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Cockerels experimentally infected with the velogenic Newcastle disease virus exhibited higher susceptibility and more severe pathological lesions than infected pullets

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Abstract

Chickens are highly susceptible to velogenic Newcastle disease virus (vNDV), but the relative susceptibility of different sexes of chickens has not yet been investigated or reported. This study compared the clinical signs and pathological lesions in cockerels and pullets experimentally infected with vNDV. Sixty cockerels and sixty pullets were used for the study. They were each randomly assigned to two groups of 30 each – infected and uninfected groups. Cockerels and pullets in the infected groups were inoculated with the Kuru duck-113 strain of the Newcastle disease virus. Clinical signs were recorded, and blood samples were collected and subjected to haematology and serum protein assay. Also gross lesions were recorded at the necropsy of dead infected birds. Sections of tissues were processed for histopathology. Results showed that, following virus infection, the pullets and cockerels exhibited comparable clinical signs, except for body weight, which was significantly ($p < 0.05$) lower in infected cockerels when compared with the uninfected. Cumulative morbidity and mortality were higher in infected cockerels than in pullets on days 2, 3 and 4 post-infection (PI). At necropsy, gross enlargement of the spleen and proventricular mucosal hemorrhages were more severe in infected cockerels than in infected pullets. The erythrocytic profile of the infected cockerels was significantly higher ($p < 0.05$) than that of the uninfected cockerels, but there was no significant difference ($p > 0.05$) in the erythrocytic profile of the infected and uninfected pullets. Serum total protein of the infected pullets was significantly higher ($p < 0.05$) higher than that of the uninfected pullets, but the serum globulin levels of both infected cockerels and pullets were significantly higher ($p < 0.05$) than those of their uninfected controls. It was concluded that vNDV-infected cockerels showed higher susceptibility and more severe pathological lesions than infected pullets.

Keywords: Newcastle disease; cockerels and pullets; chickens; pathology; susceptibility; sexual dimorphism.

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Introduction

Poultry is the largest livestock group, and poultry meat accounts for over 30% of global animal protein consumption (Permin *et al.,* 2001). Poultry production is primarily based on commercial poultry, which accounts for only 20% of the total poultry population (Conan *et al.,* 2012). In developing countries, villagers raise poultry to meet household food demands and as additional sources of income. Backyard production methods imply low biosecurity measures and a high risk of infectious diseases such as Newcastle disease (Conan *et al.,* 2012; Ashraf & Shah, 2014).

Newcastle disease (ND) is a contagious disease of birds, affecting many domestic and wild avian species (Abdisa & Tagesu, 2017). It is caused by virulent strains of Avian Paramyxovirus-1, a single-strand non-segmented negative-sense RNA virus (Ashraf & Shah, 2014). The Newcastle disease virus (NDV) is classified into three strains based on virulence – lentogenic strains that cause mild or subclinical respiratory disease in young or immunocompromised birds, mesogenic strains that induce moderate respiratory disease, and the velogenic strains that are highly virulent. The velogenic strains can localize in both the gastrointestinal and respiratory tracts and the nervous system, where they undergo systemic replication and cause organ damage and high mortality (Piacenti *et al.,* 2006; Putri *et al.,* 2017; Mariappan *et al.,* 2018). Due to the potential for causing devasting losses, the virulent strains of NDV are categorized as notifiable pathogens and must be reported to the World Organization for Animal Health (OIE) (Afonso *et al.,* 2012). The incubation period of ND is short, ranging from 2 – 15 days, with an average of 5 – 6 days (Brown & Bevins, 2017). The severity of NDV infection is dependent on host factors (species, age, and immune status), viral factors (virulence and tropism of the virus), and environmental conditions (Abdisa & Tagesu, 2017). Thus, clinical signs and pathologic lesions observed in birds infected with NDV vary. In unvaccinated flocks, morbidity and mortality rates may reach up to 100% (Hamid *et al.,* 1990). Clinical signs alone do not present a reliable basis for ND diagnosis; however, the characteristic signs and lesions associated with the virulent pathotypes can be used for tentative diagnosis of the disease (Getabalew *et al.,* 2019).

