

Counter- Pathologic Potentials of Aqueous Neem (*Azadirachta Indica*) Leaf Extracts in Cockerels Vaccinated and Experimentally Infected with Infectious Bursal Disease Virus

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Abstract: The aim of the present work was to study the effects of neem leaf aqueous extracts on the pathologic lesions associated with infectious bursal disease (IBD) and the current vaccine being used as a preventive measure against the disease. Four hundred and eighty (480) day old cockerels were used and allocated into 8 groups. The birds were grouped as vaccinated/ unvaccinated, challenged/ unchallenged, neem leaf treated/ untreated groups. The IBD vaccines (intermediate plus strain) were given at 14 and 28 days of age while the experimental infection using very virulent IBD virus was inoculated at 35 days of age and the extracts were given from day old to 6 week old. Post- mortem examination, histopathology and immunohistochemistry were conducted. The results obtained showed pathologic lesions in both the vaccinated and challenged groups but the lesions were more severe in the challenged groups. However, in the neem leaf treated groups that were challenged only mild to moderate lesions were observed and in the vaccinated groups no lesions observed. Furthermore, the immunohistochemistry revealed minimal immunoreactivity in the neem leaf treated groups when compared to the neem- untreated groups. These results indicated that both the IBD vaccinal and field strains have serious deleterious effects on tissues while the neem leaf extracts have the ameliorative potentials at both gross and histopathologic levels.

Keywords: Infectious bursal disease, neem leaf aqueous extracts, vaccine, challenge, cockerels, pathology.

1. INTRODUCTION

Infectious bursal disease (IBD) also referred to as Gumboro disease has been considered as one of the important viral disease of commercial chickens threatening the poultry industry worldwide (Müller *et al.*, 2003). It has been reported that, low return on investment has been experienced due to IBD outbreaks in the poultry industries of both developing and developed nations (Sainsbury, 2000). The disease is caused by IBD virus and is an acute and highly contagious disease in chickens at 3 weeks of age and older. There are two distinct serotypes of IBD virus namely: serotype 1 and serotype 2. Both serotypes can infect chickens and turkeys but the clinical disease is recognised only in chickens (Yamaguchi *et al.*, 1996). Only serotype 1 is virulent for chickens, replicating within the lymphoid cells of the bursa of Fabricius and destroying them, thereby inducing immunosuppression (Lukert and Saif, 2008). It causes high mortality and immunosuppression in recovered chickens leading to variety of secondary infections and decreased response to

vaccinations, which results in an important economic impact to the poultry industry worldwide (Vandenberg *et al.*, 2000). Infectious bursal disease virus (IBDV) targets the bursa of Fabricius (BF), causing damage by destroying developing B-lymphocytes. Several clinical forms are observed in the field ranging from mild immunosuppression to high mortality (90 to 100%) (Vandenberg *et al.*, 2000). Different modified live vaccines (MLVs) containing classical or variant viruses are commercially available and classified according to their degree of attenuation as mild, intermediate, intermediate plus and hot IBD vaccines (Vandenberg *et al.*, 2000; Müller *et al.*, 2003). Mild and intermediate vaccines are safer in that they cause less bursal damage than hot vaccines, but have poor efficacy in the presence of maternally derived antibody (MDA) and against very virulent IBD viruses (vvIBDVs). In contrast, less attenuated strains (intermediate plus and hot vaccines) can overcome higher levels of MDA, but cause more severe lesions in the bursal follicles, resulting in immunosuppression (Prandini *et al.*, 2008). Clinical outbreaks in vaccinated flocks are reported (Islam *et al.*, 2008). Neutralization of the vaccine viruses by the MDA has been considered as one of the factors for causing vaccination failure. To overcome this problem, relatively virulent strains of vaccine viruses with higher residual pathogenicity for example intermediate strains and intermediate plus strains have been introduced (Kouwenhoven and Van den Bos, 1994). The better protection with more virulent strains of IBD virus is due to more antigenic stimulation based on higher and longer replication in lymphoid tissues (Rautenschlein *et al.*, 2001). However, these intermediate vaccine strains may produce moderate to severe bursal lesions and immunosuppression in vaccinated chickens as reported by Franciosini and Coletti (2001) and Boudaoud and Alloui (2008).

Research has therefore been targeted at improving the immunogenicity of the vaccines by using antioxidants such as Vitamin C (Pardue 1987; Okoye *et al.*, 2000). Reports on their effectiveness or otherwise are conflicting and therefore inconclusive, possibly due to difference in the virulence of the virus strains or weather conditions (Okoye, 2005).

Researches into natural products for solving health problems have been encouraged by the World Health Organisation (WHO, 2013) and Food and Agricultural Organisation (FAO, 1995).

History reveals the long journey of omnipotent Neem tree from old legendary time to present state defining its role in the sustainability of humankind. In the old annals of the ancient Siddha medicinal system the first medicinal plant described was Neem or Margosa. In ancient immemorial period Neem has been used as a disincentive agent against highly contagious smallpox and other infectious diseases and was also regarded to defend against evil spirits from time (Kumar and Navaratnam, 2013). In old medicinal system medications and applications were compiled over palm leaves and they have been passed on from generations to generations. In the Indus civilization the use of Neem tree is as old as 4500 years during the period of Harappa culture (one among the great civilisation in the world). There the earliest of the documentation mentions the fruits and seeds; oils and leaves; roots and barks for the medical characters that are advantageous (<http://www.slideshare.net/imulla/neem-presentation-882503>). Writing palm leaf manuscript is among the oldest medium of conserving knowledge in India to store the history of herbal heritage. Centre for Traditional Medicine and Research (CTMR), Chennai. India revealed the medicinal uses of different parts viz, fruits, seeds, leaves, roots, bark etc., of Neem trees. It explains use of neem flower against bile disorders, neem leaves to prevent and treat ulcers and neem bark to brawl against paralysis and CNS disorders (Bandyopadhyay *et al.*, 2004). Old evidences obtained from two great civilisations Harappa and Mohenjo-Daro of ancient world also witnessed that *A. indica* was the prominent herb of therapeutic importance at that time not only in Indian context but in world as well. According to epic of Mahabharata, Nakul and Sahadeva used Neem oils for treatment of wounds in horses and elephants (Bandyopadhyay *et al.*, 2004).

