Research Article

The Effectiveness of Immunostimulants on NDV Genotype VII and IBDV Infection in Broiler Chickens

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Abstract | The present study was conducted to investigate the pathogeneicity of Newcastle Disease Virus (NDV) genotype VII.1.1 *"NDV-CHICKEN-EGY-ALEX-NRC-2020"* strain in combination with Infectious Bursal Disease Virus (IBDV) *"IBDV/Egypt/Qalubia/17"* and/or some commercial immunostimulants (Lector® and Orego®) in commercial broiler chickens. The pathogenicity studies parameters included the effects on protection, growth performance and clinico-pathological changes. The results indicated that, immunostimulants can keep maternal immunity longer where lector® was the best; the decline in HI titers also indicates that bird groups not contract ND natural infection. The results of performance parameters after immunostimulants administration and challenged with IBDV at 14-days of age and NDV GenotypeVII.1.1 at 21- days of age in broiler chickens proved that administration of Lector® and Orego® solution had positive effect on average body weight gain and feed conversion rates than control non medicated groups; where the Lector medicated groups showed higher rates of average weight followed by Orego® medicated groups. Regarding signs and mortality in infected groups IBD signs varied from mild in lector®, moderate in Orego® while sever signs were in non-medicated group. The mortalities began at 5th day post-challenge (pch) in non-medicated groups with total 10-20%; while the mortalities in NDV began at 3rd day pch in group non-medicated with 50% to reach 100% mortality rate in 7th pch. At necropsy both IBD and NDV infection lesions were found in dead birds of all challenged groups. Chicken groups challenged by IBDV or NDV after treatment with Lector showed milder histopathological changes than Orego solution and non-treated groups showed the most sever lesions. Chicken groups challenged with both virulent IBDV and NDV either treatment with Lector® or Orego® solution or non-treated showed no marked difference in histopathological lesions. The results of total and differential leucocytes indicated that lector had a great high effect in WBCs levels specially lymphocytes and monocytes. In conclusion the used immunostimulants are of low value against IBD and ND virulent viruses challenge. In conclusion, is recommended to apply a strict hygienic measures and suitable vaccination programmes to protect chickens against both IBD and NDV infection as the current control and prevention regimes, including vaccines and vaccination protocol are not adequate against either single or mixed infection infections with IBD and/or ND.

Keywords | Broiler chickens, NDV genotype VII.1.1, IBDV, Immune-stimulants, HI-test, H/L ratio

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INTRODUCTION

Newcastle disease (ND) and infectious bursal disease (IBD) are viral diseases of poultry causing severe economic losses in domestic bird populations due to immunosuppression, reduction of egg production and high mortalities resulting from infection with virulent strains of the two viruses. Great efforts had been done to prevent and control both of them. The most responsible factor for the outbreaks of ND and IBD among commercial poultry flocks is circulation of the causative virus among birds (Ibu et al., 2000; Oluwayelu et al., 2014; Absalón et al., 2019; Swayne et al., 2019).

ND is a contagious viral disease with highly significant clinical impact in poultry and sever economic losses all over the world including Egypt. The disease caused by Newcastle disease virus (NDV) or avian paramyxovirustype-1 (APMV-1) is a species within the genus avian orthoavulavirus-1, which belongs to family Paramyxoviridae (Rima et al., 2019). ND in chickens is characterized by respiratory, circulatory, gastrointestinal, and nervous signs (MacLachlan and Dubovi, 2011; Ahmed et al., 2017; 2022b). The severity of clinical signs depends on factors related to virus and host factors as (species, age and immune status of the host) as mentioned by McFerran and McCracken (1988).

On the other hand, IBD is one of the most important economic viral diseases that affects chickens worldwide (Müller et al., 2003). It affects chicks of 3-6 weeks of age and is characterized by whitish diarrhea, ruffled feathers and mortalities up to 30% according to virulence of virus strain and susceptibility of chickens (Eterradossi and Saif, 2013). The disease is caused by IBDV (infectious bursal disease virus), genus Avibirnavirus which belongs to family Birnaviridae (ICTV, 2017). The replication of IBDV occurs in Bursa of Fabricius particularly immature lymphocytes causing developing B-lymphocytes destruction (Wang et al., 2010) and preventing the development of immunity (Liang et al., 2015), resulting in severe immunosuppression, increased susceptibility to other infectious diseases and vaccination failure (Schat and Skinner, 2013). Allan et al. (1972) who first reported the immunosuppressive effect of IBD and Faragher et al. (1974) who observed that immune response suppression to NDV infection was greatest in chicks infected on 1 day old with IBDV, These suppressive effects includs cell mediated immunity (CMI) and humoral immunity, Giambrone et al. (1978) reported that IBD affects humoral immunity more than CMI; thus, these effects enhanced the pathogenic effects of NDV and suppress successful control of ND by vaccination due to increase rates of vaccination failure. So, it is necessary to improve the immune response against ND to produce protective antibody titers that can suppress viral replication; therefore, immunostimulants have been used as adjuvants to give prolonged cellular and humoral immune responses and improving the response of vaccines (Yu et al., 2015; Hou et al., 2016).

