Avian disease

The Protective Efficacy of Recombinant Herpesvirus of Turkey (rHVT-ND-IBD) Vaccine against Velogenic Newcastle Disease Virus Genotype VII.1.1.

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ABSTRACT

Newcastle disease virus (NDV) outbreaks have been reported worldwide in vaccinated and non-vaccinated chicken farms, causing severe economic losses despite extensive vaccination. This study assessed the effectiveness of recombinant herpes virus of turkey (rHVT-ND-IBD) expressing the F protein of genotype VII with conventional genotype II vaccines against velogenic NDV genotype VII.1. 1. in broilers. Live ND vaccine with inactivated vaccine (G1), live ND vaccine with rHVT-ND-IBD vector vaccine and inactivated vaccine (G2), live ND vaccine with rHVT-ND-IBD vector vaccine (G3) and Live ND vaccine alone (G4) were the four immunization programs that were employed. Along with the control non-vaccinated group (G5). Birds were challenged at day 28. Clinical protection, seroconversion, histological alterations, and viral shedding following challenge were used to evaluate protection. The results showed mild clinical signs in G2 and G3 than in other groups, In addition, the mortality rates were 15%, 0%, 0% and 42.5% in G1, G2, G3 and G4, respectively, vs 85% in G5. Moreover, the tracheal virus shedding in G2 and G3 was significantly ($p \leq 0.01$) reduced when compared with G1, G4 and G5. The mean lesion score (MLS) was significantly ($p \leq 0.01$) decreased in G2 and G3 compared to other groups. The conclusion was that broilers immunized with rHVT-IBD-ND and NDV genotype II vaccinations exhibited full clinical protection against NDV genotype VII.1.1. After that, it is advised to give broilers rHVT-IBD-ND together with NDV genotype II vaccines to achieve the best possible clinical protection against genotype VII.1.1, particularly in endemic nations.

Keywords: Commercial broiler, HVT-ND vector vaccine, Genotype-matched vaccine, and Genotype VII.1.1 Newcastle disease virus.

INTRODUCTION

Newcastle disease (ND) is a highly contagious disease of chickens and turkeys and one of the most important poultry diseases worldwide. The disease causes high mortality and economic losses (Ahmed et al., 2017). Because of this, the disease is notifiable (OIE, 2012). From 2013 to 2018, ND has a global impact and 109 of the 200 member countries have notified the OIE of

the disease (OIE, 2018). Since NDV infection is known to be harmful to birds' health, significant scientific research on the viruses was conducted (Alexander et al., 2012; Miller and Koch, 2013). The etiology of the disease is Newcastle disease virus (NDV), a negative-sense single-stranded RNA virus whose 15.2 kb non-segmented genome is organized into six genes encoding six structural proteins, namely nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), Fusion protein (F), hemagglutinin neuraminidase protein (HN), and the polymerase protein (L) and two non-structural proteins, V and W (Murulitharan et al., 2013). NDV outbreaks in Egypt affected both vaccinated and nonvaccinated chicken farms throughout multiple governorates. The outbreaks caused severe respiratory symptoms, nervous signs, and high mortality rates, which led to significant financial losses in broilers especially at three to four weeks old (Ewies et al., 2017; Moharam et al., 2019). NDV outbreaks in chicken farms in northern Egypt governorates are now caused by class II genotype VII strains (Ahmed et al., 2017; Moharam et al., 2019; Sedeik et al., 2019). Since genotype VII has been linked to numerous recent outbreaks in Asia, Africa, and the Middle East, it is especially significant (Awad et al., 2015). The occurrence of mutations at several locations throughout the F gene, including antigenic epitopes, N-glycosylation sites and cysteine residues, has led to the recent classification of Egyptian isolates of NDV to genotype VII 1.1 (Dewidar et al., 2021). These alterations could affect the virulence of the virus and possibly interfere with the protection provided by traditional vaccinations (Dimitrove et al., 2019; Selim et al., 2018). Hitchner B1, Clone 30, Komarov, Lasota, and other commercial NDV vaccines are all classified as genotype II, which is distinct from the majority of circulating Egyptian genotype VII. Furthermore, vaccinated chickens with Lasota vaccine and challenged with virulent genotype VII displayed symptoms and sickness, demonstrating that the vaccine cannot completely prevent viral shedding (Kim et al., 2013; Absalón et al., 2019; Sedeik et al., 2019; Sultan et al., 2022). The development of antigenically matched vaccines has proven effective for both inactivated vaccines and live vaccines developed from homologous genotypes of the challenge virus in terms of clinical protection and reduction of virus shedding titers (Miller et al., 2007; 2009; Hu et al., 2009; Dimitrov et al., 2017a, b; Absalón et al., 2019; Dewidar et al., 2022; Sultan et al., 2022). There are now two distinct genotype vaccinations available in Egypt: genotype II and the more recent genotype VII, this enables us to evaluate their degree of protection and viral shedding following a VVND-GVII.1.1 challenge (Miller et al., 2007; 2009). Genotype II vaccines include live, inactivated, and vector vaccines are available in Egypt. Genotype VII vaccines include inactivated vaccines, vector vaccines that expresses the fusion protein gene (F gene) of NDV and recombinant NDV vaccine. The recombinant NDV vaccine strain (rLasota-HN) was prepared based on the Lasota vaccine strain, which expresses the hemagglutinin neuraminidase (HN) protein of NDV genotype VII, which provides increased protection against and prevents clinical symptoms and mortality as well as lessen viral shedding compared to employing vaccinations that were not closely genotype matched (Miller et al., 2009; Dewidar et al., 2022; Sultan et al., 2022). Therefore, the current study aimed to determine whether live and inactivated vaccinations and a vector vaccine expressing genotype VII's F protein (HVT-ND) might efficiently protect broiler chickens from the

challenge with a recently identified GVII.1.1 NDV strain.

MATERIALS AND METHODS

Vaccines

Three forms of NDV vaccines have been used in this study. This included Vector vaccine (VAXXITEK® HVT+IBD+ND expressing the F protein from a genotype VII of Newcastle disease virus (NDV) bought from Boehringer Ingelheim Animal Health USA Inc.'s local agent in Egypt, the International Free Trade Company (IFT), Cairo, Egypt., Live clone 30 vaccine, vaccinal dose ≥ 6.0 $\log^{10}$ Embryo infective dose 50 (EID_{50})obtained from local agency in Egypt of MSD, Spain and killed genotype II NDV vaccine, vaccinal dose contain ND Clone 30 ≥ 50 PD_{50}also obtained from local agency in Egypt of MSD, Spain.

Challenge Virus

Velogenic NDV genotype VII strain Chicken/USC/Egypt/2015 strain (accession number MG029120) was used. The virus strain has been previously isolated and characterized (Sultan et al., 2020). 0.5ml of $10^{5.5}$ EID_{50} / bird was used to administer the ND challenge virus intramuscularly. Using Reed and Muench (1938), the EID_{50} titer was calculated and a dose of 0.5ml containing $10^{5.5}$ EID_{50} / bird was administered intramuscularly.

Experimental Design

Two hundred one-day-old commercial broiler chicks were supplied from a local hatchery. They were housed in a separate pen with food and water provided ad libitum at abio-secured experimental facility. The chicks were randomly divided into five groups (G-1 to G-5) of 40 birds. Each group was vaccinated with a different program against the NDV. G1(vaccinated by live ND and inactivated ND vaccines), G-2 (vaccinated by HVT-ND-IBD, Live ND and Inactivated NDV vaccines), G-3 (vaccinated by HVT-ND-IBD and Live ND vaccines), G-4 (vaccinated by live ND vaccines) and G-5 (control non-vaccinated challenged) as shown in Figure 1.All groups were challenged at28days old with VNDV genotype VII, strain (Chicken/ USC/Egypt /2015 with accession number MG029120), $EID_{50} = 10^{5.5}$ / 0.5 ml / bird intramuscularly. For 14 days after the challenge, the chicks were observed daily for clinical signs of NDV and deaths.

