Response of cyclophosphamide-treated broiler chickens to challenge with velogenic Newcastle disease virus

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1. Introduction

Newcastle disease (ND) is a contagious viral disease affecting wild, cage and domestic avian species of any age worldwide causing severe economic losses in the poultry sector (Alexander et al. 2012). Prior pathogenesis studies demonstrated that the disease resulting from an ND virus (NDV) infection of birds varies from mild to severe with high mortality depending on virulence of the infecting strain and immune status of the susceptible host to NDV (Brown et al. 1999; Igwe et al. 2014; Igwe and Eze 2016). All NDV strains belong to the order Mononegavirales, family Paramyxoviridae, and genus Avulavirus, are contained in one serotype known as avian paramyxovirus serotype-1 (APMV-1) (Alexander and Senne 2008). The virus is pleomorphic in shape and consists of non-segmented, single-stranded, negative sense RNA genomes (Miller et al. 2010). There are at least three different genome lengths (15,186, 15,192 or 15,198), and contain six genes encoding the six structural proteins in order from 3′ to 5′: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin–neuraminidase (HN), large protein (L) and the RNA dependent RNA (large) polymerase (L). Transcriptional editing of P produces at least one other protein, the V protein, which has anti-interferon properties (Czeglédi et al. 2006). The disease in chickens and other birds is caused by only the virulent strains of APMV-1. Outbreaks of ND may be devastating, with flock mortality approaching 100% in fully susceptible chickens (OIE 2012). Because of the severe economic consequences of an outbreak of virulent ND in commercial poultry, the disease is reportable to the World Organization for Animal Health (Office International des Epizooties (OIE), OIE 2012).

Even though all strains of NDV are contained in one serotype, there are phylogenetic differences found when comparing genome relatedness. Strains are divided into two classes: Class I and Class II, with Class II further divided into 18 genotypes (Diel et al. 2012; Dimitrov et al. 2016). Class I viruses are typically isolated from wild birds and all are of low virulence except for one strain, chicken/Ireland/1990 (Alexander et al. 1992). Class II, genotype I NDV are all of low virulence and most often isolated from different parts of the world (Courtney et al. 2013; Diel et al. 2012; Dimitrov et al. 2016; Shittu et al. 2016). Iso-lates of class II, genotype X are of low virulence and most often found in wild birds, but some have been isolated from some poultry species (Miller et al., 2009; Diel et al. 2012). These reports show that the virus has very wide host range, has been described in more than 240 bird species from 27 of the 50 orders of birds, including cage and wild birds, has the potential to infect most, if not all, bird species, and constitute one of
the major problems in the control and epizootiology of ND (OIE 2012). In many of these host species, the infection is sub-clinical, and affected birds serve as a reservoir of infection for susceptible birds (Igwe et al. 2014).

In developing and developed countries, where the majority of chickens are reared under ‘backyard’ subsistence conditions, outbreaks of ND in many locations, can drastically limit the amount of dietary protein as well as damage the microeconomy due to loss of ability to sell off extra chickens or eggs (Alexander 2001; Nwanta et al. 2008) and causing massive economic damage through control efforts and trade losses (Miller and Koch 2013).

Provision of immunological protection against ND through use of vaccines is regularly and routinely practiced by all major poultry companies (Kapczynski et al. 2013; Igwe and Eze 2016). These vaccines can protect birds from the disease but cannot prevent them from becoming infected, shedding the virulent virus in their faeces and transmitting it from infected to healthy birds, and drop in egg production in laying hens (Eze et al. 2016; Igwe et al. 2017). Therefore, ND outbreaks are still common in commercial vaccinated flocks despite the advances in vaccination against the disease (Dortmans et al. 2012). Elucidation of the immune response to vNDV still remains a top priority for the development of better control strategies in the face of reoccurring outbreaks (Kapczynski et al. 2013). It is difficult to assess the contribution of cellular (T-cell) and of humoral (B-cell) responses to protection. One approach to elucidate their respective role has been destroyed one component of the immune system leaving the other component intact (Al-Garib et al. 2003).

