stimulation and is in agreement with the observation by Rue *et al.* (2011), that a pronounced and rapid innate

immune response may be induced by virulent NDV.

Moreover, vaccination with live Newcastle disease (ND) vaccines and subsequent revaccination with an inactivated oil emulsion ND vaccine protected laying chickens against morbidity and mortality from challenge with the highly virulent Kudu-113 VNDV. All vaccinated birds displayed antibody titres against at the day of challenge and protection from disease post challenge. The protective role of antibody against NDV has been described (Reynolds and Maraqa, 2000). The results extend the findings of prior reports of protection in poultry against velogenic NDV using commercial live and inactivated oil-emulsion vaccines (Eidson *et al.*, 1982; Liljebjelke *et al.*, 2008).

Having established the longer and persistent protection (immunity) with priming with live vaccines and then inactivated oil-emulsion vaccines, the effect on protection against clinical disease, death and severe damage to the organs following challenge was examined. A positive correlation was observed between presence of antibody titres at day of challenge and protection from disease 21 day PC in the vaccinated and challenged group. In addition, it showed that the commercial industry

vaccination programs with inactivated oil-emulsion vaccines are effective at protecting commercial laying chickens from VNDV challenge. A positive correlation was also observed between the presence of antibody titres at challenge and protection from disease. These birds had no clinical signs of ND, no clear lesions grossly and there were mild histopathologic changes. This is in agreement with the study by Eidson *et al.* (1982) stating that breeder flocks revaccinated with live LaSota ND vaccine had lower egg production than the flocks vaccinated with inactivated vaccine. Similarly, Dai *et al.* (2008) reported that priming with live lentogenic vaccines, followed by boosting with inactivated oil-emulsion vaccines weeks later, induced a reasonable antibody response and provided long-lasting protection against a virulent NDV field strain.

However, these vaccines provided chickens with full protection from disease caused by VNDV, as no mortality or disease symptoms were observed in any of the vaccinated challenged chickens. Alexander (2011) reported that the clinical signs of virulent NDVs in vaccinated chickens were greatly diminished in relationship to the antibody level achieved (Allan *et al.*, 1978). The vaccines were also protective against severe damage in the organs viz, spleen, caecal tonsils and reproductive tract as these birds were resistant to challenge and had mild histopathologic changes. This is in agreement with the report of studies by Jeon *et al.* (2008) and Kapczynski and King (2005) on the protective efficacy of NDV vaccines. Also, the main lesions in the organs of the unvaccinated infected group were marked swelling, hyperaemia, haemorrhages, necrosis in the reproductive tract, lymphoid follicles and spleen observed in this study are in agreement with severe lesions observed in birds after experimental and natural infections with VVNDV (Brown *et al.*, 1999; Okoye *et al.*, 2000; Igwe *et al.*, 2014).

CONCLUSION

NDV is an economically important and frequently isolated worldwide viral pathogen whose listed status with OIE marks its importance to both commercial poultry producers and poultry trading countries. Control of VNDV through use of vaccines is regularly and routinely practiced by all major poultry companies to provide immunological protection against disease. The results of this study demonstrated the importance of the protective role of priming with live Newcastle disease (ND) vaccines and subsequent re-vaccination with an inactivated oil emulsion ND vaccine in laying chickens. This longer protective effect will offset the additional cost of the oil emulsion ND vaccine as well as the cost of administering each individual bird every 90 days with a live ND Lasota vaccine.

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