On days 7, 14, 21, 28, 35, and 42 of the trial, antibody titers were monitored using the Heamagglutination inhibition test (HI) and Enzyme linked immunosorbent assay (ELISA), before and after the challenge. Ten tracheal and cloacal swaps were collected from each group before challenge which checked by real-time reverse transcriptase polymerase chain reaction (RT-PCR) against F gene of common NDV Additionally, at 2, 4, and 7 days after the challenge, tracheal and cloacal swaps were taken from every group (10 birds per group). This was done to monitor the shedding of viruses, as shown in Table 3.
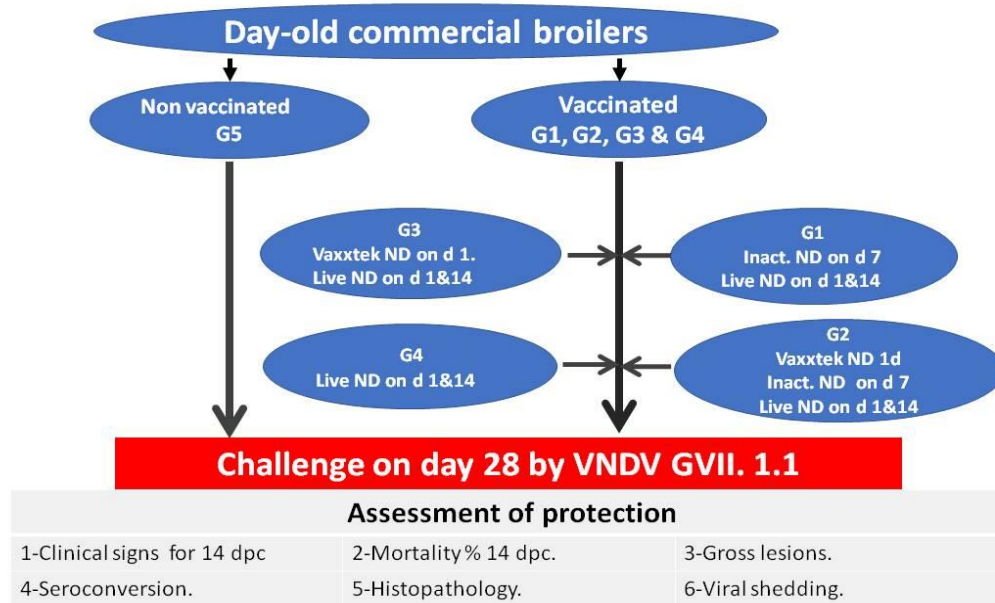
Figure 1. Experimental design of the efficacy of different vaccination programs against challenge with VNDV genotype VII.1.1 at 28 day in commercial broiler chickens.

Serology

Blood samples were collected from wing vein or by slaughtering every week at 7, 14, 21and 28 days of age before challenge and at 7 and14 days post-challenge with VNDV genotype VII.1.1. Samples were kept in slop position at 37 °C for 1 hour then kept at 4 °C overnight. Serum samples were separated by centrifugation at 3000 rpm/10_minutes and stored at -20 °C till analysis. Two methods were used to measure antibody titers: the hemagglutination inhibition (HI) test and the enzyme-linked immunosorbent assay (ELISA). HI test was applied by using four hemagglutinating units (4HAU) of genotype II, Newcastle disease virus, lasota antigen. The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2021 was followed in performing the haemagglutination inhibition (HI) test using four HA units of antigen and 1% chicken red blood cells. The guides of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2021 were followed (OIE, 2021). ELISA was applied by using

commercial ELISA kits ID.vet ID Screen NDVsupplied by ID.vet. Corporation, 310, rue louis Pasture-Grabls France.

2.1. *Virus shedding:*

At 2, 4, and 7 days after the challenge, 10 tracheal and 10 cloacal swabs were randomly collected. The swabs were then mixed with an antibiotic solution in 400 µL of Dulbecco's modified Eagle medium to detect NDV shedding. Subsequently, viral RNAs were extracted using the TRIzol reagent (Gibco, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RT-PCR was applied Using specific primer and probe sets from (QIAGEN, Valencia, CA, USA); forward; 5' -TCCGGAGGATACAAGGGTCT- 3', reverse; 5' -AGCTGTTGCAACCCCAAG- 3'and prob 5' -FAM- AAGCGTTTCTGTCTCCTTCCTCCABHQ 1- 3' (Learyet al., 2013). In the RT-PCR test; 0.25 µL of QuantiTect RT Mix, 0.25 µL of each primer, 0.125 µL of the probe, 3.625 µL of PCR-grade water, and seven µL of RNA template were used. Real time reverse

transcription was applied by 30-minute setup period at 50 °C followed by a 15-minute primary denaturation step at 94 °C, denaturation at 94 °C for 15 s for 40 cycles, annealing at 54 °C for 30 s, and extension at 72 °C for 10 s using MX3005P real-time PCR apparatus (Stratagene, La Jolla, CA, USA). RRT-PCR titers were converted into log10 EID_{50}/mL (Nolan, 2020). The usual system detection limit was previously stated as 0.5 EID_{50}/mL (Sultan et al., 2020). The Ct of unknown samples was plotted against the main standard curve to determine the amount of NDV present, which was then quantified in terms of log10 EID_{50}/mL equivalents.

Histopathological Examination

Several tissue samples (including, the brain, spleen, bursa, cecal tonsils and trachea) were collected from dead birds of groups with mortalities throughout the seven days post the challenge and from three chicks from each group after being humanely euthanized on the 7[th-] day post-challenge with VNDV including, the brain, spleen, bursa, cecal tonsils and trachea. Samples were fixed in a 10 % formalin buffered solution, processed for histology, and stained with hematoxylin and eosin (H&E) (Gibson et al., 2013). The severity of the infected tissue lesions was graded from 1 to 5 (1 for no lesions, 2 for mild lesions, 3 for moderate lesions, 4 for severe lesions, and 5 for extremely severe lesions) (Sultan et al., 2019).

Statistical evaluation

Utilizing the Chi2-test, statistical analysis was performed to study the variance incidences. Also, the ANOVA-test was used to study the level of virus titer or its amount among different days and groups. The value is significant if it is equal to or lower than (P < 0.05). The statistical analysis was made using SPSSPC+-Version 25 and the diagrams were made using Harvard graphica-5.

RESULTS

Clinical signs, postmortem gross lesions and mortalities

Chicks were monitored daily for post-challenge clinical signs and mortalities for14 days. Clinical signs differed as follows: G-2 (vaccinated with HVT-ND-IBD, Live ND, and Inactivated NDV vaccines) and G-3 (vaccinated with HVT-ND-IBD and Live ND vaccines) have no clinical signs; G-1 (vaccinated with live ND and inactivated ND vaccines) has moderate clinical signs; G-4 (vaccinated with live ND vaccines) has severe clinical signs; and G-5 (control non-vaccinated challenged) has more severe respiratory, neurological, and gastrointestinal disorders. G-5 (control non-vaccinated) showed post challenge severe clinical signs including nervous manifestation (tremors, torticollis and wing drop), respiratory signs, greenish watery diarrhea, severe depression and off food (Figure 2). Additionally, common P.M. gross lesions of VNDV included petechial hemorrhages on the mucosa of the proventriculus, severe hemorrhages on cecal tonsils, button-shaped ulcers on the intestinal mucosa, and enlarged and congested kidney (Figure 3). Mortality rates were 15%, 0%, 0%, 42.5% and 85% in G1, G2, G3, G4 and G5 respectively (Table 1).