Among all the species of birds, chickens are the most susceptible avian species to NDV (Abdisa & Tagesu, 2017a). As such, ND is responsible for devastating losses in the poultry industry (Alexander, 2000). Studies comparing the pathology and pathogenesis of ND in different types of birds have been extensively reported (Eze *et al.,* 2014a, b & c; Igwe, 2009; Igwe *et al.,* 2018; Okechukwu *et al.,* 2020). Though sexual dimorphism has been reported to play a role in the susceptibility and severity of a variety of infectious diseases in humans and animals (Vasquez-Martinez *et al*., 2018; Gay *et al*., 2021), there are no reports in available literature that compared the susceptibility and pathology of vNDV infection in cockerels and pullets. The aim of the present study was, therefore to compare the susceptibility, haematological and serum protein alterations and pathology associated with experimental vNDV infection and ND in cockerels and pullets.

Materials and Methods

Flock history: One hundred and twenty chickens (60 white cockerels and 60 brown pullets) were purchased from a local hatchery at day old, for the study. The experimental birds were not given ND vaccination. However, they received infectious bursal disease vaccine via drinking water at 10 and 21 days of age, according to the National Veterinary Research Institute (NVRI), Vom Nigeria vaccination schedule for chickens. Brooding was done on deep litter. Feed and water were provided *ad libitum*. Throughout the study, the principles of humane laboratory animal care and use were followed.

Velogenic Newcastle disease virus inoculum: The virus strain used for the study was the Kuru duck-113 (KUDU-113), isolated locally from the cloacal swab of a healthy duck, biologically characterized by Echeonwu *et al*. (1993). The virus inoculum had a median embryo lethal dose

(ELD_{50}) of $10^{6.46}$ per ml. It was procured freeze-dried and stored at -70°C.

Experimental design and virus challenge: At ten weeks of age, the cockerels and pullets were randomly assigned into four groups as follows: Group 1 (30 infected cockerels), Group 2 (30 infected pullets), Group 3 (30 uninfected cockerels), and Group 4 (30 uninfected pullets). Each bird in groups 1 and 2 were inoculated intramuscularly with 0.1 ml of the virus inoculum, while those in groups 3 and 4 each received 0.1 ml of sterile phosphate-buffered saline (PBS) as a placebo, administered intramuscularly. The infected and uninfected groups were housed and reared on deep litter in separately located experimental poultry houses.

Clinical signs, gross and histopathological examinations: The birds were observed twice daily for clinical signs throughout the five-day experimental period. Ten birds were randomly selected and weighed in each group on days 0 and 4 post-infection (PI). Morbidity and mortality were recorded. Pullets and cockerels that died during the experimental period were necropsied. Necropsies were performed immediately or as soon as possible after the death of infected cockerels and pullets, following the standard protocol as reported by Brownlie and Munro (2016). The distribution and persistence of lesions on the affected organs were studied and recorded. Samples of the spleen, bursa of Fabricius, and thymus were collected and fixed in 10% buffered formalin and prepared for histopathologic evaluation as follows: the fixed tissues were trimmed, processed by dehydration in ascending grades of alcohol and cleared in xylene. They were then embedded in paraffin wax before cutting in sections (5um thick) according to the method of Survana *et al*. (2008). The cut sections were stained with hematoxylin and eosin before examination under the light microscope, and pictures of the stained sections were captured using a camera attached to the microscope.

Haematological and biochemical assays: Blood (5 ml) was collected from ten birds in each group on day 4 post-infection (PI). Two millilitre of each of the blood samples was dispensed into a sample bottle containing potassium ethylene diamine tetra acetic acid (EDTA) for hematological evaluation. The remaining 3 ml was dispensed into plain glass bottles, allowed to stand for 45 minutes to clot, and centrifuged at 3,000 revolutions per minute for 10 minutes to separate the serum from the clot. The resulting serum was used for the assay of serum proteins.

The hematological parameters determined were packed cell volume (PCV), hemoglobin concentration (HBC), red blood cell count (RBC), total white blood cell count (WBC), and differential white blood cell count. The microhematocrit method was used to determine the PCV (Thrall and Weiser, 2002), while the cyanomethhaemoglobin method was used to evaluate the HBC (Higgins *et al*., 2008). The RBC and total WBC counts were done by the hemocytometer method using an improved Neubauer counting chamber (Campbell and Coles, 1986). The differential WBC count was done on air-dried thin smears stained by the Leishman technique (Campbell & Coles, 1986). The serum total protein was determined following the direct Biuret method, while the serum albumin levels were assayed based on the bromocresol green method, and the globulin fraction was calculated as the difference between serum total proteins and serum albumin levels (Johnson, 2008).

