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Original Research

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Comparative Efficiency of Genetically Dissimilar and Heat Stable live Newcastle Disease Vaccines against Velogenic NDV-Genotype VII

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Abstract

Newcastle disease virus (NDV) is accountable for causing one of the most contagious avian diseases in poultry, resulting in substantial financial losses worldwide. Both genotype I and II vaccines are commonly used for live NDV vaccination, particularly in broiler flocks. Recent years have witnessed a significant surge in the number of Newcastle disease outbreaks in Egyptian poultry broiler flocks, demonstrating the evolving nature of NDV infections and raising concerns regarding the effectiveness of existing NDV vaccination programs. In our study, a total of 200 one-day-old commercial chicks were divided into six groups, including unvaccinated group and five vaccinated groups. To assess the immunogenicity and protective efficacy of commonly used NDV vaccines against the prevalent Velogenic Viscerotropic Newcastle Disease Virus (vvNDV), The birds were administered live vaccines LaSota (Genotype II) and I2 (Genotype I) either as a single booster or a double booster after the initial priming with the HB1 vaccine. The comparative protective efficiency of all vaccine regimens assessment was based on clinical evaluation, observation of survival rates, and examination of viral shedding following vvNDV challenge. We also, evaluated the immune response to live vaccination by weekly monitoring of IgG specific antibodies' levels in test sera. Our findings validated that the use of multiple live NDV vaccines can offer protection against the clinical symptoms of velogenic NDV, enhance protective immunity, and reduce the virus shedding after a challenge. The inclusion of heat stable I2 vaccine could be precious in the view of earth heating and weather change.

KEYWORDS
Avian orthoavulaviruses 1, Immunogenic, Live NDV vaccine, Efficacy

INTRODUCTION

Since its initial discovery and identification in the United Kingdom in 1926, Newcastle Disease (ND) has maintained its status as one of the most significant and lethal viral diseases affecting poultry globally. This impact is particularly pronounced in developing countries (Alexander *et al*., 2012). ND is characterized by its high contagiousness, rapid spread, and its notifiable status, affecting a wide range of poultry species, including chickens, pigeons, turkeys, ducks, geese, and various other species of domestic and wild birds, leading to varying clinical manifestations (Matiur *et al*., 2015). Over the course of nearly a century, Newcastle disease virus (NDV) has been responsible for four worldwide panzootics (Hu *et al*., 2022). The first panzootic, occurring from the 1930s to the 1960s, was instigated by viruses of genotypes I, II, III, and IV. The second one, spanning the late 1960s to 1973, was primarily caused by genotype V and VI viruses. The third panzootic began in pigeons in 1975, spreading rapidly to other regions, driven by genotype VI. Finally, the fourth panzootic was triggered by genotype VII NDV, which emerged in the Far East in the late 1980s and has since expanded globally (Mase *et al*., 2002; Xu *et al*., 2017).

Egyptian research reported the first record of NDV in 1942 (Daubeny and Mansy 1947), and subsequent reports have followed (Selim *et al*., 2018; Yahia *et al*., 2021). A unified classification system for Newcastle disease viruses (NDVs) was established, based on a phylogenetic analysis of the complete F gene (Dimitrov *et al*., 2019). Class II genotypes were further divided into twenty-one genotypes with specific subgenotypes. In Egypt, both vaccinated and unvaccinated poultry flocks have experienced NDV outbreaks (Abd El Aziz *et al*., 2016; Ewies *et al*., 2017; Yahia *et al*., 2021). The primary reasons behind ND outbreaks in vaccinated flocks are believed to be inadequate NDV vaccination or alterations in avian immunity (Kattenbelt *et al*., 2006). A gradual annual increase in the mean of the environmental temperatures, is recorded globally (Yang *et al*., 2020) with special records for the African countries (Shewmake., 2008) in the last few decades and expected to be continued to the coming decades due to the climate changes factors. The immune response to NDV vaccination is affected by the environmental temperature (Beard, and Mitchell., 1987), in addition to the direct negative impact of either the fluctuation or the increase in the temperature on the applied vaccine viability and then its immunogenicity (Osman *et al*., 2021). Many studies proved either the enterotropic (Perozo *et al*., 2008), or dual pneumotropic and enterotropic (Wambura *et al*., 2006) tropism of heat stable NDV vaccinal strains could protect against field vvNDVs.

To streamline, most NDV vaccination programs utilized in the Egyptian poultry industry involve live and/or inactivated NDV genotype I, genotype II, and genetically engineered vaccines

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(Hassan *et al*., 2016; Mansour *et al*., 2021). Consequently, the aim of this current study was to conduct an in vivo assessment of the cross-protection afforded by genetically dissimilar live ND vaccine regimens against a recently identified virulent NDV genotype VII. The evaluation of vaccine regimen efficacy encompassed monitoring the humoral immune response post-vaccination, clinical protection post-challenge, as well as comparing the detection of vvNDV shedding post-challenge.