Serum antibody response

The serological immune response against NDV was monitored weekly for 6 weeks using the HI test and ELISA, starting from 7days of age until 42 days of age. The findings demonstrated that there was a significant difference (P < 0.01) in the NDV HI and NDV ELISA titers between the vaccinated groups and the non-vaccinated group at various times during the trial, both before and after the 28-day-old VNDV genotype VII.1.1 challenge. The HI titers were high at the 7 day of age due to maternal immunity then decreased in all groups at the 14 day of age then increased in

G-1,G-2,G-3 and G-4 due to the effect of vaccination at 21 day of age and increased more at 28 day to become 5, 4.6, 3.4 and 3.4 log2 respectively, but in G-5 (control non vaccinated) the HI titer at 28 day decreased to become 0 log2 (Table 2). At the 7-day post-challenge the HI titers were highly increased in all groups to become 7.2 (G-1), 10.4 (G-2), 9.8 (G-3), 8 (G-4) and 9.6 log2 in G-5 (Table 2). The results of the ELISA also were high at the 7-day of age due to maternal immunity then decreased in all groups at the 14 and 21 day of age then increased in G-1, G-2, G-3 and G-4 due to the effect of vaccination at 28 day of age to become 3048, 2721, 2052 and 2787 respectively, but in G-5 (control non vaccinated) the ELISA titer at 28 day decreased to become 234 (Table 2). At the 7-day pos- challenge the ELISA titers were highly increased in all groups to become 12399 (G-1), 10929 (G-2), 13068 (G-3), 13136 (G-4) and 12639 in G-5 (Table 2).

Virus Shedding

Tracheal and cloacal swabs were collected from each group to examine virus shedding at 1 day before challenge, 2-, 4- and 7-days post-challenge. All tracheal and cloacal swabs collected from all groups before the challenge were negative for NDV. On the 2nd DPC, G-1showed that 55% of birds were shedders and the virus titers in the tracheal and cloacal swabs, were4.25±0.52and 4.39±0.22, respectively. At the same time, G-2 showed that 20% of birds were shedders, and virus titers in the tracheal and cloacal swabs were 2.92±0.44 and 2.62±0.38, respectively; the birds in G-3 showed 35% shedders and tracheal and cloacal viral titers of 3.19±0.82 and 3.96±0.56. In contrast, G-4 showed that 80% of birds were shedders, and tracheal and cloacal virus titers were 3.78±0.84 and 3.47±0.68, respectively. The birds in G-5were 100% shedders and showed the tracheal and cloacal viral titers of 4.82±0.54

and 4.78±0.68. The 4^{th}DPC, G-1 showed 35% shedders and the virus titers in the tracheal and cloacal swabs, were 2.85±0.48 and 3.12±0.72, respectively, while in G-2 it was undetectable. The birds in G-3 showed 15% shedders and tracheal and cloacal viral titers of 1.86±0.74 and 2.36±0.31, while in G-4 were 3.78±0.75 and 3.35±0.49, respectively with 60% shedders. The birds in G-5 showed tracheal and cloacal viral titers of 5.25±0.36 and 4.33±0.74 with 90% shedders. The birds in G-1, G-2, and G-3 did not exhibit any shedding on the 7th DPC, either from the trachea or the cloaca and G-4 birds showed 30% shedders and tracheal and cloacal viral titers were 2.8±0.55 and 2.8±0.55 respectively, while, in G-5 were 5.29±0.34 and 3.13±0.76, respectively with 80% shedders (Table 3 and Figure 4 and 5).

Histopathology

Tissue Samples were collected from dead birds in G-1, G-4 and G-5 and from all groups 7days post-challenge. Dead birds in G-1 and G-4 showed sever lesions (3) in most tested organs (brain, bursa and trachea) (Figure 6). But tissue samples of G-5 collected 7 days post-challenge were the most affected compared to tissue samples of other groups (Table 4and Figure 7). The analyzed organs of G-5 (challenged non-vaccinated birds) displayed various histological alterations. The brain displayed gliosis in the cerebellum and cerebrum as well as congested blood vessels with neutrophil vacuolation. Bursa showed severe hyperplasia with depletion of lymphocytes and formation of microcysts, epithelization and interfollicular connective tissue formation. The spleen exhibited mild congestion, hemorrhage, and focal amyloidosis. Trachea showed lining epithelium hyperplasia with mucous glands activation, mucosa thickening due to edema, congestion, and mononuclear cells infiltration in lamina propria, edema and

congestion of the submucosa and muscular layer. Cecal tonsils showed focal depletion of lymphocytes with the formation of empty cavities and focal aggregation of granulocytes in lamina propria. The kidney showed denudation and degeneration of the lining epithelium of renal tubules, with intertubular congestion and mononuclear cell infiltration.

Table 1. Clinical signs and mortalities of both vaccinated and unvaccinated challenged groups post the challenge with virulent Newcastle disease virus genotype VII.1.1. at 28th day of age.

G	Birds no	Vaccination regime for Newcastle disease		1st week			2nd week			Mortality%
		Age/day	Age/day	RS	DS	NS	RS	DS	NS	
G1	40	Live[1] Inactivated[2]	1,14 7	13/40 (32.50%)	8/40 (20 %)	0/40 (0 %)	12/31 (38.7 %)	11/31 (35.5 %)	4/31 (13 %)	15%
G2	40	HVT- IBD_ND[3] Live[1] Inactivated[2]	1 1,14 7	3/40 (7.5 %)	0/40 (0 %)	0/40 (0 %)	1/37 (2.7 %)	0/37 (0 %)	2/37 (5.40 %)	0%
G3	40	HVT- IBD_ND[3] Live[1]	1 1,14	5/40 (12.5 %)	0/40 (0 %)	0/40 (0 %)	1/37 (2.5 %)	0/37 (0 %)	0/37 (0 %)	0%
G4	40	Live[1]	1,14	33/40 (82.5 %)	28/40 (70 %)	8/40 (20 %)	20/20 (100 %)	18/20 (90 %)	10/20 (50 %)	42.5%
G5	40	Control non vaccinated		35/40 (87.5 %)	25/40 (62.5 %)	20/40 (50 %)	3/3 (100%)	3/3 (100 %)	3/3 (100 %)	85%
Chi[2]-test				15.75**						

1: a live attenuated freeze-dried vaccine against Newcastle Disease. Each dose of vaccine contains 10^6 EID_{50} of NDV strain Clone 30.

2: Inactivated oil emulsion genotype II NDV vaccine against Newcastle disease virus, administrated by a dose of 0.5 ml through S/C.

3: vector vaccine against Marek's disease (MD), infectious bursal disease (IBD) and Newcastle disease (ND), expressing the F protein from a genotype VII of NDV administrated by subcutaneous injection in the hatchery.

** = Significant at ($P < 0.01$).

G: group number.

RS: respiratory sign,

DS: digestive sign,

NS: nervous sign.