Data analysis: Comparison of body weights, haematological parameters and serum proteins of the infected and uninfected groups were done statistically using Student's T-test. Morbidity, mortality and variations in the gross lesion presentations were analyzed using the Chi-square test. All the statistical analysis was performed using GraphPad Prism software. Significance was defined when $p < 0.05$. Results were presented as bar charts, tables and pictures.

Results

Post-infection, clinical signs such as ruffled feathers, depression, prostration, greenish-white

diarrhea with a soiled vent, and reduced water/feed intake were first observed on day 2 PI in both infected cockerels and pullets (Figure 1). Nervous signs such as leg and wing paralysis were observed on days 3 and 4 PI in both infected groups. There was no difference in the pattern of clinical signs in the infected cockerels and pullets. However, the cumulative morbidity in infected cockerels was higher than that in infected pullets on days 2 and 3 PI (Figure 2). Morbidity peaked at day 3 PI in infected cockerels, while the morbidity of infected pullets peaked at day 4 PI (Figure 2). Infected cockerels had significantly ($p < 0.05$) lower body weight when compared to uninfected ones, but there was no significant difference ($p > 0.05$) in body weights between the infected and uninfected pullets (Figure 3).

Figure 1: Clinical signs of prostration, depression and paralysis recorded in cockerels (A) and pullets (B) experimentally infected with velogenic Newcastle disease virus.

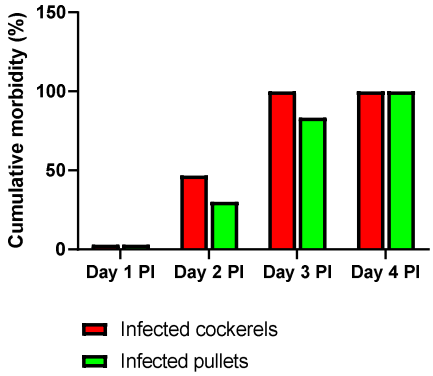

Figure 2: Cumulative morbidity pattern of cockerels and pullets infected with velogenic Newcsatle disease virus.

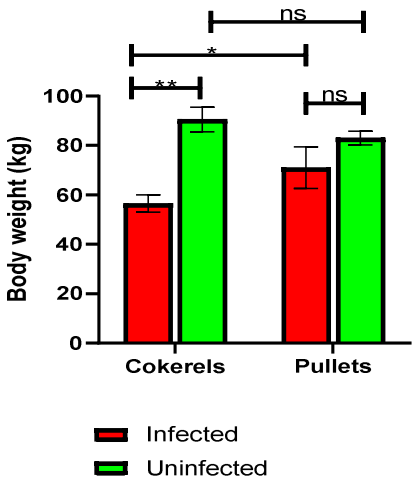

Figure 3: Mean body weight (kg) of cockerels and pullets infected with velogenic Newcsatle disease virus, compared to uninfected controls. [the error bars are for the standard error of mean; * < 0.05; **$P < 0.01$ indicates significant differences of infected/uninfected pullets and cockerels; ns = not significant].

Mortality was first observed on day 3 PI in all the infected groups (both cockerels and pullets), but the cumulative mortality was higher in cockerels on day 3 and day 4 PI (Figure 4). By day 5 PI, birds in all the infected groups had died. Overall, mortality was faster in infected cockerels than in infected pullets (Figure 4).

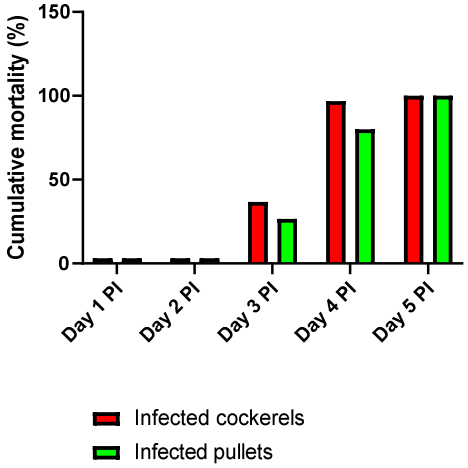

Figure 4: Cumulative mortality pattern of cockerels and pullets infected with velogenic Newcsatle disease virus.