MATERIALS AND METHODS

Ethical approval

Our protocol was reviewed and approved by ZU-IACUC committee under the number; ZU-IA-CUC/2/F/65/2021. All Institutional, national and international guidelines for the animal care have been followed.

Experimental design, and applied NDV vaccination regimens.

Two hundred one-day-old Ross commercial broiler chicks were supplied by a nearby hatchery in Obour City, Cairo, Egypt. Immediately, five blood samples were collected and sera were separated for Zero day of age - maternal-derived antibodies (MDA) screening. Then the birds were initially divided into two main groups; Fifty birds were kept in the unvaccinated, unchallenged (-Ve C) group, and one hundred and fifty birds received a priming first ND vaccination (HB1+IB). The vaccinated birds were then split into two subgroups on the 10th day. 50 birds were kept in the I2 group that received their first booster ND vaccination using the I2 live vaccine, and 100 birds received their first booster ND vaccination using the LaSota live vaccine.

Then at the 20th day of age, fifteen birds from I2 group and twenty birds from birds which received their 1st booster vaccination by LaSota live vaccine were isolated in separate rooms for evaluation humoral immune of receiving either single La booster (LaX1 group) or single I2 booster (I2X1 group) vaccination following the priming with HB1 ND vaccination. Twenty-one days post the 1st booster vaccination, ten birds from each single booster vaccinated groups (LaX1 and I2X1) in addition to ten birds from unvaccinated birds were challenged with vvNDV at 31st day old (1st challenge experiment). Both NDV vaccination and vvNDV challenge were made via oculo-nasal installation route with dose 6 log 10 EID50 / Bird. All vaccines are reconstituted and applied according to the manufacturer instructions.

At the same day ;20 of age, the remaining of each vaccinated birds(n=80) which received their 1st ND booster vaccination by live LaSota vaccine were further divided into two groups (forty birds/ group) as the following LaSota double (LaX2) group, received their 2nd booster ND vaccination again by live LaSota vaccine and LaSota + I2 (La+I2) group in addition to the remaining (thirty-five) birds from the I2 (I2X2 group) received their 2nd booster ND vaccine by live I2 vaccine. Twenty-one days post the 2nd booster vaccination, ten birds from each double booster vaccinated groups (LaX2, La+I2, and I2X2) in addition to ten birds from the unvaccinated birds be challenged with vvNDV at 41st day old (2nd challenge experiment). Later on, all existing birds were kept till 56th day when the experiment was terminated, and all birds were humanly euthanized. Different applied Newcastle disease virus vaccination protocols among the experimental groups and applied challenge experiments are summarized at (Tables 1 and 2).

Serology

The blood samples were collected weekly; since the first day till the 56th day of age (8th week), from all groups (5/each group), and sera were separated and subjected to serum hemagglutination inhibition (HI) test using LaSota strain as specific antigen (OIE, 2021).

Table 1. Summary of different applied Newcastle disease virus vaccination protocols among the experimental groups and applied challenge experiments.

Group	Vaccination			Challenge	
	Priming (1st day)	1st Booster (10th day)	2nd Booster (20th day)	1st challenge (31st day)	2nd challenge (41st day)
- Ve C	N/A	N/A	N/A	VVNDV	VVNDV
LaX1	HB1+IB	LaSota	N/A	VVNDV	N/A
I2X1	HB1+IB	I2	N/A	VVNDV	N/A
LaX2	HB1+IB	LaSota	LaSota	N/A	VVNDV
La + I2	HB1+IB	LaSota	I2	N/A	VVNDV
I2X2	HB1+IB	I2	I2	N/A	VVNDV

N/A; not applicable

Table 2. Description data of applied viruses / vaccines throughout the experiment.

Viruses / vaccines	EID50	Application / Processing		Accession No./ Batch No.	Source
		Route	Groups		
Velogenic genotype VII 1.1 (VIId) of NDV	6 log 10 EID50 / Bird.	Oculo-nasal installation	All groups For the 1st and 2nd challenge	CK/Egypt/MON-ZU/ Nov-2020	Yahia *et al*. (2021)
Thermo stable I2 Strain	9 log 10 EID50 / vial.	Mixed with (10 %) (RDSM)	N/A	N/A	Kindly provided by (AU-PANVAC).
Thermo stable I2 vaccine	6 log 10 EID50 / Bird.	Oculo-nasal installation	I2X1, I2x2, and La+I2	N/A	Provided by VSVRI
LaSota vaccine	6 log 10 EID50 / Bird.	Oculo-nasal installation	LaX1, LaX2, and La+I2	22036	Provided by VSVRI
HB1+IB vaccine	6 log 10 EID50 / Bird.	Oculo-nasal installation	LaX1, I2X1, I2X1, La+I2, and I2x2	22013	Provided by VSVRI