2. MATERIALS AND METHODS

2.1 Study Area:

The study was conducted in Sokoto, in the Poultry pen of the Faculty of Veterinary Medicine, Usmanu Danfodiyo University, Sokoto. Sokoto State is geographically located to the North Western part of Nigeria between the longitudes 4° 8'E and 6° 54'E, and latitudes 12° and 13° 58'N (NPC, 2006). The state falls within two vegetation zones: the Sudan Savannah and Northern Guinea Savannah. The climate is characterized by altering dry and wet seasons with short cold and dry period of harmattan usually accompanied by dust-laden winds and fogs which start from October and last through February. The duration and intensity of annual rain fall ranges from 60-160 days and 635-1000mm (occurring between May to October) respectively. The mean monthly temperature is generally 20-46° C, relative humidity ranges from 12-17% with the highest occurring in August (NPC, 2006).

2.2 Experimental Birds:

Four hundred and eighty (480) day old cockerels for the study which were purchased from a commercial hatchery (Farm support^R) in Ibadan. The experimental birds were raised for 8 weeks.

2.3 Housing and Feeding:

The birds were managed on deep litter system in cleaned formalin- potassium fumigated pens. One 200 watt electric bulb was fixed to provide warmth to the birds for each of the groups with additional source of heat around the pens. The pen temperature was maintained at 33 to 35⁰C for 1 to 3 weeks old and 28 to 29⁰C for the remaining 3 to 8 weeks old. They were fed on commercial feed; chick mash (Animal care^R) for the 8 weeks but the treated water was given from day old to 6 weeks. The feed and water were provided *ad libitum*.

2.4 Biosecurity Measures:

Strict biosecurity measures were taken for all the groups. Moreover, the challenged groups were separated into a different pen entirely, employing 2 well trained poultry attendants to routinely feed the birds, disinfect themselves, watering and feeding utensils as well as the environment in order to prevent the spread of microorganisms into or outside the poultry pen. Each of the attendants was responsible for particular groups of birds (unchallenged: groups A to D and challenged: groups E to H). Groups A to D were raised in the main Faculty of Veterinary Medicine poultry pen while groups E to H within a separate pen. Complete personal protective wears were used during vaccination and experimental infection which were incinerated immediately after used. At the entrance of each pen, footbath was provided containing 2% formalin and shoes were provided and hanged just at the interior side of the pens door for use specifically within the pens. At the end of the experiment, the whole pens were fumigated with formalin- potassium composition while the litter was removed and incinerated.

2.5 Preparation and Extraction of Neem (*Azadirachta indica*) leaf Aqueous Extracts:

Mature green neem leaves were used for the experiment. The leaves were obtained from Shehu Kangiwa Square and a botanist from UDUS herbarium professionally identified the Neem and labelled it (UDUH/ANS/0004). The leaves were rinsed in distilled water, air dried and pulverized. The extract was prepared following the procedure reported by (Sithisarn *et al.*, 2006).

The concentration of the aqueous Neem leaf extract used was 3.0mg/ml which was administered through drinking water for 6 weeks. The choice of the concentration was based on the earlier preliminary work done on the safety margin of the extract.

2.6 Vaccine:

A live IBD (intermediate plus strain) vaccine was used for the study which was sourced from an Agro- Veterinary Company in Kaduna, Nigeria. The vaccination was carried out at 2 and 4 weeks of age via oral route.

2.7 Challenge IBD Virus:

At day 35 the birds in groups E, F, G and H were challenged orally with 0.1ml of a live vvIBD virus containing 10^{9.76}CID/ml.

2.8 Experimental Design:

2.8.1 Grouping:

The 480 birds were assigned randomly into eight groups (A to H) with 60 birds per group. Group A was the negative control group and therefore, neither receive the aqueous extracts nor were they vaccinated against IBD. Group B was the positive IBD- vaccinated control group, thus, did not receive the aqueous extracts but was vaccinated against IBD. Group C, D, G and H were treated with 3.0mg/ml of Neem leaf aqueous extracts in drinking water from day old to 6 weeks. Group C, F and G were vaccinated against IBD while D, E and H were not vaccinated against the IBD. Birds in group E, F, G and H were challenged with vvIBD virus while those in group A, B, C and D remained unchallenged.

2.8.2 Vaccine and Vaccination:

Birds from groups B, C, F and G were vaccinated against IBD at 14 and 28 days of age with IBD intermediate plus vaccine containing $\geq 10^3$ EID₅₀/ml, 0.2ml/bird via oral route while groups A, D, E and H remained IBD-unvaccinated.

2.8.3 Experimental Infection:

Birds from group E, F, G and H were experimentally challenged orally with 0.2ml of vvIBD virus at 35 day of age (5 week old) while A, B, C and D were not challenged.

Table 1: Experimental Design

Group	IBD Vaccination Status	Experimental Challenge with vvIBDV	Neem Extract Treatment
A	Unvaccinated	Unchallenged	No Extracts given
B	Vaccinated	Unchallenged	No Extracts given
C	Vaccinated	Unchallenged	3.0mg/ml Extract
D	Unvaccinated	Unchallenged	3.0mg/ml Extract
E	Unvaccinated	Challenged	No Extracts given
F	Vaccinated	Challenged	No Extracts given
G	Vaccinated	Challenged	3.0mg/ml Extract
H	Unvaccinated	Challenged	3.0mg/ml Extract

2.9 Post- mortem Examination:

Post-mortem examination was conducted (5 birds from each group) at one week post each experimental IBD vaccinations and experimental challenge (that was, at day 21, 35 and 42) and the lesions were recorded.

2.10 Organs/ Tissues Sample Collection:

Bursa of Fabricius were taken from five birds in each group at one week post each IBD- vaccine administration and vvIBD virus challenge after being euthanized for histopathology as well as immunohistochemistry where necessary.