Immunostimulants are biological and synthetic compounds that enhance the cellular immunity by macrophages activation which stimulate the immune response such as cell killing, antigen engulfment, cytokines release, production of antibody and humoral defense mechanisms (Chan et al., 2008) that increase the immune response sustainability to the infectious agent and resistance to disease infections (Firenzouli et al., 2008; Weickert and Pfeiffer, 2008). The used immunostimulants composed of lectin, oregano solution and other ingredients had different mechanisms of action as immunostimulants due to their composition difference (Amer et al., 2017). Oregano essential oils (OEO) are extracted from *Origanumvulgare* plants (leaves and flowers). They are consisted of a lot of ingredients; most of them are thymol and carvacrol that constitute approximately 78-82% of oregano essential oil (Al-Bandak and Oreopoulou, 2007). Oregano essential oils have potent antioxidant effects (Rhee et al., 1996) and also, they increase the differentiation proportion of T lymphocytes CD4+ and CD8+ (Walter and Bilkei, 2004).

On the other hand, lectins have role in innate immunity. They act as pattern recognition receptors (PRRs) and recognize pathogen-associated molecular patterns (PAMPs). Therefore, lectins contribute to a protective immune response (Lepenies and Lang, 2019).

The pathogenicity evaluation of NDV and IBDV isolates was carried out by experimental infection of susceptible chickens depending on clinical signs, mortality, gross lesions and histopathological analysis of the target organs (Jackwood et al., 2011) as well as leucocytogram where, Esonu et al. (2002) reported that the hematological parameters can reflect the physiological conditions of an animal to certain environmental factors and microbial agents.

Thus, the current study aimed to investigate pathogenicity of local identified NDV strain related to genotype VII.1.1 to commercial broiler chickens in presence of IBDV as immune suppression virus and/or immune stimulants and studying the effects on protection, performance and clinicopathological changes.

MATERIALS AND METHODS

Ethics statement

This study was approved for experiment under the ethics of Medical Research Ethics Committee (MREC) of the

National Research Centre, Egypt with approval number (27210112021).

Chickens

Two hundred (200) commercial Cobb 500^{R} broiler chicks were provided as hatched by certified local hatchery. The chicks were divided into 10 separate groups (20 birds per each) as shown in Experimental Design in (Table 1). Each group was kept in isolated unit separately with strict hygienic and biosecurity measures. Conventional welfare regulations and animal food standards were taken into account.

Experimental design

Two hundred commercial broiler chicks were divided into 3 main groups A, B and C of 60, 60 and 80 chicks to be treated as Lector®, OREGO sol® and non-medicated group; respectively. At the 14th day birds were further grouped into 10 groups (Gps1:10), 20 birds each as follows: group A (Lector®) was divided into 3 Gps (1, 2 and6), group B (Orego sol®) into 3 Gps (3,4 and 5) while group C into Gps (7-10). At 14 days old; Gps (1-4), Gp7 and Gp 9 were inoculated with IBD virus. At 21 days old groups Gps 2, 3, 5, 6, 8 and 9 were challenged with vNDV genotype VII 1.1. Gp 10 was kept as control negative, respectively (Table 1). Birds of all groups were kept under daily observation with recording of daily feed intake, sighs and mortality as well as weekly body weight gain till the end of the 4th week of life.

Chicken ration

The chickens were fed on commercial rations according to the NRC (1994) and given pelleted starter (Crude Protein "CP" not less than 23%) and growing (CP not less than 21%) rations. The starter ration was used for the first 2 weeks and growing ration was used to the end of the experiment. Drinking water and rations were given to chickens *ad-libitum*.

Virus propagation

The challenge viruses (vNDV and IBD) were propagated via allantoic cavity inoculation of 9-days-old specific pathogen-free (SPF) embrocated chicken eggs (ECE) (OIE, 2021).

Challenge viruses

ND virus

The challenge virus was characterized by sequencing as velogenic NDV (vNDV) genotype VII1.1 designated as "NDV-CHICKEN-EGY-ALEX-NRC-2020" with an accession number of (MW580389) on GenBank (Ahmed et al., 2022a). The challenge with vNDV was carried out at 21 days of age. The dose of challenged virus was 10^{6} EID_{50} given 0.5 ml / bird by intramuscular route (I/M) according to OIE (2021).

IBD virus

The challenge virus was characterized by sequencing of VP2 gene and designated as "IBDV/Egypt/Qalubia/17" with an accession number of (MK088026) on GenBank (Elsamadony et al., 2019). The challenge with vNDV was carried out at 14 days of age. The dose of challenged virus was 10^{4} EID_{50} given 0.1ml/ bird by oculo-nasal route (OIE, 2021).

Immunostimulants

Two immunostimulants were administrated from 1st week to 4th week (first 3 days of each week) including:

Table 1: Experimental design at the 14th day of life for detection ofPathogenicity of NDV genotype VII.1.1 isolate to chicken in association with IBDV and immunostimulants.

Pathogenicity and immunostimulants assessment	Challenged virus		Immune stimulant (administrated at 1st- 4th week (1st 3 days/week)		Group no.
1- Sero-conversion 2-Clinical signs 3-Post mortem gross lesions 4-Mortality % 5-Histopathological examination 6-Total and differential leucocytes count as well as H/L Ratio. 7-Broiler performance parameters	----	IBD	----	Lector	1
	NDV	IBD	----	Lector	2
	NDV	IBD	Orego sol	----	3
	----	IBD	Orego sol	----	4
	NDV	-----	Orego sol	----	5
	NDV	-----	----	Lector	6
	----	IBD	------		7
	NDV	----	------		8
	NDV	IBD	------		9
	Control non- challenged group.				10

NDV challenge; the dose of challenged virus equal $10^{6}EID_{50}$ given 0.5 ml / bird by IM route. IBDV challenge: The dose of challenged virus equal 10^{4} EID_{50} given 0.1ml / bird by oculo-nasal route.

