

EFFECTS OF NEEM (*Azadirachta indica*) LEAF AQUEOUS EXTRACTS ON HAEMATOLOGICAL PARAMETERS OF COCKERELS EXPERIMENTALLY INFECTED WITH INFECTIOUS BURSAL DISEASE VIRUS

Abstract

The aim of the study was to identify the effects of aqueous extract of Neem (*Azadirachta indica*) leaf on some haematological parameters of cockerels vaccinated and/ or infected with infectious bursal disease virus (IBDV). Four hundred and eighty (480) day old cockerels were purchased for the study 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 (vvIBDV) was inoculated at 35 days of age and the extracts were given from day old to 6 week old. Blood samples were collected at 1 week post each IBD vaccination and vvIBD virus challenge for haematology and the results were analyzed and recorded. The results obtained showed heterophilia and lymphopenia in the vaccinated groups while very virulent IBDV strain results in panleucopenia, lymphopenia and heterophilia. However, increased lymphocytes and total white blood cell counts (TWBC) was observed following Neem leaf aqueous extracts administration with resultant decrease in heterophils. These results indicated that neem leaf aqueous extracts enhances the immune status of the birds by increasing the TWBC and lymphocyte counts.

Keywords: Haematology, very virulent infectious bursal disease virus, neem leaf aqueous extracts, vaccine, cockerels.

1.0 Introduction

Infectious bursal disease (IBD) is an infectious, acute, mild or subclinical viral disease of young chickens characterized by trembling, incoordination, inflammation followed by necrosis and atrophy of the bursa of Fabricius (BF) resulting in immunosuppression following extensive destruction of lymphocytes (Abdu, 2007). Infectious Bursal Disease Virus (IBDV) is a small, non-enveloped virus, belonging to the genus *Avibirnavirus* of the family Birnaviridae, which is characterized by a bisegmented double stranded RNA genome which are packaged into single-shelled, non-enveloped virions (Pan *et al.*, 2007). Infectious bursal disease virus is classified into two serotypes (Serotype 1 and Serotype 2) by virus neutralization test (De wit and Baxendale, 2013). These two serotypes are antigenically distinct. Serotype 1 viruses are pathogenic to chickens and differ in their virulence (Vandenberg, 2000). They cause lesions in the BF by depletion of B- lymphocytes (Aihara *et al.*, 2015). Whereas, serotype 2 viruses are avirulent to chickens and isolated mainly from turkeys and chickens (Ismail and Saif, 2012). Four pathotypes of Serotype 1 viruses have been described based on virus virulence as mild, classic, variant and very virulent IBDVs (Ceren, 2013). Until 1985, the IBDV field strain (classical strains) isolated was of relative low virulence, causing only 1-2% of specific mortality, and outbreaks were

satisfactorily controlled by vaccination. However, since 1985 antigenic and pathogenic variant strains of IBDV, distinct from these classical strains, with increased specific mortality have been described in different parts of the world (Vandenberg, 2000). In the USA, new strains responsible for up to 5% of specific mortality were described (Rosenberger and Cloud, 1986). These virus isolates were designated antigenic-variant viruses since they were antigenically different from the classical strains isolated before 1985. They infected broiler chickens possessing relatively high levels of MA and were highly immunosuppressive (Box, 1989).

Later on, after 1988, in Europe, and subsequently in Japan new classes of pathotypic variants with high mortality rates (50-60% in laying hens and 25-30% in broilers) were observed (Lin *et al.*, 2007). These isolates caused up to 100% mortality in specific-pathogen-free (SPF) chickens and were therefore designated very virulent IBDV (vvIBDV) strains (Nunoya *et al.*, 1992). They rapidly spread all over Asia, Africa and to other major parts of the world (Vandenberg, 2000). It is still a problem in many parts of the world till date (Mbuko *et al.*, 2010).

In present scenario, interest is renewed in herbal medicines due to its less side effects and being safer. Interest and demand is increasing to obtain more drugs from plant sources to alleviate the ailments of humankind. The use of different parts of several medicinal plants to cure specific ailments has been in vogue form. The green medicine has been popular since ancient times being safe and multiple benefits. Neem is rich source of different compounds having medicinal properties, so drug development programme should be started utilizing the biological and medicinal properties of neem. In modern era, emphasis should be on control of diseases of human, animals and environment using non-toxic herbal products. By making quantum of research on biological and medicinal properties of neem, some of the herbal products have been prepared but still there is lot of scope in this field for better utilization of this wonder plant (Tiwari *et al.*, 2014). Thus, the need to know the effects of this important plant and the IBDV on some haematological parameters that are believed to play a key role in fighting against infections of chickens, since no data available to that effects in and around the area of the study.