Figure 2. Clinical symptoms of chickens kept in G-5 (control non-vaccinated challenged group) on the third day following the challenge with the VNDV genotype VII.1.1 strain "chicken/USC/Egypt /2015" at day 28. **(A and B)** greenish-watery diarrhea and paralysis, **(C)** conjunctivitis, **(D)** torticollis.
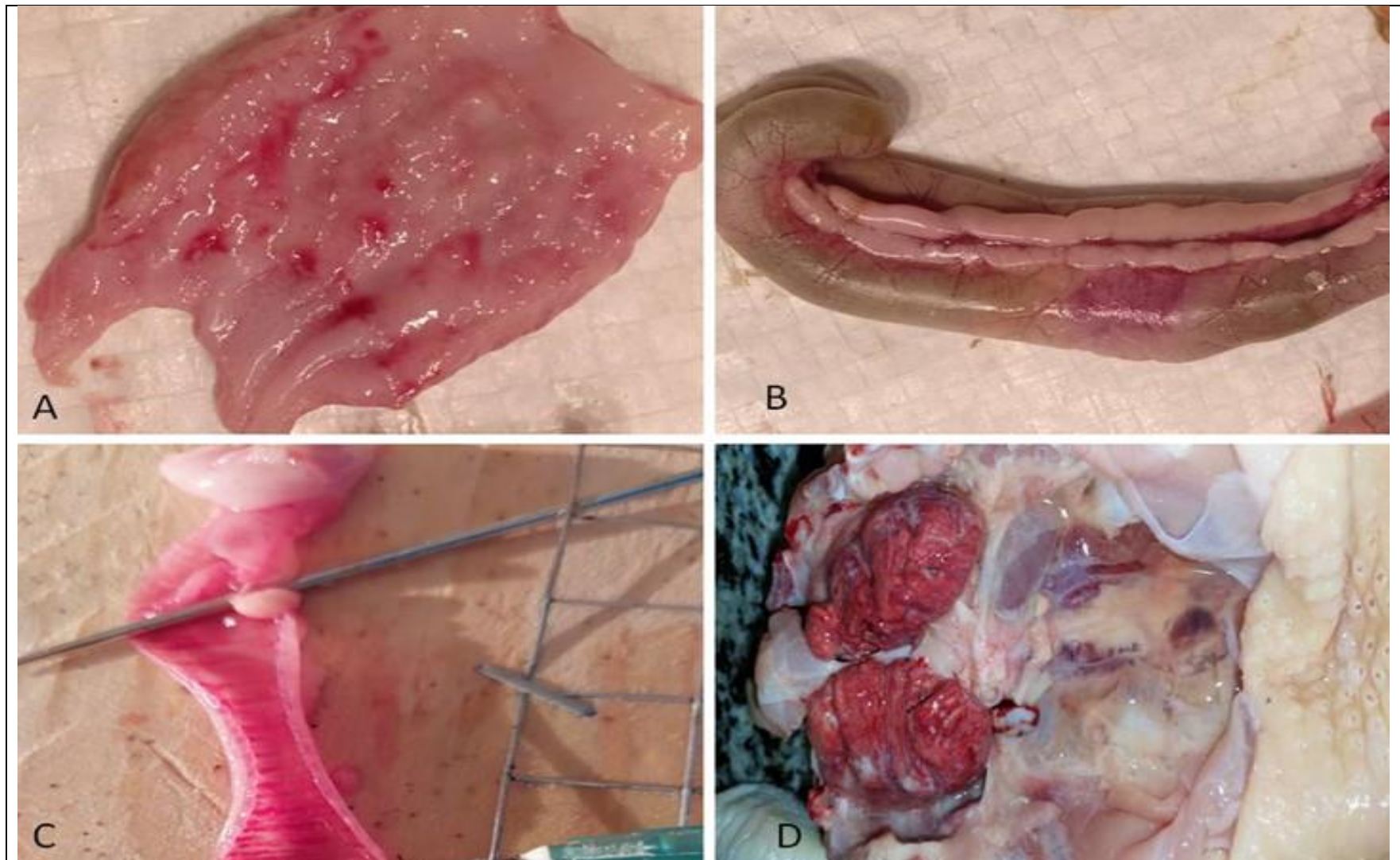
Figure 3. Characteristic postmortem lesions of the VNDV genotype VII.1.1 strain "chicken/USC/Egypt /2015" on the third day after challenge in group 5 (control non-vaccinated challenged group). **(A)** displaying petechial hemorrhagic spots on the proventricular glands, **(B)** button-like ulcer on the intestinal wall, **(C)** hemorrhagic tracheitis **(D)** Showing congestion of the lung.

Table 2. Mean $\log_2$ HI titers and mean ELISA titers of both vaccinated and unvaccinated challenged groups post the challenge with the VNDV genotype VII.1.1 strain "chicken/USC/Egypt /2015" at 28th day of age..

G.	Vaccination regime		Mean HI titer ± SD						Mean ELISA titers ± SD					
	Vaccine Type	Age / Day	7	14	21	28 (challenge	7 DPC	14 DPC	7	14	21	28 (challenge)	7 DPC	14 DPC
G1	Live[1] NDV Inactivated[2] NDV	1&14 7	5.6± 1.55[A]	2± 0.12[A]	3.8± 0.13[A]	5± 0.05[A]	7.2± 1.10[C]	11.6± 1.16[A]	10361± 10.33[A]	3635± 13.12[A]	1532± 15.44[D]	3048± 14.15[A]	12399± 12.99[D]	11875± 11.18[C]
G2	rHVT IBD_NDV[3] Live[1]NDV Inactivated[2]NDV	1 1&14 7	4.8± 1.14[B]	2.4± 0.12[A]	3.8± 0.14[A]	4.6± 0.04[B]	10.4±1.22[A]	9± 0.1.11[B]	9191± 9.22[B]	2306± 12.24[C]	2058± 12.24[B]	2721± 17.55[B]	10929± 10.94[E]	11603± 11.66[E]
G3	rHVT IBD_ND[3] Live[1] NDV	1 1 &14	4.4± 1.12[B]	2± 0.22[A]	3.8± 0.16[A]	3.4± 0.22[C]	9.8± 1.16[B]	7.8± 1.18[D]	9263± 9.24[B]	2595± 12.55[B]	2187± 12.44[A]	2052± 12.22[D]	13068± 13.20[B]	12142± 12.44[B]
G4	Live[1] NDV	1 &14	4.2± 1.44[B]	2± 0.12[A]	3.2± 0.14[A]	3.4± 0.12[C]	8± 1.70[C]	9.6± 1.16[C]	8583± 8.54[C]	3844± 13.44[A]	1631± 11.15[C]	2787± 12.44[C]	13136± 13.14[A]	13085± 13.80[A]
G5	Non vaccinated	-------	3.2± 1.12[C]	1± 0.11[B]	0.2± 0.01[B]	0[D]	9.6± 1.14[B]	9.5± 1.15[C]	7212± 7.22[D]	1373± 11.34[D]	551± 5.12[E]	234± 12.45[E]	12639± 12.64[C]	11748± 11.17[D]

1: a live attenuated freeze-dried vaccine against Newcastle Disease. Each dose of vaccine contains 10^6 EID_{50} of NDV strain Clone 30,
2: Inactivated oil emulsion genotype II NDV vaccine against Newcastle disease virus, administrated by a dose of 0.5 ml through S/C,
3: vector vaccine against Marek's disease (MD), infectious bursal disease (IBD) and Newcastle disease (ND), expressing the F protein from a genotype VII of NDV administrated by subcutaneous injection in the hatchery. G: group number. DPC: day post challenge. A, B, C, D: represent the statical analysis significance. Means within the same column of different litters significantly differ at (P < 0.01).

Table (3): Viral shedding titers $\log_{10}$ EID$_{50}$ /1 ml of both vaccinated and unvaccinated challenged groups post the challenge with the VNDV genotype VII.1.1 strain "chicken/USC/Egypt /2015" at 28th day of age.