Cyclophosphamide (CY), a tumoricidal agent, has been used experimentally as an immunosuppressant in numerous pathogenicity studies to determine some aspects of the immune response such as the role of T and B cells in protective responses to infectious pathogens in chickens (Isogai et al. 1980; Kibenge et al. 1987; Corrier et al. 1991; Okoye et al. 1992; Reynolds and Maraqa 1999). CY treatment leaves the bursa reticulum intact and destroys only the lymphoid cells. When given in large doses, CY could deplete the B- and T-cells (Winkelstein et al. 1972; Sharma and Lee 1977; Stevenson and Fauci 1980; Al-Garib et al. 2003). Velogenic ND (vND) in chickens is characterized by severe atrophy of the lymphoid organs due to severe necrosis and depletion of the lymphocytes (Igwe et al. 2014). This gives the impression that some replication by the virus might be taking place in the lymphocytes. The aim of this project was to find out if the destruction of the lymphoid organs by CY treatment before challenge had any effect on the establishment and severity of vNDV infection in broilers.

2. Materials and methods

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

2.1. Broiler chickens

One hundred-day-old White Marshall broiler chicks (Gallus gallus domesticus) procured from a reputable local commercial hatchery were used for the study. Brooding was done separately for each of the groups on deep litter and they were not vaccinated against any disease. Feed and water were provided ad libitum. The chicks were kept in isolation in the Poultry Experimental Unit of the Department under strict biosecurity measures.

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.

2.2. CY treatment

CY (Paucocyclo, Keally Pharmaceutical Pty, Ltd, Amber, India) was obtained in a dry form containing active ingredients. An aseptically prepared aqueous solution was prepared daily by reconstituting 500 mg in 30 mL of distilled water. At 4 weeks of age, the birds were randomly divided into 4 groups of 25 broiler chicks each. The groupings and their treatments were:

- Group A consisted of CY-treated and vNDV-challenged chickens (CYTI).
- Group B consisted of CY-treated and unchallenged chickens (CYTU).
- Group C consisted of CY-non-treated and vNDV-challenged chickens (CYNV).
- Group D consisted of CY-non-treated and unchallenged chickens (CYNV).

Broiler chickens of groups A and B were each given 75 mg/kg of CY each day intramuscularly (IM) in the breast muscle on days 28, 29 and 30 of age. Chemical ablation was achieved according to the methods of Winkelstein et al. (1972) and Glick and Olah (1984). The four experimental groups were housed separately.

All the groups were observed for effects of CY treatment daily. Seven birds from each group were randomly selected and weighed individually on days 0, 7 and 14 (day 0 post-challenge [PI]) post-CY-treatment. Fourteen days post-CY-treatment, five birds from each group were euthanized and examined for gross lesions. The carcasses, bursa and spleen were weighed. The bursa weight (BW) and spleen weight (SW) indices were determined for each broiler as described by Lucio and Hitchner (1979). Bursa, spleen, thymus and caecal tonsil were collected for histopathology.

2.3. ND virus challenge

The challenge velogenic virus used, duck/Nigeria/Plateau/Kuru/113/1992, class II, genotype XVII (Shittu et al. 2016), was obtained from National Veterinary Research Institute (NVR), Vom, Nigeria. It was isolated from apparently healthy duck and characterized as velogenic viscerotropic NDV (vNDV) by Echeonwu et al. (1993) and Igwe et al. (2014). The inoculum had a median embryo infective dose (EID50) of 10^6.4 per millilitre.

At 6 weeks of age, the chickens were found to be serologically negative for NDV antibodies by haemagglutination inhibition (HI) test. Each broiler chicken in groups A/CYT and C/ CYNTI was inoculated intranasally (I/N) with 0.2 mL of the viral
inoculum, while each bird in groups B/CYTU and D/CYNTU (corresponding non-infected chickens) received 0.2 mL of phosphate buffered saline (PBS) through the same route as placebo.

2.3.1. Clinical observation
After vNDV challenge at 6-week-old, all the groups were observed for clinical signs of ND daily throughout the experimental period. The daily morbidity and mortality were recorded. Seven birds from each group were randomly selected and weighed individually on days 0 and 3 PI using separate weighing balances. In the infected groups, those showing depression were picked before others.