2.0 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° 58N (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).

92 2.2 Experimental Birds

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

96 2.3 Housing and Feeding

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

104 2.4 Biosecurity Measures

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

118 2.5 Preparation and Extraction of Neem (*Azadirachta indica*) leaf Aqueous Extracts

119 Mature green neem leaves were used for the experiment. The leaves were obtained from
120 Shehu Kangiwa Square and a botanist from UDUS herbarium professionally identified the
121 Neem and labelled it (UDUH/ANS/0004). The leaves were rinsed in distilled water, air
122 dried and pulverized. The extract was prepared following the procedure reported by
123 (Sithisarn *et al.*, 2006). The concentration of the aqueous Neem leaf extract used was
124 3.0mg/ml which was administered through drinking water for 6 weeks. The choice of the
125 concentration was based on the earlier preliminary work done on the safety margin of the
126 extract.

127 2.6 Vaccine

128 A live IBD (intermediate plus strain) vaccine containing $\geq 10^3$ EID₅₀/ml was used for the
129 study which was sourced from an Agro- Veterinary Company in Kaduna, Nigeria. The
130 vaccination was carried out at 2 and 4 weeks of age via oral route.

131 2.7 Challenge IBD Virus

132 At day 35 the birds in groups E, F, G and H were challenged orally with 0.1ml of a live
133 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 $\geq 10^5$ 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.

151

Table 1: Experimental Design

153

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

154

155 **2.9 Blood Collection**

156 At week post each IBD vaccination and vvIBD virus challenge, blood samples from five
157 birds in each group were collected randomly for haematology. Using 21 gauge needle and
158 2 ml syringe blood was collected directly from the heart which was immediately
159 transferred into an EDTA bottle.

160 **3.18 Haematology**

161

162 Total leukocytes count and differential leukocytes count were determined as described by
163 Coles (1986) and Ganti (1983) respectively from the blood samples collected immediately
164 after the collection.

165 **3.18.1 Total Leucocyte Counts**

166

167 The blood was drawn up to 0.5 mark on the WBC blood cell diluting pipette. Glacial acetic
168 acid fluid was further drawn into the pipette to bring the fluid up to the mark 101 above the
169 bulb. The pipette was then shocked to mix the blood and the diluting fluid. The WBC was
170 then counted using an improved neubourhaemocytometer as described by Coles (1986) in
171 which a cover slip was placed on cleaned counting chamber and this make a space of 0.1
172 mm between the cover slip and the counting chamber. The diluted blood was then
173 introduced into the 0.1mm space of the counting chamber and was allowed to be settled for
174 a minute. The haemocytometer was then mounted on the light microscope at $\times 40$
175 magnification. The WBC in the larger 4 corner squares were counted and recorded.

176 Total leucocyte counts in μL = the sum of the cells counted in the larger 4 corner squares
177 $\times 50$.

178 **3.18.2`Differential Leukocyte counts**

179

180 A drop of blood was placed on one end of glass slide. A spreader slide would be placed in
181 front of the drop of blood at about 30° and pushed back till it just touched the drop. As
182 soon as the spreader touches the blood, it was pushed forward smoothly and quickly at an
183 angle of about 30° . The blood smears were air-dried for 2 minutes and stain with
184 Leishman's stain for 10 minutes. The smears were then gently rinsed with distilled water
185 and air-dried. The stained slides were examined under oil immersion objective in a
186 meander method to cover the entire slide. The cells (heterophils, basophils, eosinophils,
187 lymphocytes and monocytes) were counted with the aid of tally counter (Ganti, 1983).

188 **3.23 Statistical Analysis**

189

190 The haematological parameters were presented in tables and their standard deviations
191 calculated.

192 They were further subjected to one way analysis of variance (ANOVA) at 95% confidence
193 interval using GraphPad[®] instat software.

194 Result

195 **4.8 Result of Haematological Parameters**

196 **4.8.1 Effect of Neem Leaf Aqueous Extract on Haematological Parameters of** 197 **Cockerels Following Vaccination against IBD**

198
199 The haematological values for groups B and C showed that at 3 weeks of age (one week
200 post primary vaccination; PPV), the basophils (BAS) and monocytes (MON) were not
201 statistically different when compared to group A (negative control group). However, group
202 B (untreated- vaccinated group) had a significantly ($P<0.05$) lower lymphocyte and total
203 white blood cell counts (TWBC) values with higher heterophils (HET) when compared to
204 group A. Group C (treated-vaccinated group) had a significantly ($P<0.05$) higher
205 lymphocytes and TWBC when compared to group B. In addition, highest lymphocytes and
206 TWBC values were recorded in group D when compared to other experimental groups
207 (Table 4.10).