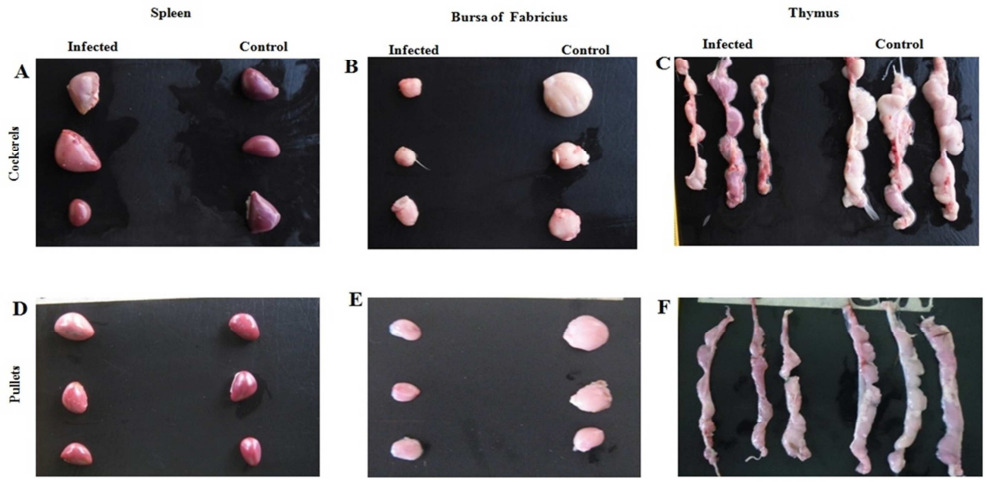

Figure 5: Gross lesions in the spleen, bursa of Fabricius and thymus of cockerels and pullets experimentally infected with velogenic Newcastle diseases virus, at day 3 post-infection (PI): A – Spleen of infected cockerels enlarged more than that of the uninfected control; D – Slight splenic enlargement in the infected pullets, compared to the uninfected control; B and E – Atrophy of bursa of Fabricius in both infected pullets and cockerels, compared to their uninfected controls; C and F – Atrophy of thymus in both infected pullets and cockerels, compared to their uninfected controls.

Necropsy of the infected dead birds revealed that all infected cockerels and pullets exhibited atrophy of the bursa of Fabricius (Figures 5 B and E). This feature was accompanied by mottling of the spleen and enlarged/congested kidney. Enlargement of the spleen and proventricular mucosal hemorrhages were observed more in cockerels than in pullets (Figures 5 and 6). The quantification of the distribution of the lesions is presented in Figure 7.

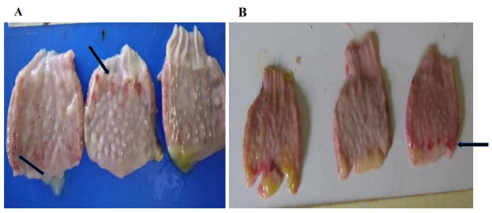

Figure 6: Gross lesions of proventricular mucosal haemorrhages in cockerels and pullets experimentally infected with velogenic Newcastle disease virus, at day 4 post infection. [A – Cockerels; B – Pullets]

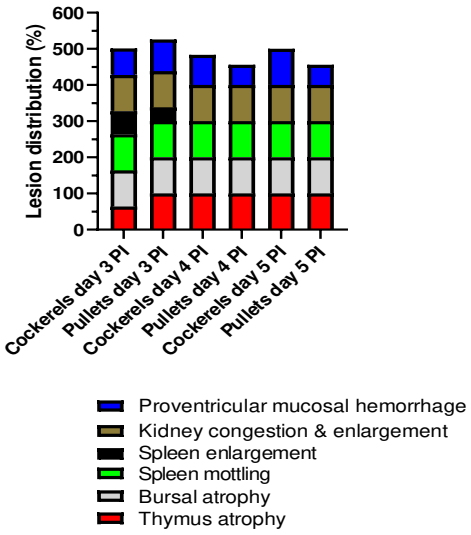

Figure 7: Distribution of gross lesions (%) in organs of cockerels and pullets experimentally infected with velogenic Newcastle disease virus.

Results of the histopathological evaluation of sections of organs harvested at necropsy on day 3 and day 4 PI are presented in Figure 8. Lymphocytic necrosis, depletion, and fibrin deposition were observed around the sheathed

arterioles in the spleen of infected cockerels and pullets (Figures 8A and D). However, hyperplasia of the follicular epithelium was observed in infected cockerel on day 4 PI, which was absent in infected pullets (Figures 8 A and D). The bursa of Fabricius in the infected groups showed lymphocytic necrosis and depletion (Figures 8 B and E). Similar lesions were observed in sections of the thymus (Figures 8 C and F).