G	0		2dpc		4dpc		7dpc	
	TR	Cl	TR	Cl	TR	Cl	TR	Cl
G1	0/10^A	0/10^A	5/10^B (4.25±0.52)	6/10^B (4.39±0.22)	3/10^C (2.85±0.48)	4/10^C (3.12±0.72)	0/10^C	0/10^C
G2	0/10^A	0/10^A	2/10^D (2.92±0.44)	2/10^E (2.62±0.38)	0/10^E	0/10^E	0/10^C	0/10^C
G3	0/10^A	0/10^A	3/10^C (3.19±0.82)	4/10^C (3.96±0.56)	1/10^D (1.86±0.74)	2/10^D (2.36±0.31)	0/10^C	0/10^C
G4	0/10^A	0/10^A	8/10^C (3.78±0.84)	8/10^D (3.47±0.68)	6.10^B (3.78±0.75)	6/10^B (3.35±0.49)	3/10^B (2.8±0.55)	3/10^B (2.8±0.55)
G5	0/10^A	0/10^A	10/10^A (4.82±0.54)	10/10^A (4.78±0.68)	10/10^A (5.25±0.36)	8/10^A (4.33±0.74)	8/10^A (5.29±0.34)	8/10^A (3.13±0.76)

Chi2= 25.45** Significant at (P < 0.01). A, B, C, D: represent the statical analysis significance. G: group number. TR: tracheal swabs. CL: cloacal swabs. Dpc: days post challenge.
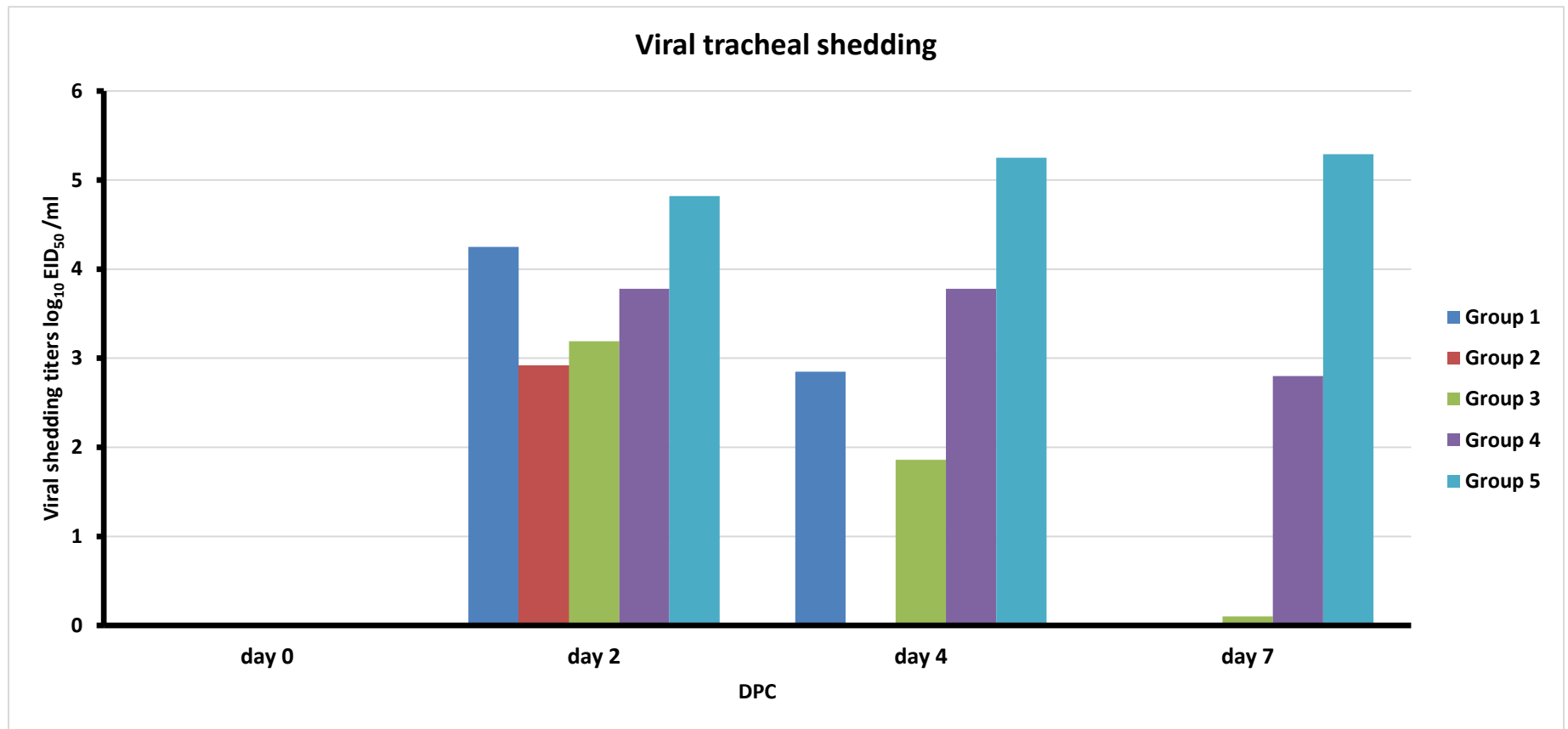
Figure 4. Tracheal viral shedding titers $\log_{10}$ EID$_{50}$ /1 ml of both vaccinated and unvaccinated challenged groups post the challenge with the VNDV genotype VII.1.1 strain "chicken/USC/Egypt /2015" at 28th day of age.
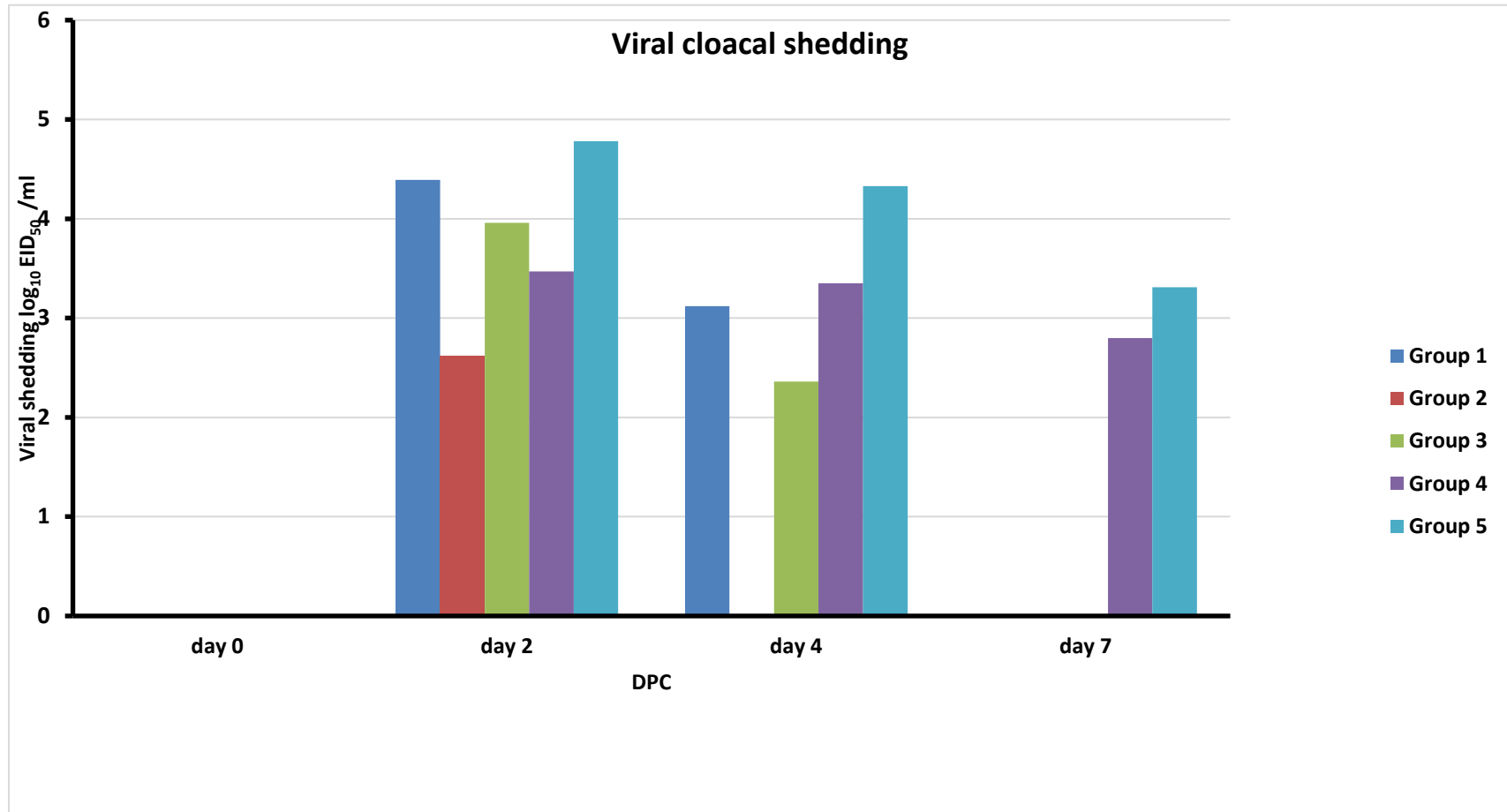
Figure 5. Cloacal viral shedding titers $\log_{10}$ EID$_{50}$ /1 ml of both vaccinated and unvaccinated challenged groups post the challenge with the VNDV genotype VII.1.1 strain "chicken/USC/Egypt /2015" at 28th day of age.