208 At 5 weeks of age (one week post booster vaccination; PBV), the lymphocyte and TWBC
209 count in group B was lower when compared to that in group A but the difference was not
210 significant. Significantly ($P<0.05$) higher value of lymphocytes and TWBC counts were
211 evidenced in group C when compared to that in group B. Highest values of lymphocyte
212 and TWBC counts was observed in group D when compared to other groups. Significantly
213 ($P<0.05$) higher value of heterophil was observed in group B and C when compared to
214 group A (Table 4.11).

215 **4.8.2 Effect of Neem Leaf Aqueous Extract on Haematological Parameters of** 216 **Cockerels Challenged and Unchallenged 6 weeks Old Cockerel Chicks**

217

218 Significantly lower lymphocytes and TWBC counts were identified in group E and F when
219 compared to those in group A. Statistically, significantly ($P<0.05$) higher values of
220 lymphocytes and TWBC counts were found in group G and H when compared to those in
221 group E and F while group G on the other hand, had significantly ($P<0.05$) higher values of
222 lymphocyte and TWBC counts when compared to group H. Significantly ($P<0.05$) higher
223 values of heterophils were observed in all the challenged groups (E, F, G and H) as
224 compared to group A. Similarly, significantly ($P<0.05$) higher values of heterophils were
225 observed in group E and F as compared to group G and H while group H had a
226 significantly higher value of heterophil as compared to group G. All the challenged groups
227 had significantly ($P<0.05$) lower monocytes counts when compared to the negative control
228 group (group A). Group G had statistically insignificant ($P>0.05$) higher values of
229 monocytes when compared to the other challenged groups (Table 4.12).

230 **Table 4.10:** Effect of Neem Leaf Aqueous Extract on Haematological Parameters of Vaccinated and Unvaccinated 3 Weeks Old Cockerels

				Haematological Parameters						
				3 Weeks Old						
GRP	Ext	Vac	Ch	LYMP	HET	EOS	BAS	MON	TWBC	
A	-	-	-	87.8±1.39 ^b	10.4±1.47 ^b	0.79±0.38 ^a	0	0.9±0.36 ^a	16.51±2.37 ^b	
B	-	+	-	82.50±1.41 ^a	14.99±1.09 ^a	0.89±0.69 ^a	0	1.61±0.7 ^a	13.22±1.93 ^a	
C	+	+	-	85.50±1.50 ^b	13.0±1.42 ^a	0.4±0.28 ^a	0	1.1±0.3 ^a	20.7±3.0 ^c	
D	+	-	-	89.8±0.26 ^c	8.10±0.9 ^c	0.9±0.1 ^a	0	1.2±0.3 ^a	21.0±3.1 ^c	

231 ^{a-c} Values within column with no common superscripts differ significantly at P<0.05

232 KEY:

233 GRP: Group LYMP: Lymphocyte MON: Monocyte

234 Ext: Extract HET: Heterophil TWBC: Total White Blood Cells

235 Vac: Vaccination EOS: Eosinophil

236 Ch: Challenge BAS: Basophil

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Table 4.11: Effect of Neem Leaf Aqueous Extract on Haematological Parameters of Vaccinated and Unvaccinated 5 Weeks Old Cockerels

				Haematological Parameters						
				5 Weeks Old						
GRP	Ext.	Vac.	Cha.	LYMP	HET	EOS	BAS	MON	TWBC	
A	-	-	-	83.20±2.1 ^a	10.8±0.89 ^a	4.8±0.52 ^a	0	1.10±0.49 ^a	13.90±1.23 ^a	
B	-	+	-	81.23±1.19 ^c	12.97±1.19 ^b	3.62±0.57 ^a	0	2.12±0.51 ^b	13.08±0.89 ^a	
C	+	+	-	83.99±1.0 ^a	12.21±0.9 ^b	1.8±0.34 ^b	0	2.0±0.9 ^b	15.3±3.2 ^b	
D	+	-	-	87.2±1.2 ^b	8.59±0.7 ^a	2.9±0.25 ^b	0	1.31±0.8 ^a	15.9±2.9 ^b	

^{a-b} Values within columns with no common superscripts differ significantly at P<0.05

KEY:

GRP: Group

LYMP: Lymphocyte

MON: Monocyte

Ext: Extract

HET: Heterophil

TWBC: Total White Blood Cells

Vac: Vaccination

EOS: Eosinophil

Ch: Challenge

BAS: Basophil

255 **Table 4.12:** Effect of Neem Leaf Aqueous Extract on Haematological Parameters of Challenged and Unchallenged 6 Weeks Old Cockerels