RDSM: Reconstituted dried skimmed milk; AU-PANVAC:The Pan African Veterinary Center of the African Union; N/A: Not applicable

VVND challenge

To evaluate the clinical protection of the different applied vaccination protocols, two challenge experiments were performed (each experiment was made 21st days post each booster vaccination) inside biological isolators in where ten birds from each (unvaccinated and vaccinated) groups were challenged by vvNDV genotype VII 1.1 (VIId) of NDV and were observed for 10 days post challenge for clinical symptoms and mortalities according to OIE (2021).Oropharyngeal swabs were collected at the, 3rd, 5th, and 7th while cloacal swabs were collected at 5th, 7th, and 10th days post challenge (DPC) from 3 birds from each challenged group. The collected swabs were immersed in Phosphate-Buffered Saline (PBS) supplemented with gentamycin (50 µg/ml) and mycostatin (1000 units/mL). Then each three swabs from each route of the same time point were pooled into a single sample at the time of RNA extraction. The descriptive data included the route, EID50, batch number, and source of applied viruses/ vaccines are summarized at (Table 2).

Detection NDV virus shedding

Viral shedding was assessed using real-time RT-PCR. The RNA was extracted from pooled samples using the Pure Link® RNA Mini Kit (Invitrogen, USA) following the provided kit instructions. The RT-qPCR for APMV-1 detection was performed using the TOPreal™ One-step - SYBR Green with low ROX - RT qPCR Kit (Enzynomics, Korea) on the CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories, USA). Specific primers targeting a conserved region of the matrix (M) gene of APMV-1, as previously described (Wise *et al*., 2004), were used for this purpose (Figure 1).

Statistical analysis

The data from the HI test were analyzed with the statistical software SPSS (Version 21, IBM, USA). The data is presented as mean titers along with the standard deviation. To determine if there were any statistically significant variations among the different chicken groups subjected to various vaccinations and the control group, an ANOVA test was employed. P-values less than 0.05 were considered statistically significant.

RESULTS

Clinical protection

After the first and second challenge trials, unvaccinated groups that were exposed to vvNDV displayed symptoms of infection, including ocular discharges in 3 out of 10 birds after the first challenge and 5 out of 10 after the second challenge, nasal discharges in 1 out of 10 birds after the first challenge and 3 out of 10 after the second challenge, respiratory distress in 8

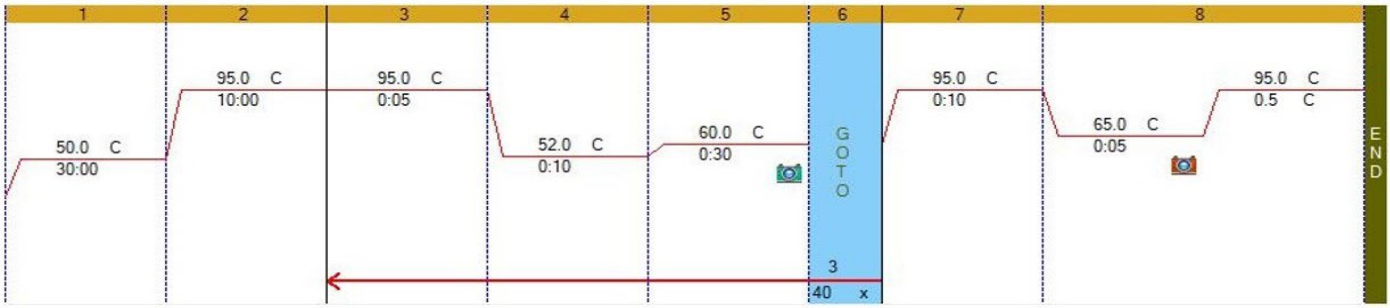

Fig. 1. Thermal conditions applied at RT-qPCR and for the melting curve. Bio-Rad CFX manager 3.1 software was used for the calculation of melting temperature (Tm) of melting curves and Cp values.

Table 3. Clinical protective efficacy of different Live Newcastle disease virus (NDV) vaccine regimens against vNDV-GVII.1.1challenge.

Group	NDV Vaccination	Protection %	Ocular discharges	Nasal discharges	Respiratory Distress	Nervous signs
- Ve C	Unvaccinated	20% 1st 10% 2nd	3/10 1st 5/10 2nd	1/10 1st 3/10 2nd	8/10 1st 9/10 2nd	2/10 1st 3/10 2nd
LaX1	HB1, and La	80%	1/10	0/10	2/10	0/10
I2X1	HB1, and I2	90%	0/10	0/10	1/10	0/10
LaX2	HB1, La, and La	100%	0/10	0/10	0/10	0/10
La + I2	HB1, La, and I2	100%	0/10	0/10	0/10	0/10
I2X2	HB1, I2, and I2	90%	0/10	0/10	1/10	0/10

Table 4. Mean log 2 HI-NDV antibody titers of vaccinated birds with different live vaccination protocols.