Lector 50®: It is a commercial product obtained from Microbiotech, USA (Batch NO 1119) and composed of lectine 5000 mg/L, Xylitol 20000 mg/L and Fructoligosaccharide 50000 mg/L, Sodium Chloride 30000 mg/L and distilled water up to 1 liter. It was used in drinking water at a rate of 1.25ml Lector /Liter water/day.

Orego Sol®: It is a commercial product obtained from CCPA International (Batch No. 2100288) and composed of Thymol (thymus vulgaris oil) 2500 mg/L, Carvacrol (oregano oil) 40000 mg/L, Glyceryl polyethylene glycol ricinoleate 220000mg/L and distilled water up to 1 liter, it was used in drinking water at a rate of 1.25 ml Orego/ Liter water/day.

Haemagglutination inhibition (HI) assay

Sera were obtained from all birds pre-challenge at designated days as shown in Table 2 and tested by HI assay. The HI assay was carried out using (LaSota strain) according to standard procedures with 4 Haemagglutinating Units' virus/ antigen in 50 µL and HI titer ≤ 2 Log_2 is considered negative (OIE, 2021).

Clinico-pathological examination

Chickens in all groups were monitored daily for clinical signs and mortality. Clinical signs observed, mortality and the pathological post mortem findings in dead birds were recorded.

Total and differential leucocyte count

Blood samples were collected on EDTA for total and differential leucocyte count at days 0, 3 and 7 post ND challenge. Total and differential leukocyte counts were done using Natt and Herrick (1952) diluting solution according to Bounous and Stedman (2000). The neutrophil (heterophil)/ lymphocyte (H/L) ratio was calculated by dividing the number of neutrophil cells by lymphocyte cells (Fidan et al., 2017).

Histopathological examination

Two chickens from each group were slaughtered at days 4th and 6th post ND challenge for histopathological examination. Collected organs including; the thymus, spleen, cecal tonsils and bursa were fixed on 10% formol saline and subjected for histopathological examination. Samples were prepared for staining by H and E, section covered with slides and examined by light microscope (Bancfort and Stevens, 1996).

Broiler growth performance parameters

Growth performance parameters were recorded for each group from 1st to 4th week of age according to NRC (1994). Feed consumption and feed conversion rate were determined using the following formula: Feed consumption (FC) g/bird = Feed intake in a replication/ No. of live birds in a replication. Feed conversion ratio (FCR) = Feed intake (g)/ Live weight (g).

Statistical analysis

One way ANOVA with Tukey's post hoc test analyzed by SPSS 21 software is used to analyze the data and calculate the averages, standard deviation of individual treatments and corresponding controls. Results were considered to be statistically significant only if the comparison to each of examined groups gave a P-value of <0.05.

RESULTS

Pathogenicity of NDV genotype VII.1.1 isolate to chicken in association with IBDV and immunostimulants

Infected groups with IBDV (Gps 1, 2, 3, 4, 7 and 9), showed clinical signs on 4th day post challenge (pch). The observed clinical signs were whitish watery diarrhea with ruffling of feathers. These signs varied from mild in Gps 1, 2, moderate in Gps 3, 4 and sever in Gps 7 and 9. The mortalities pch in Gps 2, 4, 7 and 9 with total 5%,20%, 5% and 5%, respectively, while Gps 1, and 3 show no mortalities after IBDV challenge as shown in Table 2.

Post mortem lesions in both dead and sacrificed birds were dehydration and variable bursal lesions (hemorrhages and/ or enlargement) accompanied with muscular hemorrhages and hemorrhages on junction between proventriculus and gizzard. Negative control group (Group 10) showed no clinical signs or mortalities along the experiment period.

After challenge with NDV, the observed signs started pch in Gps (2, 3, 5, 6, 8 and 9). All groups showed depression and marked decrease in feed intake with respiratory, nervous signs and greenish diarrhea. The signs were sever in Gps 2, 3, 8 and 9, while moderate signs appeared in Gps 5 and 6. The mortalities began on the 3rd day pch in groups 8 and 9 with 50% in 1st day then increase till reached 100% mortality rate in 7th pch, while the mortalities in Gps 2, 3, 5 and 6 were 68%, 95%, 89% and 84%, respectively (Table 2). At necropsy, petechial hemorrhage were found on tips of proventricular glands, ulceration of cecal tonsils, enlarged mottled spleen and hemorrhagic tracheitis. These lesions were found in dead birds of all challenged groups.

Serological response measured by HI assay

Mean HI titers results are shown in Table 2. It was found that on 7, 14 and 21 days of age, the HI titers are 4.20±1.5, 3.3±1.2 and 0.0±0.0 in lector treated groups (Group A) but are lower in Orego treated group (Group B) where it recorded 3.67±0.57, 2.1± 0.58 and 0.0±0.0, while the lowest was in non-medicated group (Group C) 3.3±0.86,

Table 2: Results of HI and mortality rates in chicken groups received immunostimulants, challenged with IBDV at 14-days and/or NDV at 21-days of age (n= 20 chickens/ group).

