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Research Article

IMMUNITY ENHANCEMENT IN LASOTA VACCINATED LAHORE PIGEONS USING *ANDROGRAPHIS PANICULATA* POWDER

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ABSTRACT

Evaluation of differential cell counts and Newcastle Disease (NCD) antibody levels after LaSota vaccination of Lahore pigeons maintained with 0, 50, 100, 150 and 200 mg doses of *Andrographis* powder along with normal feed was conducted in the animal house. *Andrographis* powder was given to pigeons by hand feeding regularly once in a week and the birds were immunized with LaSota vaccine in the ocular route one week before the beginning of the experiment. HI titre, ELISA titre, RBC, WBC, haemoglobin, heterophils, basophils, eosinophils, lymphocytes and monocytes were estimated from the blood samples taken from the birds on the 50th day of experiment. This herbal powder had increased the HI titre, ELISA titre, WBC, heterophils, basophils, lymphocytes and monocytes that give immediate protection against NCD and reduced the level of eosinophils compared to control. This study confirms that *Andrographis* powder has enhanced the cell mediated immunity as well as humoral immunity of LaSota vaccinated pigeons, and recommends the use of 100-200 mg of this herbal powder once in a week for vaccinated pigeons to improve their overall health and immunity for giving complete protection against Newcastle disease.

Keywords: LaSota vaccine, *Andrographis* powder, immunity, Lahore pigeons.

INTRODUCTION

Newcastle disease (ND) is a highly catching disease of pigeon (Asplin, 1949) that causes a rapid high-mortality characterized by loss of appetite, lethargy, gastro in testinal regurgitation and diarrhoea, neurological signs like head shaking and torticollis, and respiratory signs like cough and sneezing (Ballouh *et al*., 1985). This disease was first observed in pigeons of Java Island in the early 1926s and thereafter it had spread to Newcastle (UK), from which it had spread to most other countries in the world possibly through infected migratory birds (Alexander, 1991). Newcastle disease of pigeon is caused by Avian Paramyxovirus serotype 1 (APMV-1), which has been placed in the genus *Rubulavirus* of the sub-family *Paramyxovirinae* of the family *Paramyxoviridae*. Since the pigeon APMV-1 has retained some antigenic differences from the other serotypes of NCDVs, it is known as pigeon paramyxovirus type 1 (pPMV-1). The virulent strains of PPMV-1 may cause up to 100% mortality in some farms (Chu and Rizk, 1972). The circulating strains of NDV are capable of causing both epizootic and enzootic Newcastle diseases in many commercial farms and they account for up to 100% mortality in unprotected birds (Trang *et al*., 2016). This disease has no cure, but vaccination is the most effective method to prevent NCD in pigeons for reducing the mortality (Al Garib, 2003; Paulillo *et al*., 2009; Rahman *et al*., 2004; Spradbrow, 1997; Zeleke *et al*., 2005). Live vaccines prepared with lentogenic strains of NDV are more frequently used for poultry than the vaccines prepared from chemically inactivated strains, mixed with adjuvant (Alexander, 1991). LaSota is a freeze-dried NDV live vaccine produced on a large scale at a relatively low cost and is in most common use for poultry and pigeons under Indian conditions. This vaccine is easy to administer on a large scale, and it rapidly stimulates humoral, cell-mediated and mucosal immunity in poultry (Chandrasekar *et al*., 1989; Parry & Aitken, 1973). Even though pigeons in lofts have been continuously vaccinated with LaSota to control NDV, pockets of infection have been reported here and there in vaccinated flocks due to some sorts of vaccine

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failure (Dai *et al*., 2014; Richard *et al*., 2014). This vaccine failure may be due to breakdown in the immunity of birds caused by (a) mycotoxins in the feed (Shivachandra *et al*., 2003), (b) cold/heat stress (El Lethey *et al*., 2003), (c) velogenic strain of NCDV causing the infection (Sharif *et al*., 2014), and (d) malfunctioning of the bird's immune system (Li *et al*., 1999; Sharma *et al*., 2000). Breaking the immunity has caused many farmers to lose confidence in the use of vaccines because of doubts about their efficacy, so that immunity of LaSota vaccinated pigeons has to be enhanced further for getting the maximum protection against this disease.