Table 4. Histopathological mean lesion scores of examined organs of slaughtered birds on 7day post challenge of broiler chickens vaccinated by different vaccination programs for NDV against challenge with VNDV genotype VII.1.1 at 28th days of age.

Organs/Group	Brain	Bursa	Spleen	Trachea	Cecal tonsil
G 1	3 ± 0.03^A	2 ± 0.02^A	0^B	3 ± 0.03^A	0^B
G 2	2 ± 0.02^B	1 ± 0.01^B	1 ± 0.01^A	3 ± 0.03^A	0^B
G 3	1 ± 0.01^B	1 ± 0.01^B	1 ± 0.01^A	2 ± 0.02^B	0^B
G 4	1 ± 0.01^B	2 ± 0.02^A	1 ± 0.01^A	2 ± 0.02^B	1 ± 0.01^A
G 5	1 ± 0.01^B	3 ± 0.03^A	3 ± 0.03^A	2 ± 0.02^B	3 ± 0.03^A

Means within the same column of different litters significantly differ at (P < 0.01).
(0) normal. (1) mild lesion. (2) Moderate lesion. (3) Severe lesion. (G1) vaccinated by live ND and inactivated ND vaccines. (G2) vaccinated by HVT-ND-IBD, Live ND and Inactivated NDV vaccines. (G3) vaccinated by HVT-ND-IBD and Live ND vaccines. (G4) vaccinated by live ND vaccines. (G5) control non-vaccinated challenged.
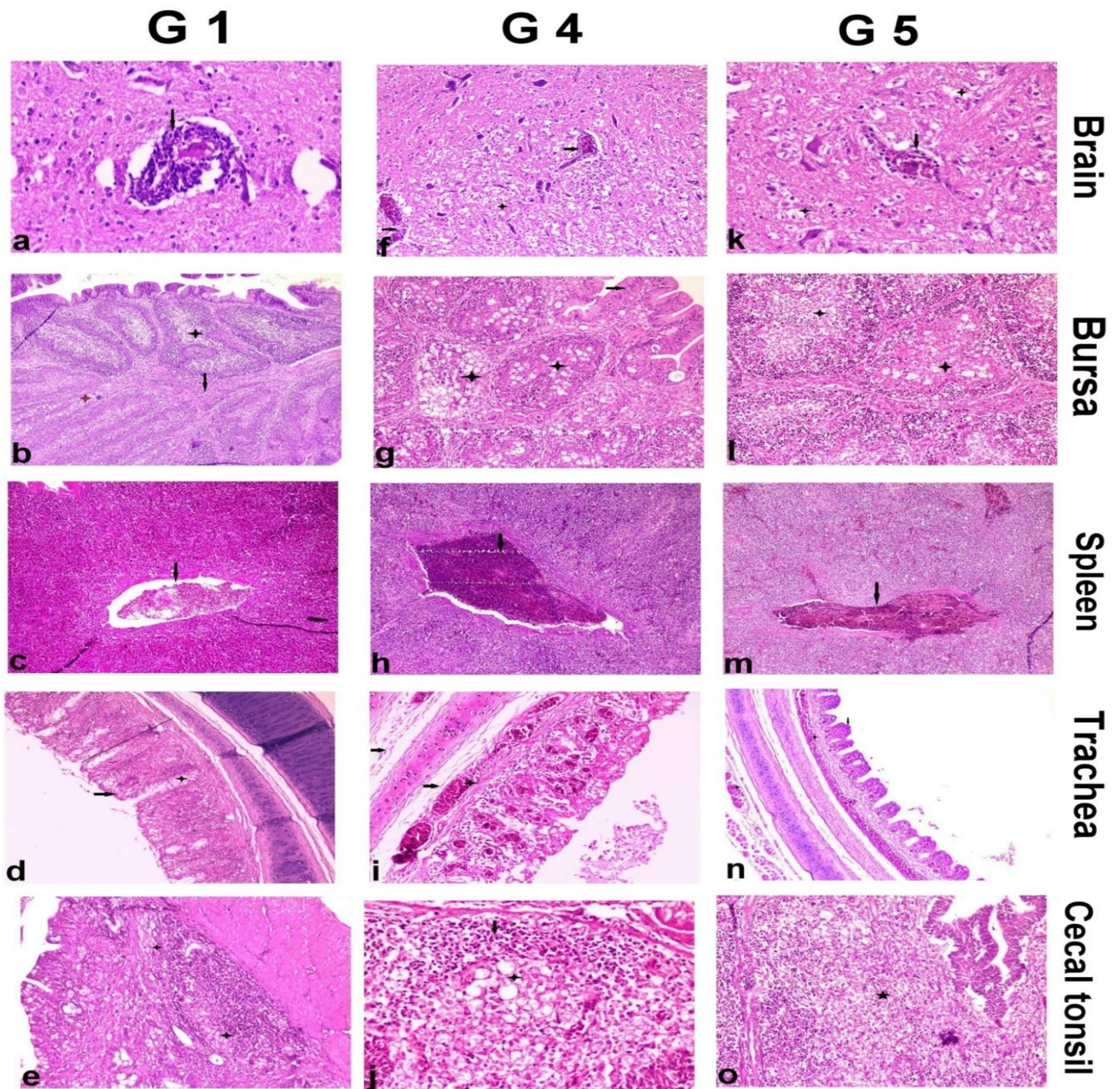
Figure 6. Histopathological changes of dead birds of group 1, 4 and 5 for 7 days post-challenge at 28th day. G1 (vaccinated by live ND and inactivated ND vaccines). (a) Cerebrum displaying perivascular cuff (arrow) H&E X400. (b) Bursa demonstrating lymphocyte depletion (black star) with microcysts formation (red star), with interfollicular connective tissue formation (arrow) H&E X100. (c) Spleen displaying blood vessels congestion (arrow) H&E X100. (d) Trachea displaying mucosal thickening