The packed cell volume (PCV), haemoglobin concentration (Hb), and red blood cell (RBC) counts of infected cockerels were significantly higher ($p < 0.05$) than those of the uninfected cockerels on day 4 PI, but there were no significant differences ($p > 0.05$) between the PCV, Hb and RBC counts of the infected and uninfected pullets, though the values for the infected pullets were relatively higher than that of the uninfected (Table 1). There were no significant differences ($p > 0.05$) between the white blood cell counts (total and differential) of the both the infected and uninfected cockerels and pullets (Table 2), but relative to the uninfected controls, there was slightly lower total white blood cell, heterophil and lymphocyte counts of the infected cockerels on day 4 PI, while for pullets, the opposite was recorded; infected pullets has a slightly higher total white blood cell, heterophil and lymphocyte counts when compared to their uninfected controls (Table 2). There was also a slightly higher monocyte count in the infected cockerels relative to the uninfected, and a slightly lower eosinophil monocyte count in the infected pullets relative to the uninfected (Table 2).

The serum total protein levels of the infected pullets was significantly higher ($p < 0.05$) than that of the uninfected pullets, but there was no significant difference ($p > 0.05$) between the mean serum total protein level of the infected and uninfected cockerels (Table 3). For both the cockerels and pullets, there were no significant differences between the serum albumin levels of both the infected and uninfected (Table 3). However, the serum globulin levels in both infected cockerels and pullets were significantly ($p < 0.05$) higher than those of their uninfected controls (Table 3).

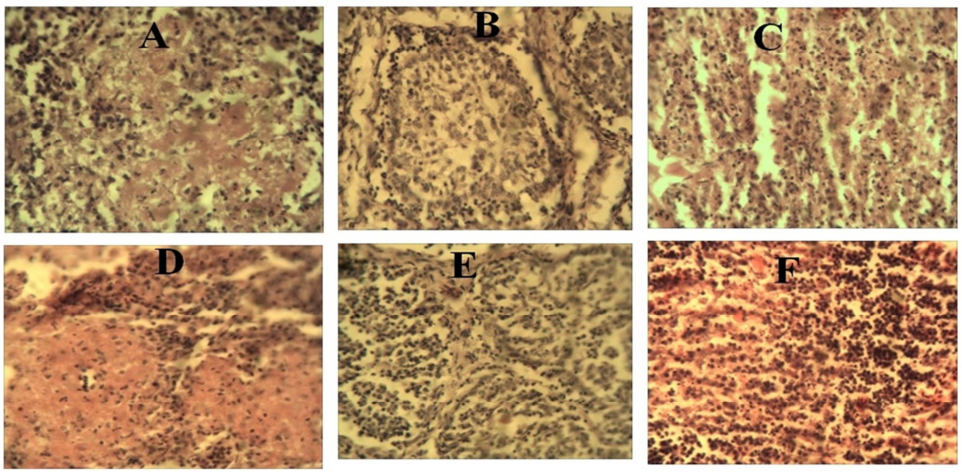

Figure 8: Histologic sections of the spleen, thymus, and bursa of Fabricius of cockerels and pullets experimentally infected with velogenic Newcastle disease virus, on day 4 post-infection (H & E, × 400). A and D - Spleen of an infected cockerel and pullet, respectively, showing lymphocytic necrosis, depletion, and fibrin deposits around the sheathed arterioles; B – Bursa of infected cockerel showing lymphocytic necrosis, depletion, and hyperplasia of follicular epithelium; C – Thymus of infected cockerel showing lymphocytic necrosis and depletion; E – Bursa of infected pullet showing lymphocytic necrosis and depletion; F – Thymus of infected pullets showing lymphocytic necrosis and depletion.

Table 1: Erythrocytic profile (means ± standard deviation) of cockerels and pullets infected with velogenic Newcastle disease virus, compared with uninfected controls, at day 4 post-infection.

Parameters	Infected Cockerels	Uninfected Cockerels	Infected Pullets	Uninfected Pullets
Packed cell volume (%)	41.00 ± 7.07 [a]	26.50 ± 3.55 [b]	34.63 ± 9.39	29.63 ± 1.55
Haemoglobin concentration (g/dl)	10.86 ± 0.74 [a]	7.93 ± 0.69 [b]	9.56 ± 2.63	9.31 ± 0.76
Red Blood Cell counts (10^6/µL)	3.02 ± 0.58 [a]	2.41 ± 0.28 [b]	2.67 ± 0.26	2.81 ± 0.23

[a, b] Superscripts represent a significant difference between the means of infected and uninfected cockerels. ($p < 0.05$).