Immunostimulation of birds is a right choice to enhance the immunity through increased antibody production, increased cellular immune responses, and increased macrophage phagocytic ability, which provide high resistance to the birds against various viral infections (Dugas *et al*., 1999). Experiments confirm that *Andrographis panculata* is an immunostimulant to tone up both the innate immunity and acquired immunity in fishes, aquatic animals, birds, cattle and man (Puri *et al.,* 1993). Dietary supplementation of this herbal powder has appreciably increased WBC's, lymphocytes, basophils, heterophil and monocytes count but lowered the eosinophyll count in pigeons (Athis Kumar, 2017). Therefore, in this study, an attempt was made to enhance the HI titre, ELISA titre, WBC, monocytes and lymphocytes of the pigeons immunised with LaSota vaccine for Newcastle disease by providing different doses of *A. paniculata*.

MATERIALS AND METHODS

Birds and Experimental Design

One-year old Lahore pigeons (*Columba livia domestica*; family: Columbidae; order: Columbiformes) were chosen as the experimental birds for this study. A total of 20 pairs were divided into 5 groups each having 4 pairs and grown in a separate loft of 5′ x 7′ x 3′ size. The lofts were constructed with wooden frame, steel plated roof and wire mesh floor and lateral sides. These lofts were kept at a height of 2.5 from the ground level for reducing dampness facilitating the rapid spreading of pathogenic germs (Athis Kumar, 2018). Feed mixture (Table-1) was given at the rate of 90 grams per pair of pigeons per day and drinking water was provided at the rate of 120 ml per pair/day. Vitamins required for the birds were provided along with the drinking water at the rate of 5ml of Vimeral® (vitamin mix)/ 1 liter water. This feed composition was maintained throughout the study period (50 days) for feed uniformity in the experimental pigeon groups. Whole plant of *Andrographis paniculata* was collected locally from Kanyakumari district (India), dried under shade, then sun dried and ground into an herbal powder. Required amount of the herbal powder was fed to the pigeons by hand feeding along with some drops of water. Pigeons in the 1st group were fed only with the conventional feed (control), and those in the 2nd, 3rd, 4th and 5th groups were fed with the normal feed as usual and 50 mg, 100 mg, 150 mg and 200 mg of *Andrographis* powder respectively once in a week. For inducing active immunity against Newcastle Disease, the birds were vaccinated with LaSota vaccine in the ocular route one week prior to the beginning of the experiment.

Table1. Composition of normal feed.

Ingredients	Percentage
Wheat grains	35 %
Finger millet	15%
Pearl millet	15%
Green pea	30%
Grid*	4.97%
Vimeral ® **	0.5 ml/pair

* Grid: I kg contains 100 g charcoal, 100g egg shell, 75g limestone, 150g table salt and 575g brick powder; ** Vimeral ®: 1ml contains vitamin A -12,000 IU; Vitamin B_{12} – 20 mcg; vitamin D_2 -6,000 IU; and vitamin E -40mg.

Collection of Blood

Blood samples were taken from the birds on the 50th day of experiment. The wing surface at the elbow joint was sterilized by wiping with cotton soaked with surgical spirit and blood sample was taken from the jugular vein through vein puncture using 23 G sterile hypodermic needle of Disproven Insulin syringe. About 2 ml of blood was taken in from a pigeon, as done by Oladele *et al*. (2008) on the day of experiment and the samples taken from a pair of birds were pooled together as one sample (4 ml) for investigation. Of this, 2 ml is stored in labeled Bijou bottles containing ethylene diamine tetra acetic acid (EDTA) at the concentration of 2 mg/ml as anti-coagulant for the study of haematological parameters and the remaining 2 ml blood was stored in yet other labeled bottle without any anti-coagulant for the preparation of serum.

Preparation of Serum

2 ml of each blood sample was taken in a test tube and its mouth was closed with a cotton plug. The test tube was kept undisturbed at 37°C for one hour and then the blood was centrifuged at 2000 g for 10 minutes. Serum in the fluid was carefully poured into a screw-cap tube and stored at -20°C for the further study.