				Haematological Parameters					
				6 Weeks Old					
GRP	Ext.	Vac.	Cha.	LYMP	HET	EOS	BAS	MON	TWBC
A	-	-	-	83.80±1.67 ^a	10.8±0.99 ^a	3.7±0.52 ^a	0	1.7±0.49 ^a	14.40±1.66 ^a
B	-	+	-	79.5±1.03 ^b	15.40±1.1 ^b	3.2±0.74 ^a	0	1.9±0.43 ^a	13.55±2.21 ^a
C	+	+	-	84.67±1.34 ^a	10.56±1.43 ^a	2.22±0.98 ^b	0	2.56±0.56 ^b	26.77±2.31 ^b
D	+	-	-	86.90±3.36 ^a	9.20±2.05 ^a	2.05±0.53 ^b	0	2.0±0.45 ^a	28.8±1.14 ^b
E	-	-	+	71.5±1.86 ^c	23.8±1.45 ^c	3.8±0.83 ^a	0	0.9±0.21 ^c	9.85±1.07 ^c
F	-	+	+	73.5±2.64 ^c	23.3±2.69 ^c	3.1±0.79 ^a	0	0.4±0.42 ^c	10.26±1.28 ^c
G	+	+	+	81.5±2.19 ^a	14.6±1.21 ^b	1.6±1.1 ^b	0	2.3±0.59 ^b	24.06±1.28 ^d
H	+	-	+	77.4±2.23 ^b	18.1±2.0 ^d	1.8±0.95 ^b	0	2.6±0.34 ^b	20.91±2.38 ^e

256 ^{a-c} Values within columns with no common superscripts differ significantly at P<0.05

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Discussion

A week post IBD vaccination (3 weeks of age), the haematological parameters of the vaccinated groups (B and F) revealed a significant reduction in lymphocytes (lymphopenia) and TWBC when compared to the negative control group (group A). However, significant increase in heterophils (heterophilia) was observed in the vaccinated groups. The lymphopenia and decreased TWBC might be associated to the viraemia induced by the IBD vaccine virus as well as the gross/ microscopic lesions present in the BF possibly due to IBD vaccine which can also lead to immunosuppression as earlier reported by [Aihara *et al.* \(2015\)](#). The tissue destruction due to the IBD vaccines administered could be responsible for the heterophilia seen. Conversely, group C and G had a significantly higher lymphocytes and TWBC when compared to group B and F while on the other hand, the group B and F had a higher heterophils when compared to group G and H even though not significant. The increased lymphocytes and TWBC observed in group C and G could be attributed to the immunostimulatory effects of Neem leaves aqueous extract used as earlier reported by [Kwawukume *et al.* \(2013\)](#) and [Linus and Lawrence \(2015\)](#), while the decreased heterophils observed in the same group C and G when compared to group B and F could be associated to the minimal tissue damage seen post IBD vaccination when compared to those in group B and F. Hence, more heterophils would be formed in the circulation in order to meet up with the demand to the target. Also, there may be a possibility that, these increased heterophils observed in group B and F was associated to the possible bacterial infection in addition to the vaccinal virus. This was because; throughout the study we did not administer any prophylactic antibiotic to any group. However, Neem leaves extract has been shown to have antibacterial effects, and as such those groups (C and G) that were on the Neem extract may have less heterophils than the others.

At 1 week post vvIBDV challenge (6 weeks of age), significantly lower lymphocytes and TWBC were recorded in group E and F when compared to group G and H and this further signifies that the Neem leaves extract has an immunostimulatory effects as earlier reported by [Kwawukume *et al.* \(2013\)](#). On the other hand, significantly higher values of lymphocytes and TWBC were observed in group G when compared to group H and this indicates that chicks in group G were more immunocompetent than those in group H and this could be attributed to the acquired immunity had following IBD vaccinations in addition to the extract administered while those in group H had no acquired immunity even though, they were also on the same extract and then both groups became challenged with vvIBDV. The significantly higher values of heterophils observed in the challenged groups (E, F, G and H) when compared to the negative control group was attributed to the viraemia due to the vvIBDV inoculation while the significant decrease in basophils observed in the challenged groups (E, F, G and H) when compared to the negative control group may also be associated to vvIBDV infection.

Conclusion

In conclusion, the IBD vaccines causes some changes on the haematological parameters (heterophilia and lymphopenia) and the very virulent IBDV has effect on some haematological parameters, resulting in panleucopenia, lymphopenia and heterophilia. However, increased lymphocytes and TWBC was observed following Neem leaf aqueous extracts administration with resultant decrease in heterophils.

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