2.3.2. Pathological examination
Three birds were euthanized in each group and necropsied along with dead ones for gross lesions on days 4, 5 and 6 PI. The carcasses, bursa and spleen were weighed and the bursa weight indices (BWIs) and spleen weight indices (SWIs) calculated on days 0 and 5 PI. Samples of the bursa, spleen, thymus, caecal tonsil and brain were fixed in 10% formal saline for 48 h and processed for histopathology as described by OIE (2012).

2.4. Serology
Three millilitres of blood were collected from each bird through the jugular vein at weeks 4 (before the CY treatment), 5, and 6 of age, immediately before vNDV infection (day 0 of challenge), and on days 3 and 21 PI from 10 birds in each group. One milliliter of the blood samples was collected into a labelled clean sample bottle containing 1 mg of ethylene diamine tetra acetic acid powder as an anticoagulant and used immediately for haematologic analysis using standard procedures, while the remaining blood samples were harvested into sterile bijou bottles and were stored overnight in the refrigerator at 4°C. The sera samples were stored in the freezer at −20°C and assayed for NDV HI antibody titres using the method previously described (OIE 2012). The antigen used for the HI test was a PBS suspension of La Sota vaccine which had 4 HA unit of antigen.

2.5. Haematology
The smears for differential leukocyte count were prepared and stained by the Leishman technique and enumerated by the battlement counting method (Thrall and Weiser 2002).

2.6. Statistical analyses
Data generated for the study were subjected to one-way analysis of variance. The mortalities data were analysed with the Fisher’s exact test. Variant means were separated post hoc using the least significant difference method (Okafor 1992). Probabilities less or each to 0.05 were accepted as significant.

3. Results
3.1. Effect of cylophosphamide treatment on the broilers
The CY-treated groups, A/CYTI and B/CYTU showed feather loss and significant (p < .05) weight loss on days 7 post-CY treatment (PCYT) and 14 PCYT (D0 PI) when compared with those of the non-CY-treated groups, C/CYNTI and D/CYNTU (Figure 1). There were highly significant differences between the mean BW index (p < .05), and also between the mean SW index of the CY-treated, A/CYTI and B/CYTU and non-CY-treated groups, C/CYNTI and D/CYNTU, on day 14 PCYT (D0 PI) (Figures 2 and 3). The sacrificed chickens in the A/CYTI and B/CYTU groups showed severe atrophy of the bursa, thymus and spleen from days 5 to 14 PCYT (Figures 4 and 5), when compared with their controls.

Histopathological sections of the bursa of Fabricius showed folded but intact epithelium, severe lymphocytic depletion in the follicles and hyperplasia of the follicular epithelium. The follicles were atrophic. Increased number of macrophages and few plasma cells were present in the plicae of the bursa. Lymphocytic necrosis and depletion with only the reticular cell network remaining were observed in the thymus, caecal tonsils and spleen.
3.2. NDV challenge

3.2.1. Clinical signs

By days 2–3 PI, 90% of vNDV-infected groups (A/CYTI and C/CYNTI) showed a drop in feed and water consumption, ruffled feathers and depression. At day 4 PI, morbidity was 100%, and all broiler chickens in the infected groups showed whitish-greenish diarrhoea which soiled the vent, and the clinical signs progressed to coughing, sneezing and nasal discharges of sero-mucous fluid at days 5–7 PI. From day 8 PI group C/CYNTI broilers that survived showed paralysis of the legs and wings, and torticollis. Mortality started from day 4 PI and involved 15.38% and 22.22% of groups A/CYTI (2/13) and C/CYNTI (4/18) broiler chickens, respectively. Peak mortality occurred on day 5 PI in the remaining broiler chickens involving 90.91% of group A/CYTI (10/11 birds) and 85.71% of group C/CYNTI (12/14 birds). Mortality started from day 4 PI and involved 15.38% and 22.22% of groups A/CYTI (2/13) and C/CYNTI (4/18) broiler chickens, respectively. Peak mortality occurred on day 5 PI in the remaining broiler chickens involving 90.91% of group A/CYTI (10/11 birds) and 85.71% of group C/CYNTI (12/14 birds). Mortality was lowest in the remaining broiler chickens by day 6 PI and involved a bird from each infected group; bringing the total mortality rates to 13/13 chicks or 100% in group A/CYTI and 17/18 chicks or 94.44% in group C/CYNTI. There was no statistical difference (p > .05) between the mortalities (Table 1). There were no clinical signs seen in the control groups of broiler chickens (B/CYTU and D/CYNTU).