Group	1st Day Week zero	8th day 1st Week	15th day 2nd Week	22nd day 3rd Week	29th day 4th Week	36th day 5th Week	43rd day 6th Week	50th day 7th Week	58th day 8th Week
- Ve C	9.80±.83 a	8.2±.83 a	5.0±1.0 b	4.2±.44 a	2.4±.54 a	2.0±.70 a	1.6±.54 a	1.2±.44 a	0.4±.54 a
LaX1	9.80±.83 a	6.6±.89 a	7.2±.44 a	7.4±.54 b	6.6±.54 b	4.6±.54 b	4.0±.54 b	3.6±.54 b	3.2±.83 b
I2X1	9.80±.83 a	6.6±.89 a	7.2±.44 a	7.6±.54 b	6.8±.83 b	5.4±.54 c	4.4±.54 b	3.6±.54 b	3.4±.54 b
LaX2	9.80±.83 a	6.6±.89 a	7.2±.44 a	5.8±.44 c	6.4±.54 b	7.8±.44 de	8.2±.44 cd	8.0±.70 c	7.8±.83 c
La + I2	9.80±.83 a	6.6±.89 a	7.2±.44 a	6.8±.83 bc	7.4±.54 b	8.8±.44 d	9.4±.89 d	9.6±.54 d	8.8±.83 c
I2X2	9.80±.83 a	6.6±.89 a	7.2±.44 a	6.6±.56 bc	6.8±.83 b	7.6±.54 e	7.8±.83 c	8.4±.54 c	7.8±.44 c

Means with different letters (a, b, c, d, and e) within the same row are significantly different at P value ≤0.05.

out of 10 birds after the first challenge and 9 out of 10 after the second challenge, and nervous signs in 2 out of 10 birds after the 1st challenge and 3 out of 10 after the 2nd challenge. Their survival rate was 20% after the first challenge and 10% after the second challenge. None of negative control group revealed any illness. Among the vaccinated groups, the birds exhibited higher survival rates, ranging from 80% in the Lax1 group to 90% in the I2X1 and I2X2 groups. Both the LaX2 and La+I2 groups demonstrated 100% survival rates up to the 10th day post-challenge. The single booster groups (LaX1 and I2X1), as well as one of the double booster groups (I2X2), displayed minimal clinical signs post-challenge. Notably, the double booster groups (LaX2 and La+I2) showed no clinical symptoms post-challenge (Table 3 and Figure 2).

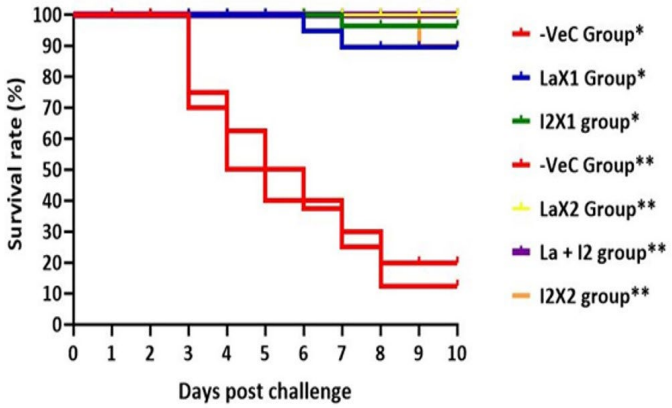

Fig. 2. Survival rate of NDV challenged birds received different booster live NDV vaccine protocols.
-Ve C group didn't receive any ND vaccination. Red line.
LaX1 group received 1st ND vaccination by live HB1+IB at 1st day, then received only one booster ND vaccination by live LaSota vaccine at 10th day old. Blue line
I2X1 group received 1st ND vaccination by live HB1+IB at 1st day, then received only one booster ND vaccination by live I2 vaccine at 10th old. Green line
LaX2 group received 1st ND vaccination by live HB1+IB at 1st day, then received two boosters of ND vaccinations by live LaSota vaccine at 10th and 20th day respectively. Yellow line
La + I2 group received 1st ND vaccination by live HB1+IB at 1st day old, then received 1st booster ND vaccination by live LaSota vaccine at the 10th day old, and then received a 2nd booster ND vaccination by live I2 vaccine at the 20th day old. Purple line
I2X2 group received 1st ND vaccination by live HB1+IB at 1st day, then received two boosters of ND vaccination by live I2 vaccine at 10th and 20th day old respectively. Orange line
*Groups submitted to the 1st challenge experiment
**Groups submitted to the 2nd challenge experiment

observed starting from the second week and onwards. By this point, the mean HI titer had dropped to 5.2 log_2. The decline continued, and a mean HI titer of 2.0 log_2 was noted by the fifth week, further dropping to 0.4 log_2 by the eighth week.