HI titer assay for NDV

Sera of all the birds were subjected to HA and HI titer assay according to the standard methods described by Hanson *et al*., (1976). The test was carried out by running two fold dilutions of equal volumes (25 µl) of Phosphate Buffered Saline (PBS) and test serum in U-bottomed micro titer plates. 4 Hemagglutination units (HAU) of the viral antigen of LaSota strain obtained from CIRAD, France was added to each well and the plates were left at room temperature for a minimum of 30 min. 25 µl of 1% (v/v) chicken RBCs collected from pathogen-free chickens older than 3 weeks and serologically negative to NCD antibody

was added to each well. After gentle mixing, the plates were allowed to settle for about 40 minutes at room temperature. The HI titer was read from the highest dilution of serum causing complete inhibition of 4 HAU of antigen. Those wells that showed sedimentation of RBC as the control wells were considered as inhibition. A titer greater than or equal to 1:8 was taken as positive.

ELISA Test

The serum samples were also utilized for estimation of ELISA titre for Newcastle Disease, as per the procedure of Office International des Epizooties (2000). Fifty microlitres of diluted (1 in 100) antigen was added to each well of a pretreated (activated) flexible poly-vinylchloride (PVC) microtitre plate (Flow Laboratories, England). After overnight incubation at 4°C, excess antigen was removed and the plate was washed three times (3 min each time). The antigen-coated wells were allowed to react with appropriately diluted test sera, followed by conjugate diluted 1 in 2000, and then substrate solution. All reagents including substrate were added in 50 µl volumes per well and incubated for 1 hour at 37°C. At each step, the excess reagents were removed by washing and the substrate degradation was finally stopped by adding 30 µl of 4N H_2SO_4 per well. The absorbance of coloured reaction products was measured at 450 nm in a Tjtertek Uniskan microelisa reader.

Haematological Parameters

Hematological parameters like red blood cells (RBC) count, haemoglobin (Hb) concentration, WBC and differential count for heterophilis, basophils, eosinophils, monocytes and lymphocytes were determined using standard techniques described by Rehman & Abbas (2003).

Statistical analysis

All the data obtained from this experiment were subjected to one-way ANOVA, using SPSS (1997) computer software. The significant differences among the means values were analyzed with the Duncan multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Dietetic treatments with *Andrographis* powder had significantly ($p>0.05$) increased the WBC, basophils, heterophils, lymphocytes and monocytes and decreased eosinophils in the blood compared to the control (Table 2). The WBC increased from 27.44 ±1.09 to 29.33 ±1.17 ($x10^3/mm^3$) instead of 24.54 ± 1.12 in the control. Heterophils count had significantly increased from 6.83 ± 0.42 to 7.21 ± 0.53% in the blood instead of 5.42 ± 0.60 % in the control. In the meantime, basophils count slightly increased from 0.45 ± 0.08 (control) to 0.47±0.11% in the blood. Lymphocytes count significantly increased from 12.84 ±1.9 to 14.75 ± 1.8 % in the blood instead of 12.46 ± 1.12 % in the control. Likewise, monocytes count significantly increased up to 1.63±0.5% from 1.22 ± 0.5 % in the control. In contrast, eosinophils count had decreased from 0.38 ± 0.08 to 0.35 ± 0.06 % in the blood instead of 0.38 ± 0.09 % in the control. However, this dietary treatment did not affect the RBC count and haemoglobin content of blood. The maximum immunostimulating effect was observed at 200 mg *Andrographis* powder at weekly intervals.