GP no.	Immune stimulant	Challenged virus		HI titer means SD Log-2 at age/ days (N = 20)			IBD challenge			NDV challenge		
				7	14	21	No	Death	%	No	Death	%
1	Lector	IBDV	--	4.20 ± 1.5	3.30 ± 1.2	1.7 ±0.52	20	0	0	20	0	0
2	Lector	IBDV	NDV				20	1	5	19	13	68
3	Orego sol	IBDV	NDV	3.67± 0.57	2.10 ±0.58	0.0 ± 0.0	20	0	0	20	19	95
4	Orego sol	IBDV	--				20	4	20	16	0	0
5	Orego sol	--	NDV				20	1	5	19	17	89
6	Lector	--	NDV	4.20 ± 1.5	3.3 ± 1.2	0.0 ± 0.0	20	1	5	19	16	84
7	--	IBDV	--	3.3 ± 0.86	2.70 ±0.57	1.7 ±0.52	20	1	5	19	0	0
8	--	----	NDV				20	1	5	19	19	100
9	--	IBDV	NDV				20	1	5	19	19	100
10	--	--					20	0	0	20	0	0

HI titer ≤ 2 Log- $_2$ considered negative (OIE, 2021). N, number of tested samples; Gp no.: Group number

Table 3: Results of performance parameter after immunostimulants administration and challenged with IBDV at 14-days of age and vNDVGenotypeVII.1.1at 21- days of age in broiler chickens.

G. no	I.S	Challenge virus		Performance parameters at age/weeks							
				1		2		3 (after IBD challenge)		4 (after NDV challenge)	
				ABW M±SD	FCR	ABW M±SD	FCR	ABW M±SD	FCR	ABW M±SD	FCR
1	Lector	IBD	----	211±9.45	1.77	569±3.6	1.34	1065±64.0	1.82	1228±130	1.87
2	Lector	IBD	NDV					1089±100.5	2.11	1018±172.6	1.97
3	Orego sol	IBD	NDV	210±5.13	1.79	540±71.2	1.46	947±143.1	1. 85	1192±321	1.94
4	Orego sol	IBD						1000±184.2	1.90	988±182.4	1.75
5	Orego sol	-	NDV					1201±93.8	1.36	1115±282.8	1.62
6	Lector	--	NDV	211±9.45	1.77	569±3.6	1.34	1129±152	1.16	1153±138.8	1.82
7	-----	IBD	----	224±15.1	1.3	557±17.3	1.68	909± 181.0	1.95	1257±196	1.83
8	-----	IBD	NDV					953±199.0	2.02	----	-----
9	------		NDV					995±178.0	1.83	------	---
10	---------							1205±78.6	1.44	1544±164.1	1.69

G.no: group number; I.S: Immunostimulant; ABW: average body weight; FCR: feed conversion ratio.

2.7±0.57 and 1.7±.52, respectively. These results indicate that immunostimulants can keep maternal immunity longer and the lector induced better results compared with orego.

Performance parameters

Average body weight (ABW) of the chickens in one day old was 52 gm for all groups before beginning the experiment. As shown in Table 3, at the 1st and 2nd week of age, there is no marked difference between ABW and food conversion rate (FCR) in Gps A, B and C, but Lector medicated Gp (A) showed higher rates of ABW (211 and 569 gm) and FCR (1.77 and 1.34) in 1st and 2nd week of age, respectively, followed by Orego treated Gp (B) where ABW (210 and 540 gm) and FCR (1.79 and 1.46), respectively, while the control Gp (C) ABW was (224 and 557 gm) and FCR (1.3 and 1.68), respectively. At the 3rd week of age (1 week after IBD infection) Lector medicated (Groups A) Gps (1, 2, and 6) showed higher rates of ABW (1065, 1089, 1129 gm) with mean (1094 gm) and FCR were (1.82, 2.11, and 1.16) respectively, followed by Orego medicated Gps (3, 4 and 5) ABW were (947, 1000, 1201) with mean (1049 gm) and FCR were (1.85, 1.90, and 1.36), respectively. While, the ABW of control non-medicated Gps (7, 8, 9 and 10) were (909, 953, 995, 1205) with mean (1015 gm) and FCR (1.95, 2.02, 1.83, 1.44), respectively. At 4th week of age (1 week after NDV infection) Lector medicated groups ABW was mean of 1133 gm, followed by ABW of Orego medicated groups mean of 1098 gm, furthermore the ABW in control non medicated group for IBD infection (Gp7) was mean of 1257 gm, and all birds were died in Gps 8 and 9. Finally, the control non-medicated and non-infected group (Gp 10) recorded the highest ABW in 4th week of age.

Table 4: Total and differential leukocyte count of chicken groups received immunostimulants, vIBDV at 14 days of age and vNDV challenge at 21 days of age at 0, and 3 days post NDV challenge.