In the case of single booster NDV vaccine groups, the mean HI log_2 titer increased until the 12th day after the booster vaccination (third week) and then gradually decreased. Notably, there were no significant differences between the genotype II (LaSota) vaccine and the genotype I (I2) vaccine, except for the 26th day post-booster (fifth week) when the HI mean titer showed a significant drop in the genotype II single booster vaccinated group (LaX1) to 4.2, in comparison to the 5.4 recorded in the genotype I single booster vaccinated group (I2).

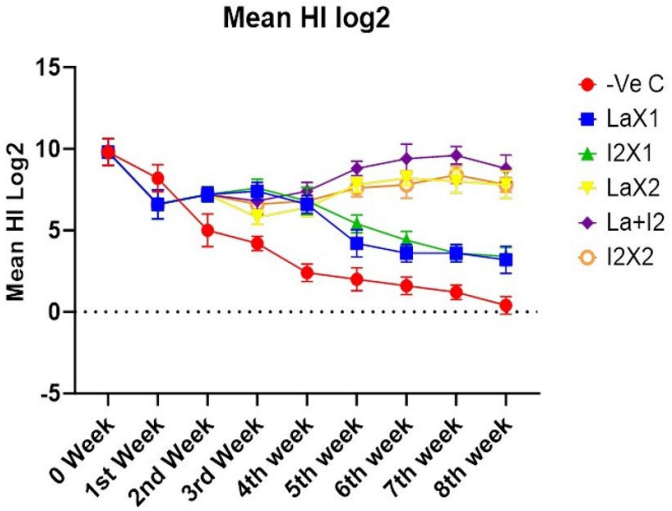

Fig. 3. Comparative mean HI titer of NDV Ab among different groups along with un-vaccinated control.
LaX1 group received 1st ND vaccination by live HB1+IB at 1st day, then received only one booster ND vaccination by live LaSota vaccine at 10th day old. Blue line
I2X1 group received 1st ND vaccination by live HB1+IB at 1st day, then received only one booster ND vaccination by live I2 vaccine at 10th day old. Green line
LaX2 group received 1st ND vaccination by live HB1+IB at the 1st day, then received two boosters of ND vaccinations by live LaSota vaccine at 10th and 20th day respectively. Yellow line
La + I2 group received 1st ND vaccination by live HB1+IB at 1st day old, then received 1st booster ND vaccination by live LaSota vaccine at the 10th day old, and then received a 2nd booster ND vaccination by live I2 vaccine at the 20th day old. Purple line
I2X2 group received 1st ND vaccination by live HB1+IB at 1st day, then received two boosters of ND vaccination by live I2 vaccine at 10th and 20th day old respectively. Orange line

Serological response following vaccination

All the birds initially possessed a high level of NDV maternal-derived antibodies (MDA), which was detected from the first day with an average HI titer of 9.8 log_2. In the unvaccinated control group (unvaccinated –Ve C) a significant decline in MDA was

Among the same vaccine double booster vaccinated groups, the LaX2 group's HI log_2 titer reached its peak at the 23rd day post the second booster at the (sixth week), reaching 8.2. The I2X2 group's HI titer reached its peak a week later (seventh week) at 8.4 log_2, with no significant differences observed throughout

Table 5. Comparison of virus shedding in of vNDV-GVII1.1 challenged birds belonging to different vaccine schemes.

	Oropharyngeal swabs									Cloacal swabs								
	3rd day PC			5th day PC			7th day PC			5th day PC			7th day PC			10th day PC		
	L	S/L	Cq	L	S/L	Cq	L	S/L	Cq	L	S/L	Cq	L	S/L	Cq	L	S/L	Cq
- Ve C	*7/10	3/7	22.97	4/10	3/4	21.6	3/10	3/3	26	4/10	3/4	26.52	3/10	3/3	23.96	2/10	2/2	24.15
	**8/10	3/8	18.02	4/10	3/4	9.3	2/10	2/2	23.16	4/10	3/4	27.03	2/10	3/3	23.42	1/10	1/1	23.72
LaX1	10/10	3/10	N/A	10/10	3/10	29.66	8/10	3/8	33.56	10/10	3/10	35.49	2/2	3/9	34.17	8/10	2/8	32.59
I2X1	10/10	3/10	35.86	10/10	3/10	32.01	9/10	3/9	35.32	10/10	3/10	37.19	8/10	3/8	31.14	9/10	3/9	30.4
LaX2	10/10	3/10	N/A	10/10	3/10	32.95	10/10	3/10	37.86	10/10	3/10	35.3	9/10	3/9	33.55	10/10	3/10	32.09
La + I2	10/10	3/10	N/A	10/10	3/10	33.09	10/10	3/10	32.95	10/10	3/10	39.43	10/10	3/10	33.44	10/10	3/10	36.03
I2X2	10/10	3/10	N/A	10/10	3/10	34.3	10/10	3/10	37.98	10/10	3/10	35.57	10/10	3/10	33.9	9/10	3/9	36.54