Table 2: Total and absolute differential white blood cell counts (means ± standard deviation) of cockerels and pullets infected with velogenic Newcastle disease virus, compared with uninfected controls, at day 4 post-infection.

Parameters	Infected Cockerels	Uninfected Cockerels	Infected Pullets	Uninfected Pullets
Total white blood cell counts (10^3/µL)	15.63 ± 0.18	17.56 ± 2.37	16.69 ± 2.50	14.79 ± 2.88
Absolute heterophil counts (10^3/µL)	6.41 ± 0.37	7.52 ± 1.16	6.92 ± 1.15	6.36 ± 1.91
Absolute lymphocyte counts (10^3/µL)	8.52 ± 0.42	9.57 ± 3.40	9.25 ± 2.22	7.79 ± 1.26
Absolute monocyte counts (10^3/µL)	0.40 ± 0.11	0.36 ± 0.17	0.31 ± 0.17	0.24 ± 0.24
Absolute eosinophil counts (10^3/µL)	0.24 ± 0.11	0.42 ± 0.17	0.21 ± 0.17	0.33 ± 0.07
Absolute basophil counts(10^3/µL)	0.08 ± 0.11	0.04 ± 0.08	0.00 ± 0.00	0.08 ± 0.09

No significant differences between the infected and uninfected groups in all the leukocytic parameters ($p > 0.05$)

Table 3: Serum total protein, albumin and globulin levels (means ± standard deviation) of cockerels and pullets infected with velogenic Newcastle disease virus, compared with uninfected controls, at day 4 post-infection.

Parameters	Infected Cockerels	Uninfected Cockerels	Infected Pullets	Uninfected Pullets
Total proteins (g/dl)	2.98 ± 0.65	2.38 ± 0.46	3.20 ± 0.40 [x]	2.38 ± 0.31 [y]
Albumin (g/dl)	1.76 ± 0.48	1.53 ± 0.54	1.88 ± 0.42	1.50 ± 0.21
Globulin (g/dl)	1.22 ± 0.29 [a]	0.84 ± 0.3 [b]	0.88 ± 0.39 [x]	1.32 ± 0.18 [y]

[a, b] Superscripts represent a significant difference ($p < 0.05$) between the means of infected and uninfected cockerels, while [x, y] superscripts represent significant differences between the means of infected pullets ($p < 0.05$).

Discussion and Conclusion

The higher cumulative morbidity and mortality recorded for the infected cockerels on days 2, 3 and 4 post-infection and the greater severity of lesions and haematological alterations recorded in infected cockerels relative to infected pullets in this study is thought to be as a result of the earlier reported sexual dimorphism between females and males in susceptibility to and severity of infectious diseases (Vasquez-Martinez *et al*., 2018; Gay *et al*., 2021). It has been reported that in general, males of all ages are more susceptible to gastrointestinal and respiratory infections while females are more susceptible to genitourinary tract infections (Vasquez-Martinez *et al*., 2018; Jaillon *et al*., 2019; Shepherd *et al*., 2021). Newcastle disease is known to be characterized by gastrointestinal and respiratory pathology (Etriwati, 2017; Rohollahzadeh *et al*., 2018). Sexual dimorphism in disease susceptibility and severity has been attributed to the differences in levels of sex hormones between males and female as well as genetic factors/chromosomal control of immunity (Vasquez-Martinez *et al*., 2018; Gay *et al*., 2021; Shepherd *et al*., 2021). It has been reported that the major female sex hormone (estradiol) confers protective immunity, while the primary male hormone (testosterone) suppresses anti-infectious responses (Marriot and Huet-Hudson, 2006; Vasquez-Martinez *et al*., 2018; Jaillon *et al*., 2019; Gay *et al*., 2021; Shepherd *et al*., 2021). Females are therefore more prone to develop enhanced innate and adaptive immune responses and also robust and potentially protective humoral and cell-mediated immune responses following antigen stimulation than males, and are thus less susceptible to a variety of infections and diseases than males (Marriot and Huet-Hudson, 2006; Jaillon *et al*., 2019; Gay *et al*., 2021).