G. no.	I..S +Ch. virus	DPC	TWBCs×10³ Mean± SD	Neutrophil Mean± SD	Lymphocyte Mean± SD	N/L ratio	Mon Mean± SD	Eson Mean± SD	Baso Mean± SD
1	Lector+ IBD	0	25±2.83	24.5±2.12	69.5±3.54	0.35	4.5±0.71	1.5±0.71	0.0±0.0
		3	22±1.41	23.5±0.71	70.5±2.12	0.33	3.5±3.25	1.5±1.25	0±0
2	Lector+IBD+ND	0	27.5±2.12	21±0.71	74±2.83	0.28	3.5±0.71	1.5±0.71	0.0±0.0
		3	24.5±2.12	21.5±0.71	74±1.41	0.29	3.5±3.75	1±1	.5±0.71
3	Oregosol+ IBD+ND	0	23.5±2.12	19.5±0.71	75±1.41	0.26	4±0	1±0	0.0±0.0
		3	25±2.83	22±2.83	75±1.41	0.29	3.5±3.25	1.5±1.75	0±0
4	Oregosol+IBD	0	22.5±2.12	23.5±0.71	70.5±3.54	0.33	4±1.41	1.5±0.71	0.5±0.71
		3	25±1.41	20.5±0.71	75.5±3.54	0.27	2.5±0.71	1±0	0±0
5	Oregosol+ND	0	24±2.83	19.5 ±2.83	74.5±0.71	0.26	4.5±0.71	1.5±0.71	0.0±0.0
		3	23.5±2.12	19±1.41	76.5±0.71	0.24	3±0	1.5±0.71	0±0
6	Lector	0	26±1.41	20.5±1.41	74.5±0.71	0.27	3.5±0.71	1±0	0.5±0.71
		3	25±2.83	17 ±0.71	76.5±0.71	0.22	4±1.41	2±0	0.5±0.71
7	IBD	0	24.5±2.12	19.5±1.41	76.5±2.12	0.25	3.5±0.71	1.5±0.71	0.0±0.0
		3	22.5±0.71	20.5±1.41	74±1.41	0.27	3.5±0.71	1.5±0.71	0±0
8	IBD+ND	0	23±1.41	20.5±2.83	74.5±3.54	0.27	4±0	1±0	0.0±0.0
		3	26±2.83	19.5±0.71	75.5±0.71	0.25	3.5±0.71	1.5±0.71	0±0
9	ND	0	25.5±2.12	22.5±1.41	72±1.41	0.31	3.5±0.71	1.5±0.71	0.5±0.71
		3	23.5±2.12	22.0±0.71	72±1.41	0.30	4.5±0.71	1.5±0.71	0±0
10	control negative	0	24.5±2.12	22.5±0.71	71.5±2.12	0.31	4.5±0.71	1.5±0.71	0.0± 0.0
		3	24±1.41	22.5±2.12	71.5±2.12	0.31	4±4	1.5±1.75	0.5±0.71

G. no: group number; I.S: Immunostimulants; DPC: days post NDV challenge; SD: standard deviation; N/L ratio: neutrophil/lymphocyte ratio.

Results of total and differential leucocytic counts

Comparing results of total leucocytic count at 0 time of NDV challenge (Table 4, Figure 1A) those reflect that Lector increased leucocytic values than Orego. The possible effect of immune stimulant and IBDV infection is clearly noticed where Gps treated with Lector have improved total leucocytic count 25±2.83, 27.5±2.12 (as in Gps1 and 2, respectively) as compared with control Gp 10 (24.5±2.12). At the 3rd day from NDV infection there is a marked decrease in total leucocytic count in both immune stimulant treated and non-treated groups. The IBDV infected Gps showed the lowest counts. Both infections resulted in higher lymphocyte count (Table 4, Figure 1B) than the control Gp 10 in treated and non-treated Gps. Neutrophils (Table 4, Figure 1B). Monocytes and eosinophils are lower in infected non-treated Gps than treated Gps than the control Gp at 0 and 3 days post infection.

IBD and NDV infections resulted in undetected basophiles at 0 and 3 days in compared with Lector treated Gp 6 and non-infected non-treated control Gp 10 (Table 4). N/L ratio at 0 time Lector + IBD Gp 1 (Table 4, Figure 1D) showed the highest value 0.35 followed by 0.33 in Oregosol+IBD Gp 4 and 0.31 in ND Gp 9 and control Gp10 while the lowest ratio 0.25 was in IBD Gp7. N/L ratio at the 3rd day post NDV challenge immunostimulants treated Gps showed the highest values (0.33) than control Gp10 (0.31), followed by Gps 9, 2 and 3 (0.30, 0.29 and 0.29).

Histopathological examination

Tissue sections of control negative non-challenged group (group 10) showed normal tissue structure in liver (Figures 2A), bursa (Figures 2B), thymus (Figures 2C), spleen (Figures 2D) and intestine (Figures 2E) H and E x200.

Lesions in NDV challenged (Gp 8): bursa including activation of Germinal cells that showing mitotic (Figure 3A), depletion of lymphoid follicles (Figure 3B) and inter follicular edema and sever depletion of lymphoid follicles (Figure 3C), mild lymphocytic infiltration in intestine (Figure 3D), liver showing congestion of central vein (Figure 3E) and necrosis of hepatocytes (Figure 3F), spleen lesions are congestion of red pulp (Figure 3G) and sever depletion of lymphoid follicles (Figure 3H), while thymus showed hemorrhage and necrosis of medulla (Figure 3I).