* Results of the 1st challenge experiment, ** results of the 2nd challenge experiment, L; live birds' number at the time of each swabbing, S/L; Swabs numbers/live birds number at the time of each swabbing, Cq; Cq value of quantification cycle

the experiment. The different vaccine double booster vaccinated group (La+I2) reached its HI peak at the 30th day post the second booster (seventh week), coinciding with the HI peak of the I2X2 group, reaching the highest HI titer obtained at 9.2 log_2. There was a significant difference between the La+I2 group and both of the same vaccine double booster vaccinated groups (LaX2 and I2X2). Additionally, the La+I2 group exhibited a significant increase in its HI response two weeks before reaching its peak, with HI titers of 8.8 and 9.4 log_2 at the fifth and sixth weeks, respectively, showing a significant difference compared to the same vaccine double booster vaccinated groups. Starting from the 16th day post the second booster (fifth week), all double booster vaccinated groups displayed a significant increase in HI log_2 titer responses compared to all the single booster vaccinated groups, which continued until the end of the experiment (Table 4 and Figure 3).

vvNDV virus shedding following challenge

The assessment of oropharyngeal and cloacal viral shedding after the challenge experiment was conducted on all challenged birds and the results are presented in Table 5, Figure 4 and 5 A, B, and C. For oropharyngeal viral shedding, swabs were collected on the 3rd, 5th, and 7th days post challenge (DPC) from both unvaccinated and vaccinated groups. All tested oropharyngeal samples were positive for NDV, except for four samples that tested negative for NDV. These NDV-negative oropharyngeal samples were collected on the 3rd DPC, from one of the single booster vaccinated groups (LaX1), and from all of the double booster vaccinated groups (LaX2, La+I2, and I2X2).

Among the single booster vaccinated groups, oropharyngeal NDV shedding showed a reduction, with higher Cycle threshold (Cq) values indicating lower viral shedding, compared to the unvaccinated challenged group at all three time points. The Cq values ranged from 29.66 to 33.56 in the LaX1 group and from 32.01 to 35.86 in the I2X1 group, while the unvaccinated challenged group had a Cq range of 21.60 to 26.0. Interestingly, although the LaX1 group showed negative oropharyngeal viral shedding on the 3rd DPC, it exhibited a higher oropharyngeal viral shedding (Cq range of 29.66 to 33.56) than the I2X1 group, which had a lower shedding range (32.01 to 35.86). In contrast, all double booster vaccinated groups had oropharyngeal samples that tested negative for NDV on the 3rd DPC, and they demonstrated a significant reduction in NDV viral shedding. The Cq range was 32.95 to 37.86 in the LaX2 group, 32.95 to 33.09 in the La+I2 group, and 34.30 to 37.98 in the I2X2 group, while the unvaccinated challenged group had a Cq range of 9.30 to 23.16.

Notably, the double booster with different vaccines (La+I2) group showed a noteworthy reduction in oropharyngeal viral shedding (Cq range of 32.95 to 33.09) compared to the double booster with the same vaccine (LaX2 and I2X2) groups. Furthermore, all three double booster vaccinated groups exhibited a reduction in oropharyngeal viral shedding, with a combined Cq range of 29.66 to 35.86, compared to the Cq range of 32.95 to 37.98 in the two single booster vaccinated groups.