2.11 Histopathological Studies:

At one week post each IBD- vaccine administration as well as one week post vvIBDV challenge, 5 bursae from each group were collected following humane killing and preserved in 10% formal-saline for histopathology and immunohistochemistry where applicable.

The collected samples were trimmed to the thickness of 5mm in size using microtome machine (Type 300, serial number 8706364, Anglia Scientific, Cambridge, England), fixed and dehydrated in a series of alcohol concentrations and embedded in paraffin wax. Sectioning of the tissue was done to a thickness of 5 micrometer on a microtome. The tissue was mounted on glass slide, dewaxed and stained with Haematoxylin and Eosin (H and E).

The tissues were examined using x4, x10 and x40 objectives for histopathological changes.

The B. F, after observing on the glass slides were subjectively graded as previously reported by (Babiker *et al.*, 2008) as follows:

0: Normal follicles

1: Follicles showing scattered lymphoid depletion in the cortex and medulla

2: Follicles showing lymphoid depletion and necrosis in less than 50% of the cells and mild interfollicular fibrosis

3: Follicles showing lymphoid depletion and necrosis in more than 50% of the cells and moderate interfollicular fibrosis

4: Follicles showing glandular transformation of epithelium, cystic changes and marked interfollicular fibrosis

2.12 Immuno- Histochemistry:

The viral antigen in the bursa of Fabricius at one week post challenge (that was at 42 day old) was detected by immunohistochemistry (IHC) using IHC kit sourced from BioChain® Catalog Number: K3181100, USA in the year 2015

while the primary antibody used was Mouse monoclonal anti- IBD (standard) antibody ab31672 sourced from abcam[®] company, USA in the year 2015 . Briefly, the tissues of the BF were mounted on silane coated slides obtained from Muto Pure Chemicals Co., LTD, China. The tissues on the slides were soaked in xylene twice, each for 15 minutes for deparaffinization. The slides were then incubated in a graded series of ethanol (100%, 95%, 90%, 80% and 70%) 5 minutes for each solution for rehydration. They were incubated in water for 5 minutes. The slides were immersed in 0.3% H₂O₂ for 30 minutes at room temperature and then rinsed with water followed by PBS once and the tissue sections were the circled with Pap Pen obtained from Dako[®], Denmark. The slides were incubated with 1% normal serum/ PBS (3.5ml PBS + 35µl normal serum in a tube) for 30 minutes at room temperature. The normal serum on the slides were dropped and then incubated with PBS diluted Primary antibody (1: 200) in a humidified chamber overnight. Slides were rinsed with PBS 3 times for 5 minutes each and incubated with PBS diluted biotinylated anti- mouse IgG (secondary antibody) (1.4ml: 35µl) for 30 minutes which were rinsed with PBS 3 times each for 5 minutes thereafter, detection solution was added to the tissue sections which were incubated for 30 minutes and finally rinsed using PBS for 3 times, each for 5 minutes. Development solution was then added to cover the tissue for 5- 30 minutes and the tissues were then soaked in water to stop the reaction and then counter stained using Harris' Haematoxylin. The tissues were soaked in graded series of alcohol (70%, 90% and 100%) for dehydration, 3 minutes for each solution, incubated in Xylene twice, 10 minutes each. The slides were then mounted on microscope.

2.13 Statistical Analysis:

The obtained data from P. M. Lesions, histopathology and IHC and were recorded and tabulated as well as analysed using descriptive statistics where necessary.

3. RESULTS

3.1 Gross Pathology:

3.1.1 Gross Pathology of IBD Vaccinated Groups:

There was petechial haemorrhage on the thigh muscle of the vaccinated control group B. Also found in the group was the oedematous bursa of Fabricius with petechial haemorrhage. However, no gross lesions observed in the treated- vaccinated group C.

3.1.2 Gross Pathology of vvIBDV Challenged Group:

Dehydrated carcasses, creamy- whitish diarrhea and severe ecchymotic haemorrhages on the keel and medial aspect of the thigh muscle were observed in group E. Severe ecchymotic haemorrhage was seen at the lateral aspect of the thigh muscle in group F. Pin point haemorrhagic lesion was observed in group G while severe ecchymotic and moderate petechial haemorrhages were found in the carcass of group H.

Atrophic, congested and haemorrhagic bursa of Fabricius as well as enlarged and friable liver was observed in group E.



Plate 1: Petechial haemorrhages on the thigh muscle (arrow) of chicken in the untreated- vaccinated control group B



Plate 2: Severe ecchymotic haemorrhages (arrows) and creamy- whitish faeces (arrow head) on the carcass of chicken in group E following challenge with vvIBDV at 5 weeks



Plate 3: Ecchymotic haemorrhages (arrows) on the carcass of chicken in group F following challenge with vvIBDV at 5 weeks



Plate 4: Pin point haemorrhage (arrow) on the thigh muscle of chicken in group G following vvIBDV challenge at 5 weeks



Plate 5: Moderate ecchymotic (arrow head) and petechial haemorrhages (arrows) of chicken in group H following challenged with vvIBDV at 5 weeks

Table 2: Frequency (%) of Observed Postmortem Lesions Following Experimental Challenge of Five Weeks Old Chickens with vvIBDV

Postmortem Lesions	Groups							
	A	B	C	D	E	F	G	H
Enlarged BF	0	0	0	0	20	0	0	0
Atrophic BF	0	0	0	0	80	100	0	20
Oedema BF	0	40	0	0	60	100	0	20
Haemorr BF	0	40	0	0	100	100	20	30
Skeletal Musucular Haemorrhages	0	12	0	0	100	100	20	60
Enlarged Liver	0	0	0	0	40	20	0	0
Haemorrhagic intestine	0	0	0	0	80	20	0	20
Increased mucus Intestine	0	0	0	0	40	60	40	60
Enlarged Spleen	0	0	0	0	0	0	0	0

3.2 Microscopic Lesions:

3.2.1 Microscopic Lesions Observed in Experimental IBD Vaccinated Chickens (Group B and C):

Moderate cortico- medullar follicular lymphoid depletion, interfollicular oedema and haemorrhages were observed in the BF of chickens in group B.