The groups, A/CYTI and B/CYTU showed a significant (p < .05) weight loss on day 14PCYT (D0 PI) when compared with C/CYNTI and D/CYNTU (Figure 1). At D5 PI, A/CYTI had a significant (p < .05) weight loss when compared with B/CYTU, C/CYNTI and D/CYNTU, while A/CYTI and C/CYNTI weight values were significantly (p < .05) lower than that of D/CYNTU.

3.2.2. Gross lesions

The mean BWI of groups, A/CYTI and B/CYTU were highly significantly (p < .05) lower than those of C/CYNTI and D/CYNTU on days 0 and 5 PI (Figure 2). The mean SWI of A/CYTI and B/CYTU were significantly lower than those of C/CYNTI and D/CYNTU on day 0 PI while those of A/CYTI, B/CYTU and C/CYNTI were significantly lower than that of D/CYNTU on day 5 PI (Figure 3).

Chickens in groups A/CYTI and C/CYNTI showed congestion of the muscles of the breast, thighs and legs. The bursa was consistently atrophic in groups A/CYTI and B/CYTU, but in the C/CYNTI group moderate atrophy and congestion of the bursa were observed from day 4 PI (Figures 6 and 7). The group A/CYTI chickens showed moderate swelling and mottling of the spleen on days 3 and 4 PI and atrophy on days 5 and 6 PI while group C/CYNTI chickens showed marked swelling and mottling on day 3 PI and atrophy on day 5 PI. The thymus of group A/CYTI was consistently atrophic, but in group C/CYNTI moderate atrophy was observed from day 5 PI. Enteritis,
haemorrhages on the proventricular mucosa, and haemorrhagic ulcers in the intestines were seen from days 4 to 6 PI (Figures 8 and 9). The caecal tonsils were swollen, ulcerated and haemorrhagic and contained some cheesy debris. The kidneys were swollen and congested. The lesions in the proventriculus, intestines and caecal tonsils were more severe in the CY-untreated than the treated broilers. Tracheal lesions varied from serous to catarrhal exudates in the lumen. The lungs were swollen, oedematous, congested, and had foamy exudates. Proventricular haemorrhages and intestinal ulcers were prominent.

### 3.2.3. Histopathology

The bursa showed mild oedema and severe depletion of lymphocytes in A/CYTI, but in the C/CYNTI moderate oedema and severe necrosis of lymphocytes at day 4 PI. There was severe depletion of the follicular lymphocytes, hyperaemia and interfolllicular fibroplasias at days 5 and 6 PI. The bursal epithelium was folded, and there were necrotic debris in the lumen of bursa. The follicles of the infected groups developed cavities in the medulla that could be associated with ballooning degeneration of the reticular cells (Figure 10); while evidence of lymphocytic regeneration was observed in few follicles in the group B/CYTU chickens. In both challenged groups, the thymus of the chickens that died on day 4 PI showed necrosis of the lymphocytes in the lobules and fibrin deposition while those from chickens that died on days 5 and 6 PI showed marked depletion of lymphocytes in the lobules, congestion of blood vessels and haemorrhages throughout the parenchyma. Regeneration of lymphocytes was seen in the thymus of group B/CYTU chickens at day 6 PI. There were severe necrosis and depletion of lymphocytes in the spleen of group A/CYTI and necrosis of lymphocytes in the spleen of group C/CYNTI chickens with deposition of fibrin in the spleen at days 4 to 6 PI (Figure 11). Vascular reactions in the CNS were characterized by hyperaemia, endotheliosis, spongiosis and perivascular cuffing (Figures 12 (B,C) and 13 (A,C)). There were degeneration and necrosis of neurons and purkinje cells, neuronophagia and multifocal gliosis in the brain. Lesions in the lungs included hyperaemia and oedema. The group D/CYNTU chickens had no lesion (Figures 10(A), 11(A) and 12(A)).