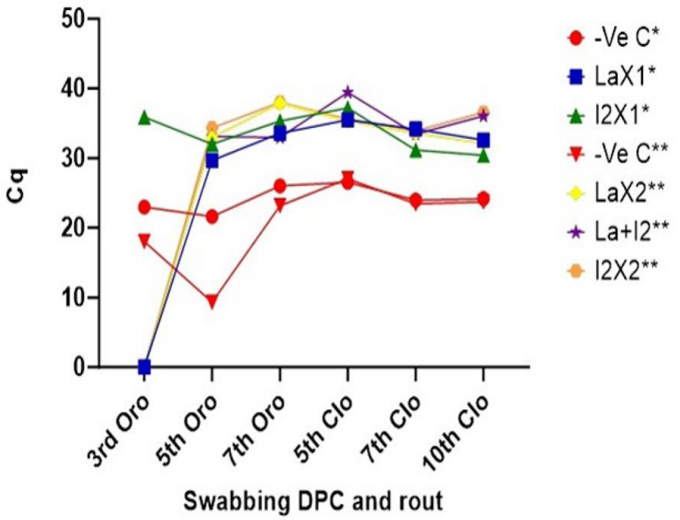

Fig. 4. Comparative Cq values representing viral shedding NDV among different groups along with un-vaccinated control. *Groups submitted to the 1st challenge experiment. **Groups submitted to the 2nd challenge experiment.
LaX1 group received 1st ND vaccination by live HB1+IB at 1st day, then received only one booster ND vaccination by live LaSota vaccine at 10th day old. Blue line
I2X1 group received 1st ND vaccination by live HB1+IB at 1st day, then received only one booster ND vaccination by live I2 vaccine at 10th old. Green line
LaX2 group received 1st ND vaccination by live HB1+IB at 1st day, then received two boosters of ND vaccinations by live LaSota vaccine at 10th and 20th day respectively. Yellow line
La + I2 group received 1st ND vaccination by live HB1+IB at 1st day old, then received 1st booster ND vaccination by live LaSota vaccine at the 10th day old, and then received a 2nd booster ND vaccination by live I2 vaccine at the 20th day old. Purple line
I2X2 group received 1st ND vaccination by live HB1+IB at 1st day, then received two boosters of ND vaccination by live I2 vaccine at 10th and 20th day old respectively. Orange line
*Groups submitted to the 1st challenge experiment
**Groups submitted to the 2nd challenge experiment

For cloacal viral shedding, samples were assessed on the 5th, 7th, and 10th DPC from both unvaccinated and vaccinated groups. All tested cloacal samples were positive for NDV. Among the single booster vaccinated groups, cloacal NDV shedding showed a reduction compared to the unvaccinated challenged group at all three time points. The Cq values ranged from 23.59 to 35.49 in the LaX1 group and from 30.40 to 37.19 in the I2X1 group, while the unvaccinated challenged group had a Cq range of 23.96 to 26.52. Similar to oropharyngeal shedding, the LaX1 group exhibited higher cloacal viral shedding (Cq range of 23.59 to 35.49) than the I2X1 group, which had a lower shedding range (30.40 to 37.19).

All double booster vaccinated groups demonstrated a reduc-

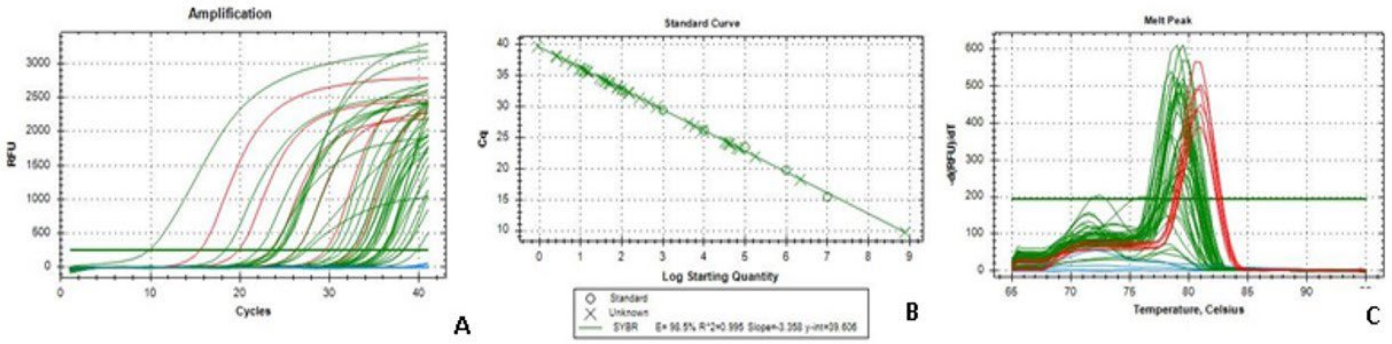

Fig. 5. (5A) Threshold cycles of tested samples, (5B) standard curve, and (5C) Melting peak of tested samples, Red lines (n=6) represent positive control samples (standard curve samples), the green lines (n=38) represent positive for Avian avulavirus samples, and blue lines (n=4) represent the negative for Avian avulavirus samples.

tion in cloacal NDV viral shedding, with a Cq range of 32.09 to 35.30 in the LaX2 group, 33.44 to 39.43 in the La+I2 group, and 33.90 to 36.54 in the I2X2 group, while the unvaccinated challenged group had a Cq range of 23.42 to 27.03. Similar to oropharyngeal shedding, the double booster with different vaccines (La+I2) group exhibited a significant reduction in cloacal viral shedding (Cq range of 33.44 to 39.43) compared to the double booster with the same vaccine (LaX2 and I2X2) groups. Furthermore, all three double booster vaccinated groups showed a reduction in cloacal viral shedding, with a combined Cq range of 32.09 to 39.43, compared to the Cq range of 23.59 to 37.19 in the two single booster vaccinated groups.