Mild follicular lymphoid depletion was observed in group C. However, no lesion observed in both group A and D.

3.2.2 Microscopic Lesions Observed in Experimental vvIBD Challenged Chickens (Group E, F, G and H):

Interfollicular oedema, severe lymphoid depletions and glandular transformation in the BF of chickens in group E.

Follicular atrophy, severe follicular lymphoid depletion, interfollicular oedema and follicular cyst were seen in the BF of chicken in group F.

Mild to moderate follicular lymphoid depletions were observed in group G.

Follicular atrophy, moderate lymphoid depletion and haemorrhages were observed in group H.

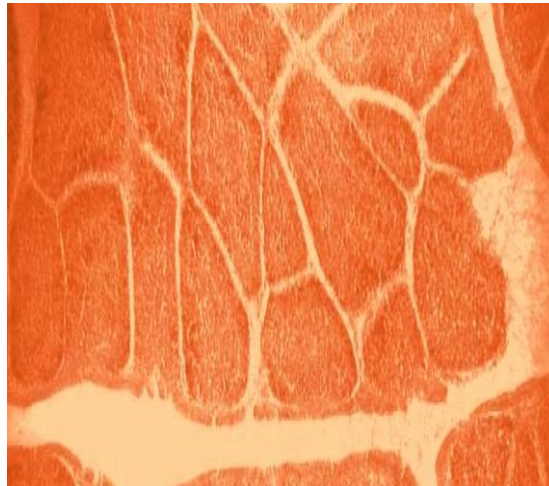


Plate 6: Showing normal lymphoid follicles in bursa of Fabricius of chickens in group A (untreated- unvaccinated and unchallenged negative control group). H and E $\times 100$

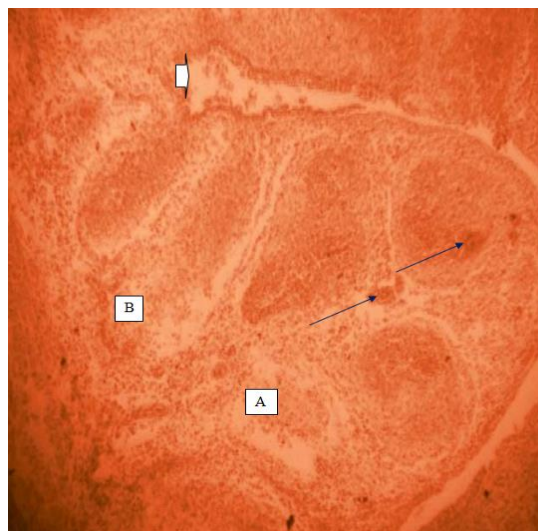


Plate 7: Showing cortical and medulla follicular destruction (A), lymphoid depletion (B), interfollicular inflammatory oedema (arrow head) and haemorrhages (arrows) in the BF of chicken in group B (untreated-vaccinated and unchallenged positive control group). H and E $\times 200$

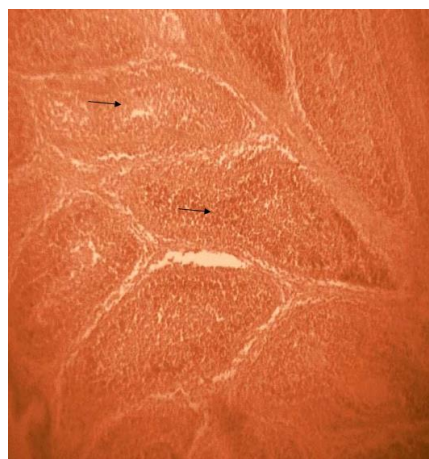


Plate 8: Showing mild follicular lymphoid depletion (arrows) in the BF of chickens in group C (treated- vaccinated and unchallenged group). H and E $\times 100$



Plate 9: Showing normal bursa of Fabricius of chickens in group D (treated- unvaccinated and unchallenged group). H and E $\times 100$

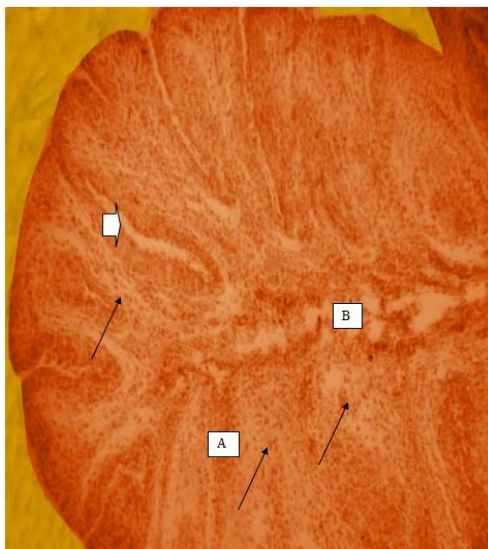


Plate 10: Showing interfollicular fibrosis (A), interfollicular oedema (B), severe lymphoid depletion (arrows) and glandular transformation (Arrow head) in BF of chickens in group E (untreated- unvaccinated challenged positive control group). H and E $\times 200$

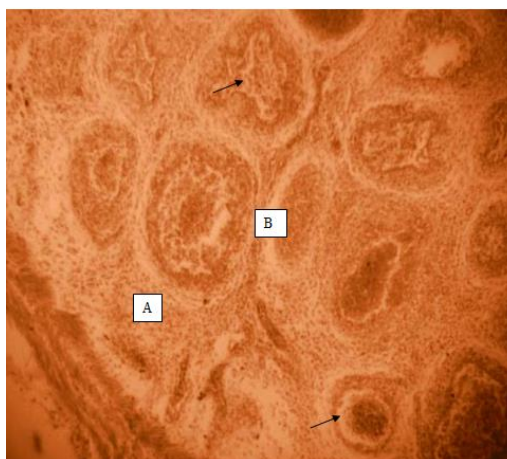


Plate 11: Showing severe follicular lymphoid depletion (A), interfollicular oedema (B) and follicular cyst (arrows) in the BF of chickens in group F (untreated- vaccinated challenged group). H and E $\times 200$