### 3.3. Serology

All the samples collected at 4 and 5 weeks of age and on days 0 and 3 PI had no detectable HI antibody to NDV. All the chickens in the infected groups were dead by day 6 PI with the exception of one surviving broiler in group C/CYNTI which had an HI antibody titer of 1024 (10 log2) at day 21 PI.

### 3.4. Effect of CY treatment on absolute lymphocyte count

At day 0 of commencement of CY treatment, the lymphocytic values did not show any significant difference ($p > .05$) between the CY-treated and CY-non-treated groups and their controls. The absolute lymphocytes counts in the CY-treated groups were significantly ($p < .05$) lower than those of CY-non-treated groups on days 7, 14 PCYT (D0 PI) and 17 PCYT (3 PI) (Table 2). The lymphocyte counts of the C/CYNTI group were significantly ($p < .05$) higher than those of A/CYTI, B/CYTU and D/CYNTU at day 3 PI.

### 4. Discussion

CY treatment caused severe atrophy of the bursa, thymus and spleen and significant reduction in the BW and SW indices of the broilers in this experiment. These were due to the severe necrosis and depletion of the lymphocytes observed in the bursa, spleen, thymus and caecal tonsil. These observations have been reported by earlier workers (Okoye et al. 1992; Kim et al. 2003) who reported that CY treatment caused suppression of humoral antibody response. CY treatment has been used as a specific suppressor of B-cell dependent humoral immunity in order to determine the role of T and B cells in protective responses to infectious pathogens (Kibenge et al. 1987; Okoye et al. 1992; Sadeyen et al. 2015). Most evidence indicates that CY suppresses B and T-cells in chickens temporarily and regeneration occurs within two weeks following CY treatment (Sharma and Lee 1977). However, evidence of
multifocal regeneration occurred in few follicles in bursa and diffused regeneration in thymus and spleen by days 19–20 (14 + 5 or 6) post-CY treatment in this experiment. CY inhibits cell division by alkylating nucleic acids and has been used in experimental studies to determine some aspects of the immune response such as the role of cell mediated immunity in protection (Kibenge et al. 1987; Corrier et al. 1991; Okoye et al. 1992). The mechanism of CY action is based on destroying lymphoid cells in both central and peripheral lymphoid organs that have been seeded with bursa-derived cells during embryo development. The severe lesions observed in the lymphoid organs in the present study showed that both the humoural and cellular immune systems might have been destroyed in the CY-treated broilers without evidence that the disease was more severe in the CY-treated broilers. The total mortalities were 100% and 94.44% in CY-treated and untreated broilers respectively. All the mortalities occurred between days 04 and 06 PI, and there was no statistically significant difference between them. The lesions in the proventriculus, intestines and caecal tonsil were more severe in the CY-untreated broilers because of the depletion of lymphocytes in these sites in the CY-treated broilers. An important factor that has played out is that vND being an acute disease caused very high mortalities in both challenged groups before the production of protective antibody level in the CY-untreated broilers could be achieved. The haematology results showed that while CY treatment caused lymphopenia, the vNDV challenge increased the lymphocyte levels in the broilers.

Figure 8. (A) Severe haemorrhages on the mucosa of the proventriculus of group C/CYNTI, (left), compared with no lesion in group D/CYNTU chickens (right) on day 6 PI. (B) Moderate haemorrhages on the mucosa of the proventriculus of group A/CYTNI, (left) compared with no lesion in group B/CYTI chickens (right) on day 6 PI.

Figure 9. Haemorrhagic ulcers (X) in the mucosa of the intestine seen from the serosal surface on day 6 PI in groups A/CYTNI (left) and C/CYNTI chickens (right).