Bone marrow which is the site of haematopoiesis contains all the cytokines required for the proliferation and differentiation of haemopoetic cells via positive and negative regulation of various cytokines, cytokine receptors and other regulatory peptides (Bloom, 1938). A combination of more than one cytokine in small concentrations may up regulate or down regulate the various lineages of haemopoetic precursors to produce characteristic cell types (Fan *et al*., 2007). (Bagby, 2000) clearly reviewed that in humans IL-3, IL-9, IL-11 and GM-CSF are required for the production of erythrocytes from myeloid progenitors, IL-3, GM-CSF, M-CSF and G-CSF are necessary for the production of neutrophils, IL-3, IL-5 and GM-CSF are inevitable for the production of eosinophils, IL-3 and TGF promote the production of basophils, IL-1, IL-6,TNF and GS-CSF are required for the production of monocytes, and IL-2, IL-7, IL-4, IL-10, IL-12, IL-13, IL-14 and IL-16 required for the formation and proliferation of lymphocytes from lymphocytes progenitors. Further, IL-1 and TNF act synergistically to stimulate the myeloid progenitors to produce red blood cells. Therefore, *Andrographis* powder might have up regulated the expression of IL-1, IL-2, IL-3, IL-10, IL-12, IL-13, IL-14, IL-15, IL -16, TGF and TNF while down regulated the expression of IL-5, IL-9, IL-11 and GM-CSF in pigeons. However, it needs further confirmation by RT-PCR with known probes. In the same line of invention, (Choi *et al*., 1999) had already proved that Lactobacilli up regulate the expression of IFN-γ, IL-1, IL-12, IL10 and IGF-β in domestic fowls.

Innate immune system, which is mainly driven by cell mediated immunity, serves as a scavenger system that fights invading pathogens immediately after they enter the body either by means of phagocytosis or by lysis of pathogens (Reid *et al*., 2016). Phagocytosis is associated with heterophils, basophils and monocytes while lysis is associated with lysozymes and other molecular cascade systems. Heterophils have phagocytic capability due to oxidative burst (Montali, 1988). The activation of heterophils by pathogens or by cytokines induces the expression of various pro-inflammatory cytokines such as IL-1, IL-6 and IL-8 (Kogut *et al*., 2005). Monocytes derived macrophages and basophils are induced to engulf the pathogenic microbes by TLR7/8 proteins which are often stimulated by feed supplements (Philbin *et al*., 2005). Expression of defenses has been reported with heterophils and macrophages to destroy pathogens (Sugiarto & Yu, 2004; Zhao *et al*., 2001) which another innate protection of the organism. In laying hens, increase in heterophil-lymphocyte ratio provides much innate immunity to the fowl (Campo *et al*., 2005).

Table 2. Changes in cell-mediated immunity of LaSota vaccinated Lahore pigeons fed with different dosages of *Andrographis paniculata*. (n=8).

Blood component	Control	Dosage of *Andrographis powder*			
		50mg	100 mg	150mg	200 mg
RBC ($x10^6/mm^3$)	2.8 ± 0.34^a	2.8 ± 0.36^a	2.8 ± 0.37^a	2.8 ± 0.39^b	2.8 ± 0.41^b
WBC ($x10^3/mm^3$)	24.54 ± 1.12^a	27.44 ± 1.09^b	28.68 ± 1.21^b	29.21 ± 1.25^a	29.33 ± 1.17^b
Haemoglobin (g %)	9.83 ± 0.72^a	9.87 ± 0.73^a	9.89 ± 0.33^a	9.89 ± 0.23^b	9.88 ± 0.32^b
Heterophils (%)	5.42 ± 0.60^a	6.83 ± 0.42^a	7.10 ± 0.51^a	7.21 ± 0.53^b	7.21 ± 0.27^b
Basophils (%)	0.45 ± 0.08^a	0.45 ± 0.08^a	0.45 ± 0.09^b	0.47 ± 0.08^b	0.47 ± 0.11^b
Eosinophils (%)	0.38 ± 0.09^a	0.38 ± 0.08^a	0.37 ± 0.05^b	0.36 ± 0.04^a	0.35 ± 0.06^b
Lymphocytes (%)	12.46 ± 1.12^a	12.84 ± 1.9^a	14.39 ± 1.8^b	14.72 ± 1.13^b	14.75 ± 1.8^b
Monocytes (%)	1.22 ± 0.5^a	1.35 ± 0.3^a	1.61 ± 0.4^b	1.63 ± 0.5^a	1.61 ± 0.7^b

* ^{a} is the significance $p<0.05$; ^{b} is the significance value $p>0.05$.