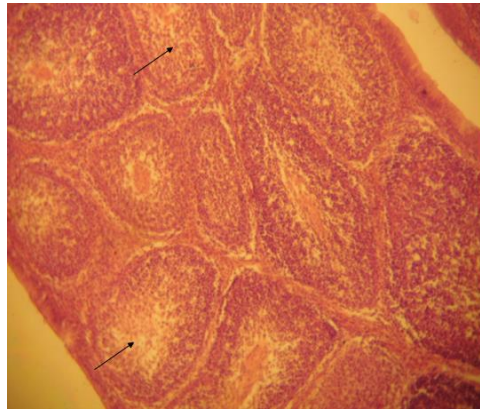


Plate 12: Showing mild to moderate follicular lymphoid depletion (arrows) in the BF of chickens in group G (treated- vaccinated and challenged group). H and E×200

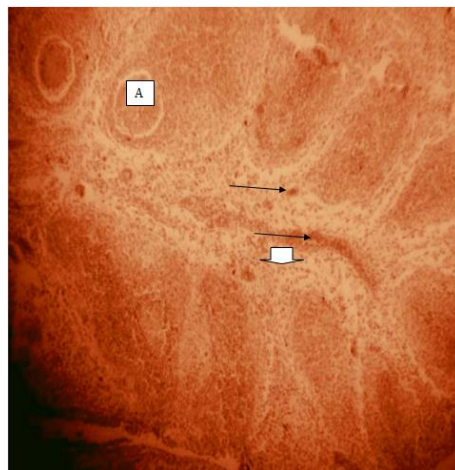


Plate 13: Showing cystic follicle (A) and mild to moderate follicular lymphoid depletion (arrow head) and haemorrhages (arrows) in the BF of chickens in group H (treated- unvaccinated and challenged group). H and E ×200

Table 3: Mean Bursal Histopathological Lesion Scores (BHS) of Chickens at One Week Post Primary and Booster IBD Vaccinations and One Week Post vvIBDV Challenge

				BHS		
				Age (Days)		
Group	Extract	Vac	Chal	21(1wk ppv)	35(1wk pbv)	42(1wk pc)
A	-	-	-	0.0	0.2	0.0
B	-	+	-	2.2	3.1	1.3
C	+	+	-	0.0	1.2	1.4
D	+	-	-	0.0	0.0	0.0
E	-	-	+	0.0	0.0	4.0
F	-	+	+	1.2	2.5	3.5
G	+	+	+	1.3	1.2	2.0
H	+	-	+	0.0	0.2	3.1

KEY:

- Group A: Untreated, unvaccinated and unchallenged (negative control group)
- Group B: Untreated, vaccinated and unchallenged (vaccinated control group)
- Group C: Treated, vaccinated and unchallenged
- Group D: Treated, unvaccinated and unchallenged
- Group E: Untreated, unvaccinated and challenged (challenged control group)

Group F: Untreated, vaccinated and challenged
 Group G: Treated, vaccinated and challenged
 Group H: Treated, unvaccinated and challenged
 Wk ppv: Week post primary vaccination
 Wkpbv: Week post booster vaccination
 W pc: Week post challenge
 Vac: Vaccination
 Chal: Challenge

3.3 Infectious Bursal Disease Viral Antigen Detection by IHC:

The distribution and intensity of immunoreactivity of IBDV antigen using mouse monoclonal primary antibody in formalin- fixed- paraffin- embedded bursa of Fabricius tissue sections were detected by IHC staining at 1 week post challenged with vvIBDV. Group A and D showed negative immunoreactivity, group B showed moderate immunoreactivity, group C showed mild to moderate immunoreactivity, group E and F showed severe immunoreactivity while group G and H showed moderate immunoreactivity.

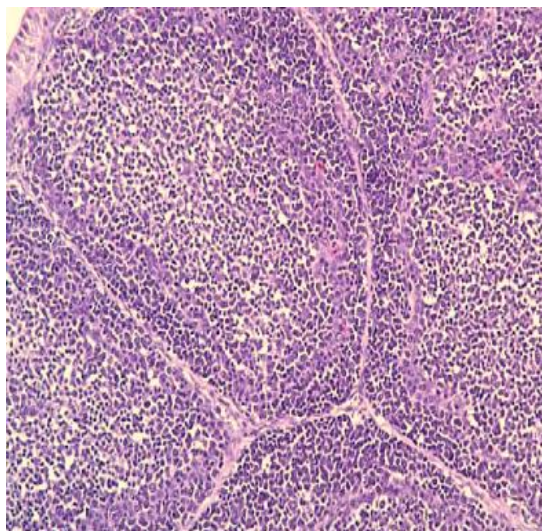


Plate 14: Showing normal bursal follicles of BF with negative immunoreaction of chicken in group A; untreated, unvaccinated and unchallenged (IHC counter with Harris Hematoxylin x 100)

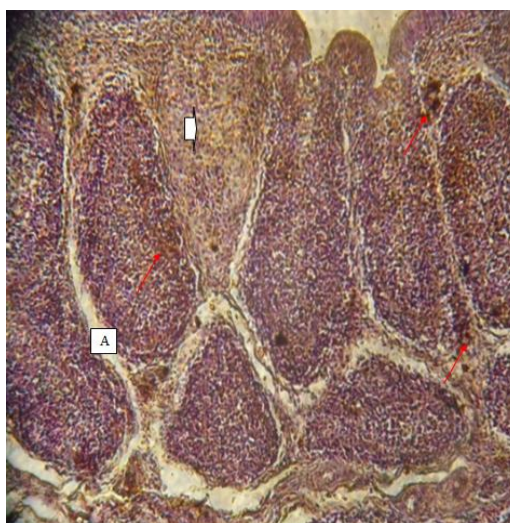


Plate 15: Showing prominent septum (A) with moderate viral immunoreactions (arrows) and follicular lymphoid depletion (arrow head) in BF of chicken in group B; untreated, vaccinated and unchallenged (IHC counter with Harris Hematoxylin x 200)