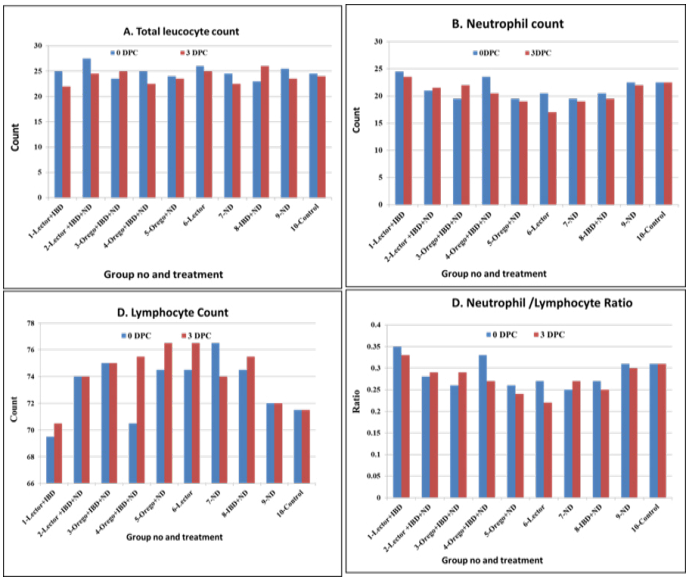

Figure 1: levels of total leucocyte and neutrophil, lymphocytes counts as well as neutrophil/lymphocyte ratio of chicken groups received immunostimulants, vIBDV at 14 days of age and vNDV challenge at 21 days of age at 0, and 3 days post NDV challenge. IBD and NDV infections resulted in undetected basophiles at 0 and 3 days in compared with Lector treated Gp 6 and non-infected non-treated control Gp 10 (table 4). N/L ratio at 0 time Lector + IBD Gp 1 (**Table 4**, **Figure 1D**) showed the highest value 0.35 followed by 0.33 in Oregosol+IBD Gp 4 and 0.31 in ND Gp 9 and control Gp10 while the lowest ratio 0.25 was in IBD Gp7. N/L ratio at the 3rd day post NDV challenge immunostimulants treated Gps showed the highest values (0.33) than control Gp10 (0.31), followed by Gps 9, 2 and 3 (0.30, 0.29 and 0.29).

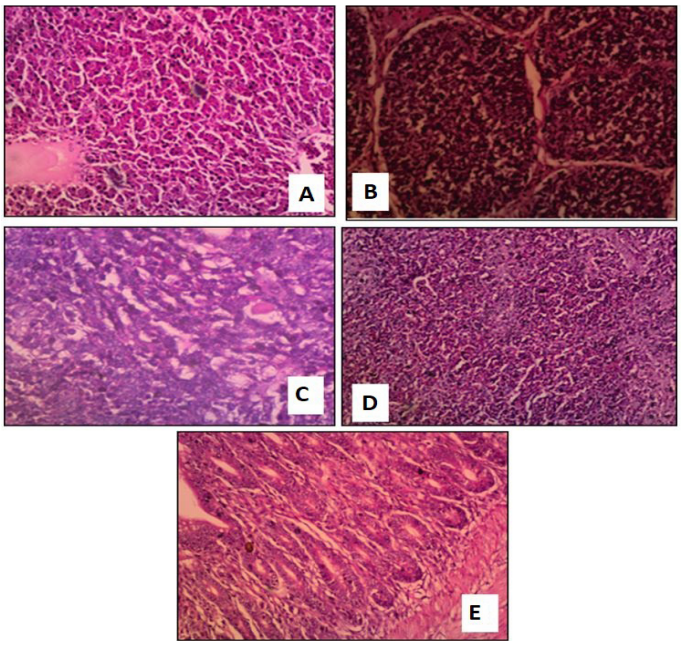

Figure 2: Tissue sections of control negative non-challenged group showing normal structure. A: Normal liver; B: Normal bursa; C, Normal thymus; D, Normal spleen; E: Normal intestine.

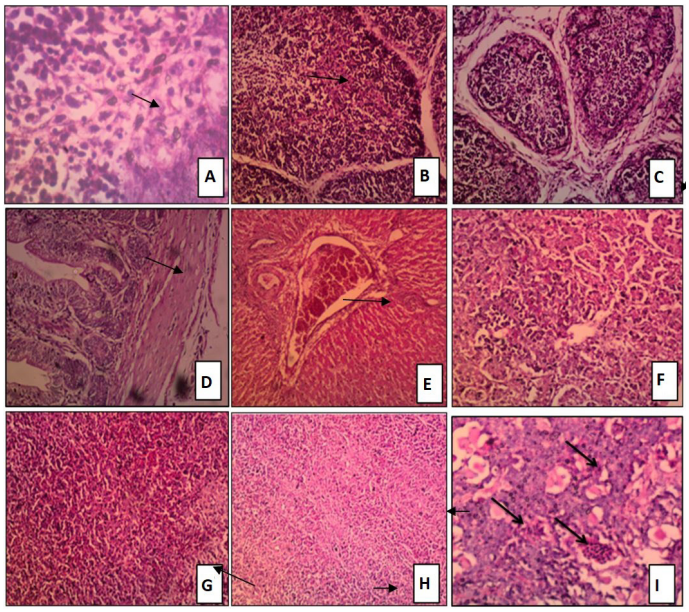

Figure 3: Tissue sections stained with H and E of chicken groups received challenged by NDV only.
A: Bursa: activation of germinal center that showing mitotic figure H and E x 400. B: Bursa: depletion of lymphoid follicles H and E x 200. C: Bursa: inter follicular edema and severe depletion of lymphoid follicles. D: Intestine: mild lymphocytic infiltration of the mucosa H and E x200. E: Liver congestion of central vein H and E x100. F: Liver: focal area of liver necrosis characterized by lymphocytic infiltration H and E x 200. G: Spleen: congestion of red pulp H and E x 200. H: Spleen; sever depletion of lymphoid follicles H and E x100. I: Thymus sowed hemorrhages and necrosis of medulla H and E x 200.

Chicken Gps challenged by NDV after treatment either with Orego solution (Gps 3, 5) or Lector (Gps 2, 6) or non-treated (Gp 8) showed histopathological changes in examined sections as mild in Lector, moderate in Orego and sever in non-treated.

Chicken Gps 1, 2, 3, 4 and 7 challenged with virulent IBDV showed bursal depletion of lymphoid follicles and hypertrophy of mucous membrane (Figure 4A) with edema, and fibrosis (Figure 4B) as well as and deposition of intra-follicular connective tissue (Figure 4C). Mild lymphocytic infiltration in intestinal mucosa (Figure 4D), liver necrosis (Figure 4E), congestion of portal vein (Figure 4F) and hydropic degeneration (Figure 4G), spleen showed congested red pulp (Figure 4H), thymus: hemorrhage and necrosis of medulla (Figure 4I).