DISCUSSION

Active immunization through vaccination, combined with other biosecurity measures, is the primary and widely adopted approach for managing Newcastle Disease (ND), particularly in regions where NDV is an endemic concern (Bhuvaneswari *et al*., 2017). The main objective of vaccination is to stimulate a robust and durable immune response (de Cassan *et al*., 2011). Recent studies have revealed greater variability between serogroups and genotypes in NDV (Miller *et al*., 2007). In recent years, NDV outbreaks have been attributed to the production of ineffective immune responses, exacerbated by significant genetic disparities between the field strains and the administered vaccines (Munir *et al*., 2012; Yahia *et al*., 2021). The Egyptian poultry industry typically follows rigorous NDV vaccination protocols, which include the use of live and/or inactivated NDV genotypes I and II, as well as genetically modified vaccines (Mansour *et al*., 2021). The present study targeted to assess the protective efficacy and immunogenicity of various vaccination regimens, utilizing live heterologous genotypes of lentogenic NDV, either as a single booster or a double booster, employing either the same strain or a different one. The assessment criteria included the observation of clinical protection, evaluation of antibody responses, and the detection of viral shedding. Notably, the study was conducted on commercial birds with relatively high maternal antibody (MDA) levels at the time of primary vaccination.

Our results revealed that unvaccinated birds challenged with velogenic NDV displayed typical signs of NDV infection, such as ocular and nasal discharges, respiratory distress, and nervous symptoms, consistent with several previous studies (Hossain *et al*., 2023). Post-challenge survival rates for unvaccinated groups ranged from 10% to 20% during the first and subsequent challenge experiments. These survivals are little more than those reported in prior studies (Zhao *et al*., 2012; Abd El-Moneam *et al*., 2020), where unvaccinated groups showed no survival post-challenge. This highlights the partial protective role of MDA and the influence of the challenge infection route or the difference in challenge virus and bird breed. All vaccinated groups exhibited a significant reduction in clinical disease post-challenge, with two double booster vaccinated groups showing no clinical symptoms at all. Moreover, the post-challenge survival rates in all vaccinated groups ranged from 80% in one of the single booster vaccinated groups (Lax1) to full protection (100% post-challenge survival) in the genotype II double booster vaccinated group (LaX2) and in the group double booster vaccinated with both genotypes I and II (La+I2). These findings are in line with previous research results (Cornax *et al*., 2012; Dimitrov *et al* 2017).

Regarding HI antibody levels; unvaccinated birds exhibited high MDA levels, noted from day one. This interfered with the response to the primary vaccination, as vaccinated groups showed a mean $\log_2$ HI titer of 6.6 at one week of age compared to the unvaccinated group, which showed a mean $\log_2$ HI titer of 8.2 at the same time point. This underscores the role of high MDA levels in relation to the timing of the primary vaccination and its impact on immune response levels (Hossain *et al*., 2023). For single booster vaccinated groups, HI titers peaked on the 12th day post-booster vaccination (third week) and then gradually declined, with a significant increase in the genotype I single booster vaccinated group (I2X1) on the 26th day post-vaccination (fifth week). Double booster vaccinated groups achieved their peak protective log 2 HI titers between the 23rd and 30th day's post-booster vaccination (sixth and seventh weeks). The La+I2 group had the highest titer at 9.2 and also showed a significant increase in HI titers two weeks before reaching its peak. These findings are consistent with previous findings (Rahman *et al*., 2017).

As for viral shedding following the challenge, live NDV vaccination provided complete clinical protection but did not prevent the shedding of the challenge virus. This aligns with previous research (Miller *et al*., 2007; Cornax *et al*., 2012). However, double booster vaccinated groups exhibited a reduction in both oropharyngeal and cloacal viral shedding, consistent with the work of (Dimitrov *et al*, .2017).

The inclusion of genotype I (I2 strain) as either a single booster or a double booster, following the same genotype or genotype II vaccination, resulted in a notable reduction in both oropharyngeal and cloacal viral shedding. This can be attributed to the higher and more sustained HI titers observed in the I2 vaccinated groups due to its heat stability properties. Another explanation based on the dual pneumo and entero tropism of the I2 strain (Wambura *et al*., 2006) can be also considered in intestinal protection since there this positive correlation between the virus replication in tissues and IgA production (Perozo *et al*., 2008). Additionally, the genetic differences between genotype I (I2) strains and widely used genotype II strains, along with their similarity to velogenic NDV strains at the F protein gene, contribute to these superior results. This includes amino acid substitutions and properties along the HN protein gene that align with circulating velogenic NDV (Yahia *et al*., 2021).

CONCLUSION

Based on our findings, it can be inferred that within the context of the poultry industry, implementing multiple booster vaccinations using live vaccines of genotype I or/and II can offer protection against clinical Newcastle Disease (ND). As the inclusion of various genotypes in a unified vaccination strategy yields superior outcomes in terms of clinical protection, immune response, and reduction in viral shedding. Moreover, adding heat stable vaccines is encouraged in the view of earth heating and weather change to avoid abridged virus dose.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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