Plate 16: Showing minimal follicular depletion (A) with mild to moderate viral immunoreactions (arrows) in group C; treated, vaccinated and unchallenged (IHC counter with Harris Hematoxylin × 100)

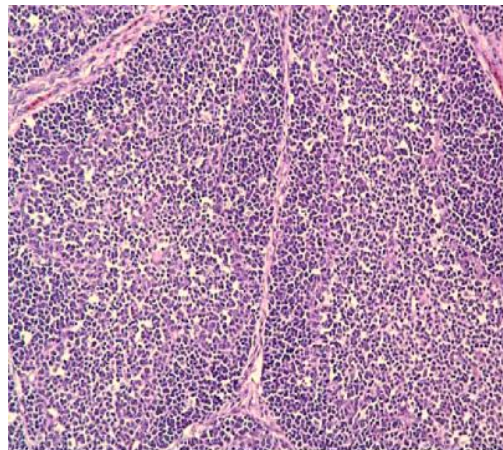


Plate 17: Showing normal bursal follicles with negative immunoreactions in group D; treated, unvaccinated and unchallenged (IHC counter with Harris Hematoxylin × 100)

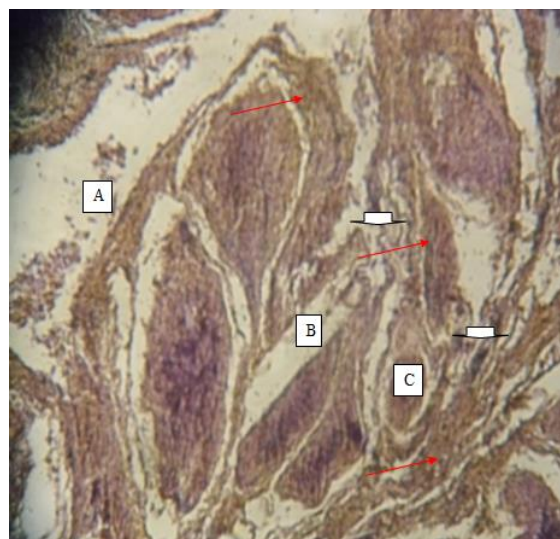


Plate 18: Showing severe follicular depletion (A), epithelial sloughing (arrow head), prominent septum (B), follicular atrophy (C) with moderate to severe viral immunoreactions (arrows) in group E; untreated, unvaccinated and challenged (IHC counter with Harris Hematoxylin × 400)



Plate 19: Showing severe follicular lymphoid depletion (A), prominent septum (B), follicular atrophy (arrow head) with severe viral immunoreactions (arrows) in group F; untreated, vaccinated and challenged (IHC counter with Harris Hematoxylin × 400)

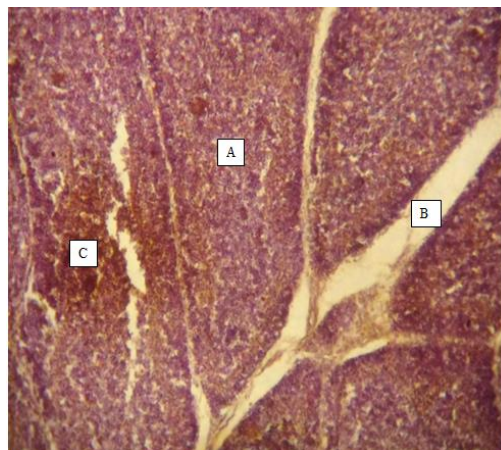


Plate 20: Showing minimal follicular depletion (A), prominent septum (B) with moderate viral immunoreactions (C) in group G; treated, vaccinated and challenged (IHC counter with Harris Hematoxylin × 200)

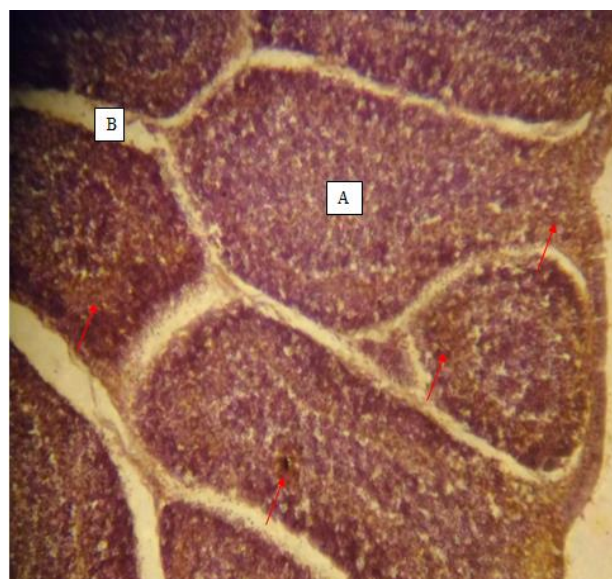


Plate 21: Showing moderate follicular depletion (A), prominent septum (B) and moderate viral immunolocalization (arrows) in group H; treated, unvaccinated and challenged (IHC counter with Harris Hematoxylin × 200)

4. DISCUSSIONS

In this research, the counter- pathologic potentials of aqueous extract of Neem (*Azadirachta indica*) leaf in cockerels vaccinated and/ or infected with IBD virus (IBDV) was studied.

The gross and histopathologic lesions observed in the challenged groups during this study were the same as reported by other researchers on IBD infections (Abdu, 2007; Cereno, 2013; De Wit and Baxendale, 2013).