The clinical signs recorded in this study for both infected groups (cockerels and pullets) and the rapid onset of morbidity after infection with the Newcastle disease virus is consistent with earlier reports in experimental Newcastle disease (Okoye et al., 2000; Igwe, 2009; Igwe *et al*., 2018; Onyema *et al*., 2019; Okechukwu *et al*., 2020). Onyema *et al*., (2019) reported rapid onset of morbidity when pullets and broilers were challenged with vNDV, and the greenish-white diarrhea (indication of a gastrointestinal lesion) recorded in the infected groups in this study is in agreement with a previous report in chickens by Okoye *et al.,* (2000) and Onyema *et al*. (2019). The nervous signs such as staggering, leg and wing paralysis, shivering, and drooling of saliva which were observed at the later phase of infection was an indication of virus replication in the brain and had been earlier reported by Okechukwu *et al.* (2020).

The significantly lower body weights of the infected cockerels is believed to be due to the greater severity of the disease in them, as lower body weights or weight losses are commonly reported in septicaemic and viraemic diseases, in association with loss of appetite and concomitant poor feed and water consumption (Summerbell *et al*., 1993; Nakada *et al*., 2022). Such body weight reductions in Newcastle disease virus infection has been previously reported by Okoye *et al*. (2000), Eze *et al*. (2014a) and Onyema *et al*. (2019).

The early onset of mortality in the infected groups and the 100% mortality recorded in the study is consistent with earlier studies that reported that morbidity and mortality of the velogenic Newcastle disease outbreak could be up to 100% in non-immunized birds (Okoye *et al.,* 2000; Ashraf and Shah, 2014; Ganar *et al.* 2014).

The significantly higher mean PCV, Hb and RBC counts recorded for the infected cockerels and the relative higher values for these parameters recorded for the infected pullets is believed to be as a result of dehydration that resulted in haemoconcentration-induced polycythemia (Popkin *et al*., 2010; El-Sharkawy *et al*., 2015), as the infected sick birds were not eating and drinking. The greater severity of the relative polycythemia in the cockerels (in which the parameters were statistically significant between the infected and uninfected) is a reflection of the severity of the disease in this group, in comparison to the pullets in which the effects on

the erythrocytic indices were not statistically significant between the infected and uninfected groups. Our findings contrast with previous studies that reported no significant difference in the mean PCV over a six weeks post-infection period in vaccinated red and white cocks (Rwuaan *et al.,* 2009). The difference in the outcome of the study by Rwuaan *et al*. (2009) compared to this present study could be due to the fact that cocks used in their study were vaccinated, which prolonged the infection/disease period.

Though there were no statistically significant alterations in the total and differential leukocyte counts, it is noteworthy that while the total white blood cell, lymphocyte, heterophil and eosinophil counts of the infected cockerels were relatively lower than that of the uninfected, the opposite was recorded in pullets: the total white blood cells, lymphocyte, heterophil and monocyte counts of the infected pullets was higher than that of the uninfected. Increase in total white blood cell count, lymphocyte, heterophil and monocyte counts are positive/favourable prognostic signs, while decrease in these cell numbers are negative/unfavourable prognostic signs that may bother a clinician (Campbell, 1994; Wakenell, 2010).

The higher serum total protein levels that was recorded in the infected groups, which was mainly due to their significantly higher serum globulin levels is believed to be part of the body's immune response in terms of increase in immunoglobulin levels to the viral infection (Rasmussen *et al*., 1982; Philips *et al*., 2015). Higher serum total proteins and globulin levels had earlier been reported in chickens infected with Newcastle disease virus (Eze et al. 2014a), though a report by Rwuaan *et al* (2009) on vaccinated cocks that were infected with the Newcastle disease virus showed no significant differences in the serum protein profile of infected cocks when compared with uninfected ones.

In conclusion, this study has shown that unvaccinated cockerels infected with Newcastle disease virus exhibited greater susceptibility, higher cumulative morbidity and mortality, and more severe pathological lesions and haematological alterations when compared to unvaccinated infected pullets. The outcome of this study further re-enforces the concept and idea of sexual dimorphism in the susceptibility to and severity of infectious diseases in animals and humans.

Animal ethics approval

Approval for this research work was obtained from the University of Nigeria Animal Care and Use Committee. Throughout the study, the principles of humane laboratory animal care were followed.

Declaration of conflicting interests

The authors declare no potential conflicts of interest with respect to the research, authorship and publication of this article.

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