(line), hyperplasia of lining epithelium (arrow) edema with activation of mucous glands (star) H&E X100. (e) Cecal tonsil displaying localized depletion and necrosis of lymphocyte in the lamina propria with microcysts formation (star) H&E X200. G4 (vaccinated by Live ND vaccines) (f) Cerebrum displaying few lymphocytes bordered the blood vessels, and (arrow) vacuolation of neuropil (star) H&E X400. (g) Bursa displaying lining epithelium hyperplasia, with depletion and necrosis of lymphocytes with microcysts formation H&E X200. (h) Spleen displaying blood vessels congestion (arrow) H&E X100. (i) Trachea displaying mucosal thickening, edema (arrow), and severely congested blood vessels in the lamina propria (star) H&E X100. (j) Cecal tonsil displaying depletion of lymphocytes with microcysts formation (star), and few inflammatory cells infiltration (arrow) H&E X400. G5 (control non-vaccinated challenged) (k) Cerebrum displaying perivascular cuff (arrow), with vacuolation of neuropil (star) H&E X400. (l) Bursa displaying degeneration and necrosis of lymphocytes with microcysts formation (star) H&E X200. (m) Spleen displaying blood vessels congestion (arrow) and thickening of its wall H&E X100. (n) Trachea displaying edema and congestion in the mucosa and submucosa (star), as well as lining epithelium hyperplasia (arrow). H&E X100. (o) Cecal tonsil demonstrating a decrease in lymphocytes and their necrosis in the lamina propria, together with the formation of microcysts (stars) H&E X200.
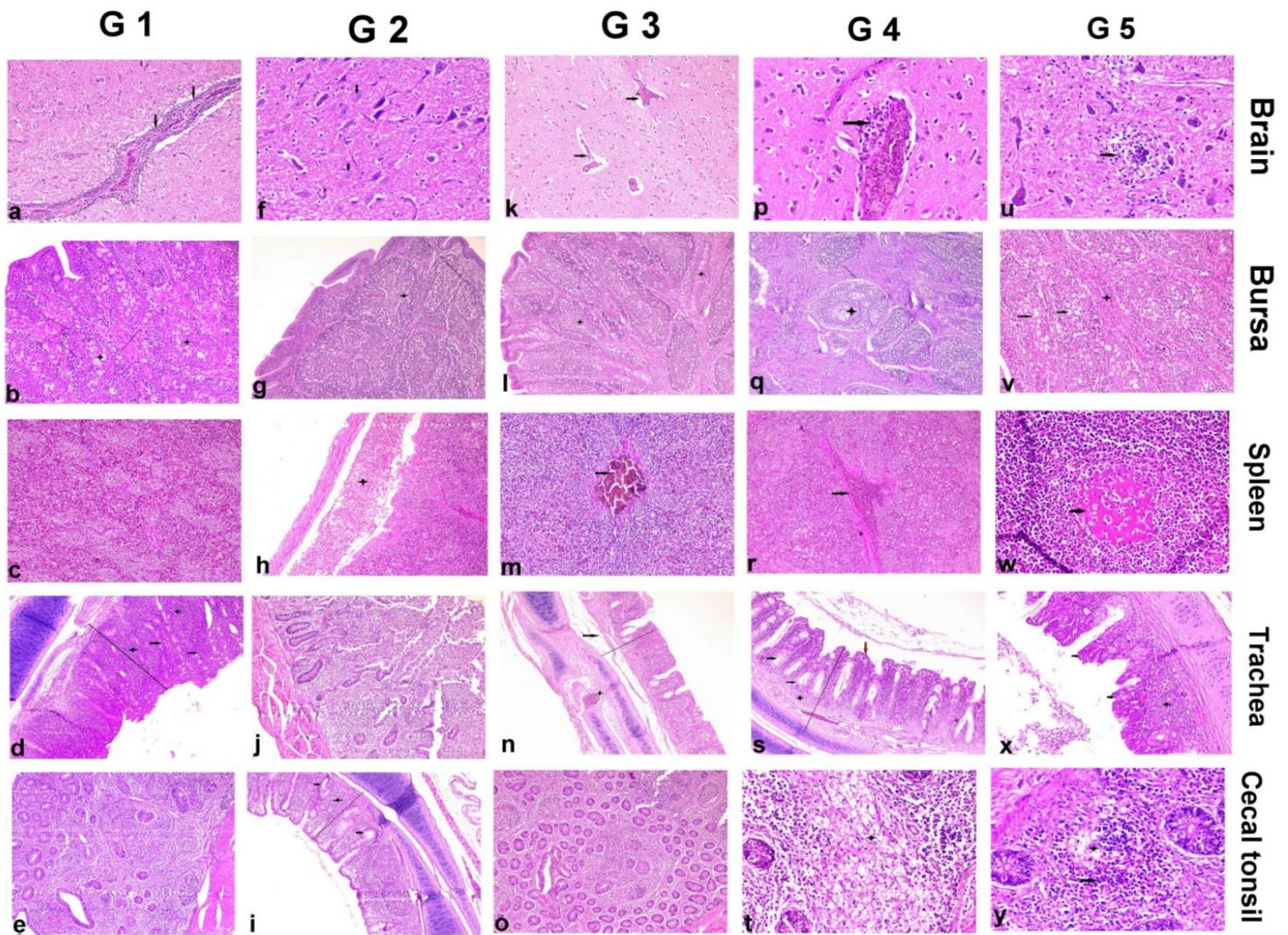
Figure 7. Histopathological changes of all groups at 7th days post challenge at 28th days of age. G1 (vaccinated by live ND and inactivated ND vaccines) (a) Cerebellum displaying perivascular cuff (arrow) H&E X400. (b) Bursa displaying lymphocyte necrosis and depletion together with the formation of microcysts (star) with interfollicular connective tissue formation (line) H&E X200. (c) Spleen displaying apparently normal structures H&E X100. (d) Trachea displaying mucosal thickening (line), with hyperplasia of lining epithelium and activation mucous glands (arrow) and mononuclear cells infiltration in lamina propria (star) H&E X100. (e) Cecal tonsil displaying apparently normal structures H&E X100.G 2 (vaccinated by HVT-ND-IBD, Live ND and Inactivated NDV vaccines) (f): Cerebrum displaying degenerated neurons (arrow) H&E X400. (g) Bursa displaying mild depletion of lymphocytes H&EX100. (h) Spleen showing subcapsular hemorrhage H&E X100. (i) Trachea displaying mucosal thickening (line), with hyperplasia of lining epithelium and activation mucous glands (arrow) and mononuclear cells infiltration in lamina propria (star) H&E X100. (j) Cecal tonsil displaying apparently normal structures H&E X100.G 3 (vaccinated by HVT-ND-IBD and Live ND vaccines) (k): Cerebrum showing perivascular edema (arrow) H&E X200. (l) Bursa displaying slight lymphocyte

decrease (star) H&E X100. (m) Spleen displaying blood vessels congestion H&E X200. (n): Trachea displaying mucosal thickening (line), with lining epithelium hyperplasia and mucous glands activation, congested blood vessels (star) and edema (arrow) in mucosa and submucosa H&E X100. (o) Cecal tonsil displaying apparently normal structures H&E X100. G4 (vaccinated by live ND vaccines) (p) Cerebrum displaying perivascular lymphocytes infiltration (arrow) H&E X200. (q) Bursa displaying depletion of lymphocytes with microcysts formation (star) and interfollicular connective tissue formation H&E X100. (r) Spleen displaying blood vessels congestion H&E X100. (s) Trachea displaying mucosal thickening (line), with hyperplasia of lining epithelium (red arrow) and activation of mucous glands (black arrow), congested blood vessels (star) in lamina propria H&E X100. (t) Cecal tonsil displaying depletion and necrosis of lymphocytes with microcysts formation H&E X400. G5 (control non-vaccinated challenged) (u) Cerebrum showing gliosis (arrow) H&E X400. (v): Bursa displaying depletion and necrosis of lymphocytes with microcysts formation (star) and interfollicular connective tissue proliferation H&E X200. (w) Spleen displaying focal amyloidosis (arrow) H&E X100. (x)Trachea displaying mucosal thickening, with lining epithelium hyperplasia (arrow) and infiltration of mononuclear cells in lamina propria (star) H&E X200. (y) Cecal tonsil displaying depletion of lymphocytes with microcysts formation (star), and few granulocytes infiltration (arrow) H&E X400.