The gross and histopathologic lesions (petechial haemorrhage on the thigh muscles, oedematous bursa of Fabricius with petechial haemorrhage, cortical and medulla follicular destruction, interfollicular inflammatory oedema, haemorrhages, follicular atrophy) observed post IBD vaccinations in group B further confirmed the adverse effects of live IBD vaccines on the BF and to a lesser extent on the skeletal muscles as the integrity of especially BF which is the primary lymphoid organ became compromised while the lesions observed in the BF of the treated groups were very minimal. Grossly, no lesions observed post IBD vaccine administrations in the liver, thymus, spleen and caecal tonsils in all the vaccinated groups. The lesions observed are in consistent with the work of other authors (Giambrone *et al.*, 2011; Moura *et al.*, 2007).

A week post challenge, the gross lesions observed in the challenged groups (E, E and H) are in consistent with the findings reported by Santos *et al.*, (2011) and Oluwayelu *et al.*, (2002). While for the challenged group G which had only pin point haemorrhages on the thigh muscles and congested BF could be attributed to the acquired immunity the group had following previous exposure through both primary and booster IBD vaccinations as well as the possible immunomodulatory effects of the extract given.

The histopathologic lesions observed post challenge, seen in the BF is in agreement with the findings as reported by Oluwayelu, (2002). The lower histopathologic lesions observed in group G is also attributed to the acquired immunity and the extract administration. The lesions in the BF so far reported in this study revealed that, the target organ for IBDV is BF as also been reported by Mahgoub, (2012) who stated that, the unique sensitivity of bursal lymphoid cells to IBDV suggests the possibility of specific viral receptors on a B- cell sub- populations that is most abundant in the BF. Moreover, Ingrao *et al.*, (2013) had shown that only non- immunoglobulin- bearing B- lymphoblast or IgM- bearing B- lymphocytes support viral replication whereas stem cells and peripheral B- cells do not. The lymphocyte depletions observed in this study could be as a result of phagocytosis of necrotic lymphocytes by macrophages in the bursal follicles. The cystic cavities formed in the BF post challenge following the necrosis and clearance by phagocytic cells and their eventual contraction might be the leading factor to the bursal atrophy which has been reported in most cases of IBD (Mahgoub, 2012).

Immunohistochemistry (IHC) staining from the present study indicates that, negative immunoreactivity was found in group A and D and this could be attributed to the fact that both groups had not been previously vaccinated with IBD vaccine and had not been exposed to field IBD virus. As such, there will not be reaction between the primary and secondary antibodies used during the technique on prepared bursal tissues. Group B that had moderate immunoreactivity could be associated to the IBD vaccine administered at 2 and 3 weeks of age. The mild to moderate immunoreactivity observed in group C indicates that even though the group had been vaccinated with IBD vaccine, the extract given might have interfered with the vaccinal viral replication within the BF and thus, the immunoreactivity was not as intense as that of group B. The severe immunoreactivity observed in groups E and F could be due to the vvIBDV effects on the BF while the moderate immunoreactivity seen in group G and H could be associated to the Neem leaves extract given which might have interfere with the vvIBDV replication and effects on the BF and hence low vvIBDV antigen expression on the BF when compared to chicks in group E and F. Tanimura *et al.*, (2013) reported that, the higher the presence of IBD viral antigen distribution and replication in the bursa of Fabricius, the more virulent strain of the IBD virus.

Conclusively, infectious bursa disease vaccines and vvIBDV used in this study causes more pathologic lesions on the bursa of Fabricius/ skeletal muscles when compared to those groups vaccinated/ challenged but were on the neem leaf extracts. Furthermore, severe immunoreactivity has been observed in vvIBDV challenged groups than those groups challenged with the same vvIBDV but were on Neem leaf aqueous extract. The extract ameliorates the IBD vaccinal and vvIBDV strain pathologic lesions in both the skeletal and smooth muscles in cockerels. The extract may serve as a good source of quality raw materials for food and pharmaceutical industries. Hence, we recommend that the Neem leaf aqueous extract should be given to birds pre and post vaccinations. Also, in the case of outbreaks, the extract can be used in our poultry industry as a therapeutic agent.