This herbal powder has increased the heterophil: lymphocyte ratio in pigeons, which indicates that it has considerably enhanced the innate immunity of pigeons. Lymphocytes essential for generating immune responses and retaining memory of previous exposure to an antigen (Burnet, 1971), take part in the innate immunity as well as in the adaptive immunity to fight against the invasion of pathogens. This finding is in agreement with the results of previous workers (Athis Kumar, 2017; Dugas *et al*., 1999; Puri *et al*., 1993), that powder of *Andrographis* has modulated the expression of various cells involving in the innate immunity of pigeons.

Table 3. Changes in the HI titre and ELISA titre of LaSota vaccinated Lahore pigeons fed with different dosages of *Andrographis paniculata*. (n=8).

Antibody titre	Control	Dosage of *Andrographis powder*			
		50 mg	100 mg	150 mg	200 mg
HI titre (log_2)	4.3 ± 0.30^a	4.9 ± 0.23^b	5.43 ± 0.24^b	6.12 ± 0.31^a	6.91 ± 0.23^b
ELISA (log_2)	2.42 ± 0.05^a	2.64 ± 0.04^a	2.88 ± 0.06^a	3.18 ± 0.05^b	3.43 ± 0.04^b

* ^{a} is the significance $p<0.05$; ^{b} is the significance value $p>0.05$.

The HI test is the most widely used serological method for measuring anti-NDV antibody levels in bird's sera and is still a standard laboratory method for diagnosis of NDV (Jestin *et al*., 1989). On the 50th day of experiment, in pigeons vaccinated with LaSota vaccine, the HI titre was 4.3 log 2, which was further increased to 4.9 -6.91 log2 by *Andrographis* powder (Table 3). This powder might have induced the formation of cytokines that involve in the antibody production. The HI titre value increased with increasing dosages of these supplements, reaching the peak at the highest dosage used in the experiment (200 mg dosage). Allan & Gough, (1974) have evaluated in chicken that HI titre value higher than 5.2 log2 can give adequate protection against NCDV infections. 100, 150 and 250 mg dosages of *Andrographis* powder had raised the HI titre above 5.2 log 2, and hence they are enough for providing adequate protection to birds against the Newcastle disease. This result is in the same line of investigation made by Ojiezeh *et al*. (2013) who highlighted the relevance of vaccination in disease prevention, control and management of NDV disease.

The mean ELISA titres of pigeons fed with *Andrographis* powder were significantly ($p>0.05$) different from the control. On the 50th day, in pigeons vaccinated with LaSota vaccine, the ELISA titre was 2.42 log 2, which was further increased to 2.64 -3.43 log2 by *Andrographis* powder (Table 3). ELISA titres increased from 50 mg dosage to 200 mg dosage, which represents the higher production of NCDV-antibodies in the pigeons while increasing the dosage of the herbal supplement. The ELISA titre was linearly correlated with HI titre. The protection rate did not differ significantly at different doses of supplements, but without doubt there was rise in the humoral immunity as reported by De Roos & Katan, (2000); Qubih & Mohammadamin, (2010); Zhang *et al*. (2007).

CONCLUSIONS

It could be concluded from this study that although LaSota vaccine is a safe live vaccine to give protection to pigeons against NCDV, the simple vaccination alone cannot provide complete protection to the immunized birds because of the inadequate amount of anti-NCDV antibodies produced immediately after the vaccination. Occasional outbreaks that appear in the vaccinated flocks can be prevented by providing about 100-200 mg of *Andrographis* powder to a pigeon regularly once in a week, without affecting the growth and reproductive attributes of the birds. This herbal powder has stimulated humoral immune

response of vaccinated pigeons to produce some additional amount of anti-NCDV antibodies for HI titres above 5.2 log2 giving complete protection against the NCD. Further, *Andrographis* powder enhances the production of WBC, heterophils, lymphocytes and monocytes, which give innate immunity to the pigeons to fight against the invasion of NCDV and some other dreadful infections. Reduction in the eosinophils count indicates improvement in the general health of pigeon. This study therefore recommends the use of 100-200mg of *Andrographis* powder to LaSota vaccinated pigeons once in a week to improve their overall health and immunity, which would definitely give complete protection against Newcastle disease.

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