Figure 10. (A) Normal bursa of group D/CYNTU chickens. H&E × 200 (left). (B) Congestion, inter-follicular fibroplasias, severe lymphocytic depletion in the follicles, hyperplasia of the cells of the follicular epithelium and ballooning degeneration in the bursa of group C/CYNTI chickens on day 5 PI, H&E, ×200.
Figure 11. (A) Normal spleen of group D/CYNTU chickens, H&E × 200. (B) Spleen of group C/CYNTI chickens on day 4 PI showing necrosis, depletion of lymphocytes and marked fibrin deposition, H&E, ×200.

Figure 12. (A) Normal cerebrum of D/CNTU chicken, H&E × 200. (B) Cerebrum of group A/CYTI chicken showing perivascular cuffin (P) and spongiosis (cerebral oedema) (S) on day 5 PI, H&E × 200.

Figure 13. (A) Normal cerebrum of D/CYNTU chicken, H&E × 200. Cerebrum of group C/CYNTI broiler showing endotheliosis (E) on day 5 PI, H&E, ×200.
One major impetus for this work is the striking similarities between the lesions of vND and those of infectious bursal disease (IBD) of chickens. In both diseases, there are proventricular haemorrhages, caecal tonsil haemorrhagic ulcers, enteritis and severe atrophy of the bursa, spleen and thymus due to severe necrosis and depletion of the lymphocytes in the lymphoid organs (Okoye 1984; Mahgoub 2012; Ezema et al. 2016; Sá e Silva et al. 2016). In fact, in both diseases, the lymphocytes are the major cells that are destroyed. The B lymphocytes are the target cells of IBD. IBD cannot establish in bursectomized young chickens and in older chickens where bursal regression has either partly or completely occurred (Okaye and Uzoukwu 1990; Okaye et al. 1992). IBD suppresses humoral antibody response in surviving chickens (Okaye 1984; Hoerr 2010; Mahgoub 2012). Evidence that vNDV infection is also immunosuppressive is gradually emerging as Ezema et al. (2016) reported that experimental vNDV infection suppressed HI antibody response to La Sota vaccination in surviving chickens. It becomes interesting to find out if like in IBD the lymphocytes play any role in the establishment and severity of vNDV infection. However, despite the close similarities in the lesions of the two diseases, the results of this experiment showed that the lymphocytes are not important in the establishment of vNDV infection in young broilers. Ojok and Brown (1996) reported that immunohistochemical labelling in NDV infection was confined to large mononuclear cells while Cattoli et al. (2011) reported that the vNDV replicated in macrophages. So, the two viruses use different target cells for their replication inside the chicken host even though they end up causing massive destruction of lymphocytes in the lymphoid organs.

5. Conclusion

Based on the results of this study, it was concluded that CY treatment may not have an effect on the susceptibility of broiler chickens to an acute disease like vND. This suggests that other cell types and not only lymphoid cells play an essential role in the pathogenesis of vND.

Compliance with ethical standards

Ethical standards

All animal studies were approved by the Institutional Committee on Medical and Scientific Research Ethics and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Table 2. The absolute lymphocyte counts of broilers treated with CY and challenged with velogenic NDV.

| Days post-cyclophosphamide (CY) treatment | Group A (CY-treated, vNDV-challenged) | Group B (CY-treated, non-challenged) | Group C (non-CY-treated, vNDV-challenged) | Group D (non-CY-treated non-challenged) |
|------------------------------------------|---------------------------------------|-------------------------------------|------------------------------------------|----------------------------------------|
| 0                                        | 25.25 ± 2.13                          | 27.94 ± 2.36                        | 21.97 ± 1.26                             | 26.27 ± 2.86                           |
| 7                                        | 12.74 ± 0.95*                         | 9.56 ± 1.63*                        | 31.65 ± 4.07*                            | 32.28 ± 5.28*                          |
| 14                                       | 20.97 ± 3.90*                         | 12.68 ± 1.84*                       | 33.41 ± 3.83*                            | 34.17 ± 5.14*                          |
| 17                                       | 25.73 ± 0.87*                         | 22.70 ± 4.16*                       | 36.57 ± 4.30*                            | 28.78 ± 4.60*                          |

Note: Different superscripts in a row indicate significant differences between the groups, p < .05.

Disclosure statement

No potential conflict of interest was reported by the authors.

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