Chicken groups challenged by IBDV after treatment with Lector (Gps 1, 2) showed histopathological changes in examined sections milder than Orego solution (Gps 3, 4) and non-treated infected (Gp 7) showed the most sever lesions.

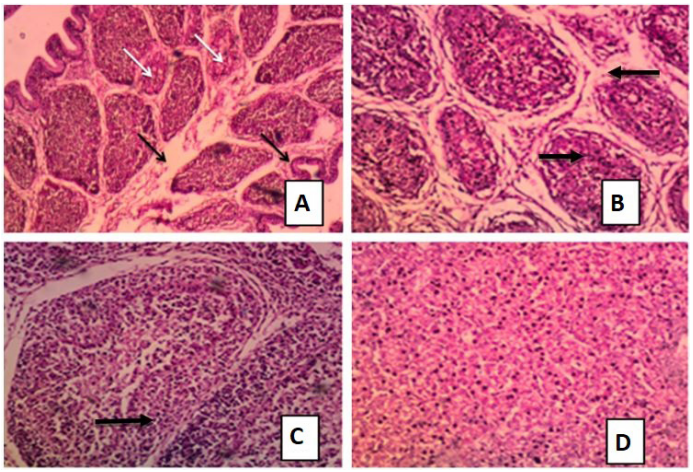

Figure 4: Tissue sections H and E stained of chicken groups challenged with virulent IBDV showing: A: Bursal depletion of lymphoid follicles and hypertrophy of mucous membrane wit inter follicular edema H and E x 100. B: Bursa: sever depletion of the lymphoid follicle accompanied with inter follicular edema and fibrosis H and E x100. C: Bursa: sever depletion of lymphoid follicle H and E x 200. D: Liver: hydropic degeneration of the hepatocytes cytoplasm H and E x 200.

Chicken Gps 2, 3 and 9 challenged with virulent NDV and IBDV; bursa showed sever depletion of lymphoid follicles, connective tissue deposition and finger like projections (Figure 5A), with intra follicular hemorrhage (Figure 5B), necrosis and C.T deposition of lymphoid follicles (Figure 4), intestine sections have hemorrhage in sub mucosa (Figure 5C) and lymphocytic infiltration (Figure 4D). Hydropic degeneration and necrosis of hepatocytes of liver (Figure 5), vacuolar degeneration and hypertrophy of bile duct (Figure 5E), congestion of central vein (Figure 5E), spleen has sever fibrosis (Figure 5F), depletion of lymphoid follicles of white pulp (Figure 5G), thymus showed focal area of hemorrhage, congestion of medulla, congestion of thymic artery and depletion of medulla (Figure 5H), also depletion of cortex was recorded. Chicken groups challenged with both virulent IBDV and NDV treatment either with Lector (Gp 2) or Orego solution (Gp 3) or non-treated (Gp 9) showed the no marked difference in lesions.

DISCUSSION

Newcastle Disease (ND) is a highly contagious disease which causes high morbidity and mortality rates in poorly vaccinated commercial chicken's flocks, in addition drops in egg production in well-vaccinated layers (Alexander et al., 2004; Miller et al., 2010; Ahmed et al., 2017; 2022b; Amer et al., 2018a). Also, Infectious Bursal Disease (IBD) causes high mortality rate and immunosuppression which leads to severe economic losses in poultry industry in Egypt (Shehata et al., 2017; Samy et al., 2020).

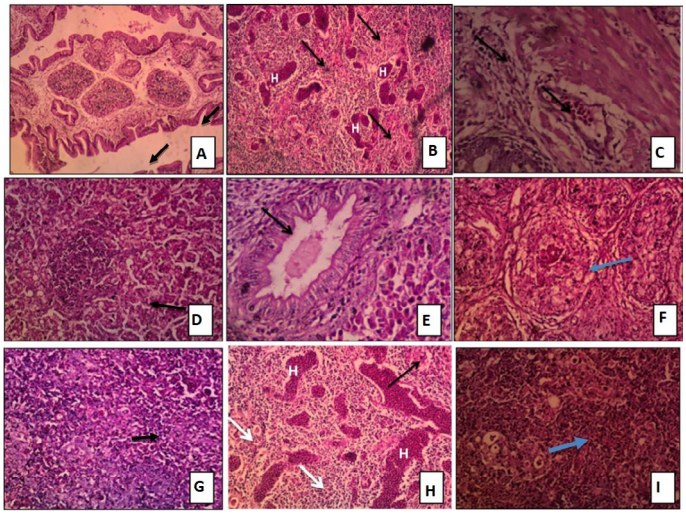

Figure 5: Tissue sections stained with H and E of Chicken groups challenged with virulent NDV and IBDV showing: A: Bursa: sever depletion of lymphoid follicles accompanied with inter follicular connective tissue deposition and hypertrophy of the mucous line forming finger like projections H and E x100. B: Bursa: sever depletion of lymphoid follicles with intra follicular hemorrhage H and E x100. C: Intestine: hemorrhage in sub mucosa with lymphocytic infiltration H and E x200. D: Liver: Hydropic degeneration in hepatocytes cytoplasm and focal area of coagulative necrosis infiltrated with lymphocytes H and E x 200. E: Liver: vacuolar degeneration in the cytoplasm of hepatocytes and hypertrophy of bile duct lining epithelium forming finger like projection in the lumen H and E x 200. F: Spleen: fibrosis of the lymphoid follicles H and E x200. G: Spleen: depletion of lymphoid follicles of white pulp H and E x 200. H: Thymus: focal area of hemorrhage, congestion of medulla, congestion of thymic artery and depletion of medulla H and E x 200. I: Thymus: depilation of cortex H and E x200.