DISCUSSION

Most recent outbreaks in Egypt have been linked to Class II Genotype VII strains, circulating in poultry farms throughout Egyptian governorates (Dewidar et al., 2012; Ahmed et al., 2017; Moharam et al., 2019; Sedeik et al., 2019). Newcastle disease (ND) is mostly controlled by immunization farms against the disease and by killing infected and at-risk birds, in addition to strict biosecurity procedures (Gallili and Ben-Nathan, 1998; Dimitrove et al., 2017a). Despite using many vaccination strategies based on conventional or non-genotype-matched vaccines, numerous ND outbreaks continue to occur with high levels of viral shedding and significant economic losses due to deaths or decreased egg production (Fawzy et al., 2020). The use of antigen-matched vaccines (vaccines developed from seeds of vaccine viruses of the same genotype as the challenge virus) has been shown to be effective for both inactive vaccines and live vaccines developed from homologous challenge virus genotype to increase efficacy against virulent challenge strains circulating in the field and, importantly, to reduce viral

shedding (Dimitrove et al., 2019; Dewidar et al., 2022 and Sultan et al., 2022).

The incidence of clinical signs varied significantly (P < 0.01) among the groups in our study following the VNDV genotype VII.1.1 challenge at 28 days of age. G-5 (control non-vaccinated) showed severe clinical signs including nervous manifestation (tremors, torticollis and wing drop), respiratory signs, greenish watery diarrhea, severe depression and off-food Post-challenge (Figure 2). Additionally, common P.M. gross lesions of VNDV include petechial hemorrhages on the mucosa of the proventriculus, severe hemorrhages on cecal tonsils, button-shaped ulcers on the intestinal mucosa, and enlarged and congested kidney (Figure 3) (Susta et al., 2011 and 2014; Miller and Koch, 2013).Versus no clinical signs in G-2 and G-3, moderate in G-1 and severe in G-4 (Table 1). similar results to our study by Hu et al., (2009) concluded that no obvious clinical signs were observed in birds receiving either inactivated NDV genotype VII or genotype II vaccines after challenge with vvNDV genotype VII (ZJ1 strain). This result indicated that, the vaccination regimes of G-2 and G-3 gave higher protection against

clinical signs than other vaccination regimes. So, groups that had received HVT-ND-IBD expressing F protein of genotype VII (genotype-matched NDV vaccine) with live or live and inactivated NDV vaccines exhibited considerably greater levels of defense against the ND clinical symptoms than groups that had received the live or live and inactivated NDV genotype II vaccines (non-genotype-matched vaccine) when challenged with velogenic NDV GVII (Dewidar et al., 2022; sultan et al., 2022). In addition, it agreed with Palya et al., (2012), who studied the effect of virulent NDV challenge on chickens vaccinated with the HVT-ND vaccine and saw partial protection at 20 days of age and complete protection at 27 and 40-days of age. Also, Esaki et al., (2013), mentioned that after in-ovo or intramuscular vaccination of one day, the protective immunity starts to develop between the ages of 2nd and 3rd week of age and is complete after the 4th week. So HVT-ND vaccine is capable of protecting against the velogenic Newcastle disease virus.

The frequency of death percent varied significantly (P < 0.01) depending on the vaccination program for NDV post-challenge with VNDV genotype VII. At the age of 28, 1.1. G5 broiler chickens without immunizations had a greater mortality rate of 85% after the challenge at 28th days of age, and showed the challenge virus's velogenic properties; viscerotropic, neurotropic, and pneumotropic affinities (Ayoub et al., 2019 and Moharam et al., 2019). While the mortality % in the G-4reached 42.5%, G-1 reached 15% andG-2 and G-3their mortality % was 0 % (Table 1). This result indicated that, the vaccination program of G-2 and G-3 gave a high protection against mortalities than the vaccination program of G-1 and G-4. As a result, after being challenged with NDV-VII, G-II ND vaccinations offer less

protection to broiler flocks than G-VII ND vaccines (Fawzy et al., 2020).

Regarding gross lesions, the dead birds in G-5 had typical postmortem lesions, such as ulceration of the cecal tonsils, tracheal congestion, and petechial hemorrhages on the tips of proventricular glands. In contrast, the immunized groups (G-1 and G-4) had milder gross lesions (figure 3) (Sultan et al., 2022).

Blood samples were collected from chicken groups before and post-challenge; HI and ELISA quantified antibodies to NDV. According to Kapczynski and King (2005), the HI test is one of the main methods used to evaluate humeral response and the degree of protection provided by NDV vaccinations. In comparison to the non-vaccinated group (G-5), all chicken in the vaccinated groups develop titers.

Before being challenged with VNDV genotype VII.1.1 at age 28, there was also a significant difference (P < 0.01) in the NDVF ELISA titer and NDV HI titer between the various vaccinated groups at different times during the trial. The results showed that, the titer level of all vaccinated groups decreased with the advanced of the experiment as its level showed a higher level at 7 days then decreased at 14 days then began to increase at 21 days and increase more at 28 days of the experiment. The lower titer level was observed in G-5 (control group). Regarding the serological response to the VNDV challenge, there were a significant difference (P < 0.01) of NDV HI titer and NDV ELISA titer among different vaccinated groups at different period of experiment post challenge with VNDV genotype VII.1.1 at 28 days of age (Table 2). The results showed that the titer level increased with the advance of the experiment post-challenge as its level showed a higher level at 7th day pot-challenge and a lower titter level observed at 14 days of the experiment.

NDV shedding into the environment by vaccinated birds is considered an essential parameter to evaluate the efficacy of the NDV vaccine (Miller et al., 2007; 2009). ND Virus shedding detection was applied by Quantitative real-time PCR which has recently been developed and validated for NDV shedding detection (Perozo et al., 2012). Tracheal and cloacal viral shedding of VNDV in broiler chickens vaccinated by different vaccination programs for NDV against challenge with VNDV genotype VII.1.1 at 28 days of age differ significantly in its incidences among different groups at different periods (P < 0.05).On the day of the challenge, no tracheal or cloacal viral shedding was detected in any of the groups, indicating that none of the groups had been exposed to a field infection with 10 virulent NDV. The RRT-PCR data for virus shedding demonstrated that G-5 (control non-vaccinated) displayed viral shedding (100% shedders) at 2, 4, and 7 dpc, confirming the challenge virus's velogenic nature. The higher level of virus shedding observed in G-5 followed by G-4 and G-1 while, the lower level of virus heeding observed in G-2 and G-3. Also, a higher level of virus shedding was observed on day 2 and day 4, In contrast, day 7 following the challenge showed a reduced level of viral shedding (Table 3, figure 4 and 5). In general, birds vaccinated with HVT-ND-IBD (expressing F protein of genotype VII)) vaccines showed significantly lower virus shedding levels. This result agrees with several studies (Sultan et al., 2020; Sultan et al., 2022; Dewidar et al., 2022).

The histopathological lesions are considered reliable for evaluating the vaccination program's protective effectiveness. The analyzed organs of G-5 (challenged non-vaccinated birds) displayed various histological alterations and showed the higher lesion scores in comparison to other groups (Payla et al., 2012).

The birds in G-2 and G-3 showed significantly lower lesion scores than the birds in G1 and G4; this could help to explain why the mortality rates in G-2 and G-3 were lower than those in G-1 and G-4 birds (figure 6 and 7). Similar to other findings, this study highlights the significance of vaccinations with matched genotypes for preventing VVNDV challenges. Since they considerably increase internal organ protection and decrease the infection's severity and associated financial costs (Sultan et al., 2020).

CONCLUSIONS

The use of rHVT-ND-IBD, a genotype-matched NDV vaccine, along with traditional vaccines provide adequate protection against infection with the recently emerging NDV VII genotype in a poultry field in Egypt. Using vector vaccine (expressing F protein of genotype VII) with genotype II vaccines (live alone or live and killed) provide better protection based on clinical symptoms, mortality rate, severity of histopathology, and reduction in NDV shedding compared to genotype II vaccine alone. So, NDV genotype VII vaccines antigenically matched to the current epidemic NDV genotype VII allow better control of virus shedding, which is an important point in reducing the spread and danger of ND.

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