REFERENCES

- [1] Abdu, P.A. (2007). Viral Diseases. In: Manual of important poultry diseases in Nigeria. MacChin Multimedia Designer, Zaria, 15–24.
- [2] Babiker, M. A. A. and Tawfeeq, E. (2008). Role of administration routes of anti- IBD virus vaccines on immunization of chicken. *International Journal of Poultry Science*, 7 (3): 279- 282.
- [3] Bandyopadhyay, U., Biswas, K., Sengupta, A., Moitra, P., Dutta, P., Sarkar, D., Debnath, P., Ganguly, C. K. and Banerjee, R. K. (2004). Clinical studies on the effect of Neem (*Azadirachta indica*) bark extract on gastric secretion and gastroduodenal ulcer. *Life Sciences*, 75(24): 2867–2878.
- [4] Boudaoud, A. and Alloui, N. (2008). Evaluation of the safety of live attenuated vaccine viruses against infectious bursal disease (Gumboro disease) in conventional broiler chicks. *Revue scientifique et technique (International Office of Epizootics)*, 27(3): 793–802.
- [5] Cereno, (2013). Infectious bursal disease, causative agent, diagnosis and prevention. Poultry Industry Council 2:1–4. Available at: http://www.poultryindustrycouncil.ca/pdfs/factsheets/fs_146.pdf [Accessed September 13, 2015].
- [6] De wit, J. J. and Baxendale, W. (2013). Gumboro: Vaccination. *International Poultry Production*. <http://www.gumboro.com/control/vaccination/index.asp> [Accessed September 13, 2015].
- [7] Franciosini, M.P. and Coletti, M. (2001). Serological, Histological and Immune- Histochemistry Studies on Infectious Bursal Disease Vaccine Strains with Residual Pathogenicity. In Immunosuppressive viral diseases in poultry, *Proceedings 1999, European Commission Cost Action 839*: 199– 206.
- [8] FAO (1995). Medicinal Plants for Conservation and Health Care. Available at: <http://www.fao.org/publications/en/> [Accessed September 14, 2015].
- [9] Giambone, J. J., Partadiredja, M., Eidson, C. S., Kleven, S. H. and Wyatt, R. D. (2011). Interaction of aflatoxin with infectious bursal disease virus infection in young chickens. *Avian diseases*, 22(3): 431–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/212001>.
- [10] Ingraio, F., Rauw, F., Lambrecht, B., Vanden Berg , T. (2013). Infectious bursal disease: A complex host –Pathogen interaction. *Dev. Comparative Immunology*, 41: 429–438. [PubMed]
- [11] Islam, M. N., Rashid, S. M. H., Hoque, M. F., Juli, M. S. B. and Khatun, M. (2008). Pathogenicity of Infectious Bursal Disease virus related to outbreaks in vaccinated flocks and the causes of vaccination failure. *Journal of Innovation and Development Strategy*, 2: 22– 30. Available at: <http://ggfjournals.com/assets/uploads/22-30.pdf> [Accessed September 15, 2015].
- [12] Kouwenhoven, B. and Vanden Bos, J. (1994). Control of Very Virulent Infectious Bursal Disease in the Neitherslands with more Virulent Vaccines. In International Symposium on Infectious Bursal Disease and Chick Infectious Anaemia. *Rauischholzhausen, Germany*: 262– 271.
- [13] Kumar, V. S. and Navaratnam, V. (2013). Neem (*Azadirachta indica*): Prehistory to contemporary medicinal uses to humankind. *Asian Pacific Journal of Tropical Biomedicine*, 3(7): 505–514.
- [14] Lukert, P.D. and Saif, Y.M. (2008). Infectious Bursal Disease. In Y. M. Saif *et al.*, eds. Diseases of Poultry. Iowa: Iowa State Press, Ames, 161– 179.
- [15] Mahgoub, H. A. (2012). An overview of infectious bursal disease. *Arch. Virology*, 157: 2047–2057. [PubMed]
- [16] Moura, L., Vakharia, V., Liu, M. and Song, H. (2007). *In ovo* vaccines against IBD. *International Journal of Poultry Science*, 6 (11): 770- 775.
- [17] Müller, H., Islam, R. and Raue, R. (2003). Research on infectious bursal disease- the past , the present and the future. *Veterinary Microbiology*, 97: 153–165. Available at: www.elsevier.com
- [18] NPC (2006). National population commission: *Census report*, 106.

- [19] Okoye, J. O. A., Agu, A. O., Chineme, C. N. and Cheonwu, G. O. N. (2000). Pathological Characterization in Chicken of a Velogenic Newcastle Disease Virus Isolated from a Guinea Fowl. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, 53: 325–330.
- [20] Okoye, J.O.A. (2005). The Changing Faces of Infectious Bursal Disease in its Surveillance and Control. *In Proceedings of Workshop on Improved Disease Diagnosis, Health, Nutrition and Risk Management Practices in Poultry*. Zaria: Department of Veterinary Surgery and Medicine and Vet. Teaching Hospital, A. B. U., Zaria, 22–24.
- [21] Oluwayelu, D. O., Emikpe, B. O., Ikheloa, J. O., Fagbihum, O. A. and Adeniran, G. A. (2002). The pathology of IBD in cross breed of Harco cocks and indigenous hens. *Journal of Clinical Experimental Microbiology*, 3: 2
- [22] Pardue, S.L. (1987). Recent findings on vitamin C supplementation in poultry. In The role of vitamins on animal performance and immune response. Florida: *Roché Tech. Symp., Daytona Beach*, 4: 18–33.
- [23] Prandini, F., Bublot, M., Le Gros, F. X., Dancer, A., Pizzont, L. and Lamichlane, C. (2008). Assessment of the immune response in broilers and pullets using two ELISA kits after in ovo or day-old vaccination with a vectored HVT + IBD vaccine (VAXXITEK® HVT+IBD). *World Poultry Journal*, 19, p.21.<http://www.zootecnicainternational.com/article-> [Accessed October 24, 2015].
- [24] Rautenschlein, S., Yehand, H.Y. and Sharma, J.M. (2001). A comparison of the immunopathogenesis of different Infectious Bursal Disease virus strains. In Proceedings of the 11th International Symposium on Infectious Bursal Disease and Chick Infectious Anaemia. Rauschholzhausen, Germany: *Rauschholzhausen* 3: 311–323.
- [25] Sainsbury, D. (2000). Infectious Bursal Disease. In *Poultry Health and Management*. Blackwell Publishers, Oxford, UK: 125–126.
- [26] Santos, B. M., Realpe, M. I. and Periara, C. G. (2011). Occurrence of infectious bursal disease after vaccination with a low- passage attenuated virus. *Latin American Poultry Congress*, 5: 123.
- [27] Sithisarn, P., Supabphol, R. and Gritsanapan, W. (2006). Comparison of free radical scavenging activity of Siamese neem tree (*Azadirachta indica* A. Juss var. *siamensis* Valetton) leaf extracts prepared by different methods of extraction. *Medical Principles and Practice*, 15(3): 219–222.
- [28] Tanimura, N., Tsukamoto, K., Nakamura, K., Narita, M. and Maeda, M. (2013). Association between pathogenicity of infectious bursal disease virus and viral antigen distribution detected by immunohistochemistry. *Avian diseases*, 39(1): 9–20.
- [29] Vandenberg, T P., Eterradossi, N., Toquin, D. and Meulemans, G. (2000). Infectious bursal disease (Gumboro disease). *Revue scientifique et technique* (International Office of Epizootics), 19(2): 509–543.
- [30] WHO (2013). World Health Organisation Traditional Medicine Strategy, Geneva, Switzerland. Available at: www.who.int.
- [31] Yamaguchi, T., Kondo, T., Inoshima, Y., Ogawa, M., Miyoshi, M., Yanai, T., Masegi, T., Fukushi, H. and Hirai, K. (1996). *In vitro* attenuation of highly virulent infectious bursal disease virus: some characteristics of attenuated strains. *Avian diseases*, 40(3): 501–9. <http://www.ncbi.nlm.nih.gov>.