The obtained decline HI titers against NDV as shown in Table 2 indicated that these bird groups were not naturally infected and the applied immunostimulants had no effect on antibody titers. Similar results were recorded by Sadeghi et al. (2013) who stated that dietary supplementations of prebiotic-based mannan-oligosaccharide and β-glucan has no significant effect on immune parameters on chicks in the non-infected group.

The recorded signs and post mortem lesions in chicken groups challenged with virulent IBDV were similar to those previously reported (Amer et al., 2007; Eterradossi and Saif, 2013; Wagari, 2021; Ghetas et al., 2022). While, the recorded signs and post mortem lesions in chicken groups challenged with NDV VII.1.1 were the same to those previously mentioned by Amer et al. (2018b, 2019) and Ahmed et al. (2022b).

Results of performance parameters after immunostimulants

administration and challenged with IBDV at 14-days of age and vNDV Genotype VII.1.1 at 21-days of age in broiler chickens proved that administration of Lector and Orego solution had positive effect on average body weight (ABW) gain and feed conversion rates (FCR) than control Gps (7, 8, 9 and 10) at the first 3 weeks of age; where the Lector medicated Gps (1, 2 and 6) showed higher rates of ABW followed by Orego medicated Gps (3, 4 and 5), these finding agreed with those obtained be Amer et al. (2017) and Zain-El-Deen et al. (2022).

Total and differential leucocytic counts showed higher values of eosinophil in immunostimulants treated groups than control ones. In the current study Lector had a great high effect in WBCs levels specially lymphocytes and monocytes and these results relatively matched with Kong et al. (2004) and Zhao et al. (2013). On the other side, the infection with IBD and/or NDV induced variation in the detected values of total and differential leucocytic counts; there were biphasic lymphopenia, eosinophilia and heterophilia in chicks infected with IBDV (Oladele et al., 2005). Faeji et al. (2019) reported a significant reduction in values of monocytes in NDV in the infected birds. However, no significant changes in the eosinophil and basophil values as compared to uninfected birds, where the leucocytic variations indicate that velogenic NDV exert significant depression on leucogram. The calculated H/L ratios indicate the severity of IBD and/or NDV infections especially in non-vaccinated birds; the neutrophil-to-lymphocyte ratio (NLR) in peripheral blood reflects the balance between systemic inflammation and immunity and is emerging as a prognostic biomarker in many diseases (Gross and Siegel, 1983). Also, Malik et al. (2018) observed that ND virus has significantly affected hematology parameters in broiler birds as compared with non-infected. Leukopenia is a characteristic of viral diseases (Jain, 1986). Chineme and Cho (1984) reported increased mean hematocrit values, as well as lymphocytopenia, in IBDV-infected chickens. Oladele et al. (2005) reported eosinophilia showed no characteristic bittern. Eosinophil counts in chicks initially increased to a peak of 1.93 x 10^3/µl at 6 h pi and subsequently declined.

Histopathologlogial lesions in IBDV infected groups showed severe depletion of the lymphoid follicle with activation of germinal center, deposition of intrafollicular connective tissues in examined bursa; while examined intestines showed depletion of the lymphoid follicle tissue and hypertrophy of the epithelial lining making finger like projection. These results agree with Dash et al. (1991), Inoue et al. (1994), Amer et al. (2007), Singh et al. (2015) and Ghetas et al. (2022).

Histopathological findings in Gps (2, 3, 5, 6, 8 and 9) due to vNDV infection were sever depletion of lymphoid follicles of white bulb of spleen, lymphocytic infiltration in cecal tonsils, sever congestion and vacuolar degeneration in liver accompanied with focal areas of coagulative necrosis and thymus showed hemorrhage with necrosis in medulla. These finding were previously recorded by Mohammadamin and Qubih (2011), Etriwati et al. (2017) and Amer et al. (2018b).

Infections of chickens with vIBDV followed by vNDV induced more sever lesions in presence or absence of immunostimulants. Also, the results supported by that vIBDV infection induces immunosuppression and increases susceptibility to other infections including NDV (Wang et al., 2010; Schat and Skinner, 2013; Liang et al., 2015).

Generally, the obtained results showed that the using of immunostimulants has no marked effect on restoring immunity in vIBDV or improve protection against vNDV genotype VII infection in infected broiler chickens. Also, losses in vIBDV infected due to challenge with VNDV are more severe**.** This result disagree with previous work where velogenic NDV or classical IBDV infections were used (Chan et al., 2008; Firenzouli et al., 2008; Weickert and Pfeiffer, 2008; Yu et al., 2015; Hou et al., 2016; Amer et al., 2017).

CONCLUSIONS AND RECOMMENDATIONS

Therefore, immunostimulants only are of low value against IBD and ND virulent viruses challenge. It is recommended applying strict hygienic measures and suitable vaccine to protect chickens against both IBD and NDV infections as the current used prevention and control methods, including vaccines and vaccination protocols are not adequate against either single or combined infections with IBD and/or ND.

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NOVELTY STATEMENT

Our study concluded that, immuno-stimulants are of low value when used alone against NDV infection, as well as, the virulence of NDV is increased when combined with IBDV infection. Furthermore, our study raise the importance about more effective vaccination programs that must be

implemented to control both NDV and IBDV outbreaks.

AUTHOR'S CONTRIBUTIONS

All authors participated in design, experimental procedure, writing, revised, and reviewing the manuscript equally.

Consent for publication

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Competing interests

The authors have declared no conflict of interest.

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