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EVALUATION OF DIFFERENT NEWCASTLE DISEASE VIRUS VACCINATION REGIMES AGAINST CHALLENGE WITH RECENTLY ISOLATED GENOTYPE VII VIRUS FROM EGYPT

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Abstract: Newcastle disease virus (NDV) genotype VII is incriminated in the currently circulating NDV outbreaks in the Middle East region. In this study, evaluation of different vaccination regimes including genetically matched or mismatched vaccines to the currently circulating field virulent NDV (vNDV) genotype VII was performed. One-day-old Arbor Acres broiler chicks were divided into nine groups; groups 1 to 3 were vaccinated with live or inactivated genetic mismatched vaccines (genotype II) or both of them. Groups 4 to 6 were vaccinated with either live or inactivated genetic-matched vaccine to vNDV genotype VII or combination of them. Group (Gp) 7 was vaccinated with a combination of inactivated genetic-matched and live genetic-mismatched vaccines to vNDV genotype VII while groups 8 and 9 were kept as control non-vaccinated. The groups that received a combination of live and inactivated vaccines from either genetically-matched or mismatched origins had the highest serological responses and protection against mortality which was 100%. The two groups received a combination of inactivated genetic matched vaccine and live vaccines of either genetic-matched or mismatched origins had the lowest clinical index and were nearly completely protected against vNDV clinical signs. The virus tracheal and cloacal shedding titers and number of shedders were significantly reduced or nearly neglicable in the instance of application of inactivated genetic-matched vaccine to the challenge virus either alone or boosted with live genetic-matched or mismatched vaccine. In consistent inactivated genetic-matched vaccine inhibited the transmissibility of the challenged virus to contacted birds. We concluded from our results that application of NDV vaccination regimes included a combination of inactivated NDV genotype VII vaccine and live vaccine regardless of its genotype provides better clinical protection and minimize virus shedding and subsequently decrease transmissibility and virus load to the surrounding environment.

Key words: Newcastle disease virus; genotype VII; virus shedding; vaccine programs

Introduction

Poultry sector is the fastest growing industry, in Egypt. It offers the nation various forms of animal protein in the form of eggs and meat, as well as employment prospects for almost 3 million workers with investments totaling over 90 million Egyptian pounds.

Several economic losses occur in this industry as a result of various pathogenic agents, particularly those associated with respiratory issues such as Avian Influenza (AI), Newcastle disease, and Infectious Bronchitis (1-4). Newcastle disease (ND) is a highly contagious infectious disease that has a negative impact on poultry production across six continents, resulting in significant economic losses, prompting the Office International des Epizooties to classify it as list A disease (5). Newcastle disease was initially described in Egypt for the first time in 1948 by (6) as a real threat to the Egyptian poultry industry. Even in vaccinated flocks, the disease causes significant economic losses due to high mortality, decreased egg production, and decreased body weight of broiler chickens (7). The causative agent

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Newcastle disease virus (NDV) is an enveloped, single stranded, non-segmented RNA of negative sense virus. The NDV is also known as the avian Orthoavulavirus 1 (AOAV-1) and has been classified in the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Avulavirinae*, and genus *Metaavulavirus* (8, 9). Within a single serotype, NDV strains have been categorized into class I and II based on the size of the genome and the nucleotide sequences of the F and L genes (10). Class II NDVs have greater genetic diversity, with 20 newly identified genotypes named I to XXI (excluding XV because it contains recombinant viruses), which include virulent and avirulent strains and are widely distributed across the African continent (9, 11). However, discrepancy in clinical signs in infected birds are primarily determined by the pathotype of the infected strain, whether velogenic, mesogenic, lentogenic, or asymptomatic. The velogenic (extremely virulent) strains can produce severe clinical symptoms such diarrhea, neurological and respiratory signs, egg difficulties, hemorrhagic lesions in the intestinal tract and proventriculus, and significant mortality rates (7, 12). Although there is no cure for ND infection, culling infected birds, combined with strict biosecurity and aggressive vaccination protocols, are the most effective ways to control NDV outbreaks. There are three types of NDV vaccines: live, inactivated and recombinant vaccines. Many such studies have demonstrated the ability of different types of NDV vaccines to reduce or prevent clinical disease and mortality, reduce virus shedding into the environment, and increase the amount of virus that necessary to infect vaccinated birds (10, 13). Even in well-vaccinated farms, ND outbreaks continue to occur frequently despite the application of intensive vaccination programs and intense usage of ND vaccines. The former has raised questions about the protective efficacy of the commercially applied vaccines and the role antigenic variations of NDV in the NDV vaccination defect phenomena. LaSota and Hitchner B1 NDV strains used to produce the most commercially available NDV vaccines, both have the same genotype of viruses isolated in the 1940s and are classified within class II as genotype I or II (14). Nowadays, both strains are genotypically different or mismatched from strains causing the recent outbreaks of ND. These conventional vaccines could partially or completely protect chicken from mortalities and clinical signs but not able to prevent viral dissemination from infected birds to the environment through its dropping and respiratory secretions which represent source of infection to non-vaccinated birds (15). Genotype VII of NDV was firstly isolated by (16) then it was reported many times in concurrent outbreaks among Egyptian poultry flocks with high mortality percentage (17-20). Recent investigations on the usefulness of genotype VII-based vaccinations in the prevention of NDV epidemics indicated that they offered improved protection and decreased viral shedding from infected birds against challenge with vNDV genotype VII (21-23). In Egypt, even with strict preventive measures, NDV genotype VII outbreaks have been still occurred which makes it mandatory to improve the applied vaccination strategy. Therefore, the goal of this study was to evaluate the efficacy of different NDV vaccination programs composed of genetic-matched or mismatched vaccine or even combination of them in experimentally infected broiler against the velogenic genotype VII strain.

Materials and methods

Experimental birds

One hundred seventeen Arbor Acres broiler chickens, one day of age, were obtained from EL-Abrar Company for poultry production at Ismailia Governorate. The birds were raised in a floor-based system in the animal house experimental facility at Faculty of Veterinary Medicine, Zagazig University. Throughout the experiment, all birds had free access to food and water. The birds were observed for any changes that could indicate the presence of infection. All the procedures were carried out under strict biosafety conditions that comply with local animal welfare regulation on experiments with live birds. The study was approved by the head of Institutional Animal Care and Use Committee (ZU-IACUC) under acceptance number ZU-IACUC/2/F/ 187/2019, Zagazig University

Vaccines

1. IZOVAC B1 Hitchner (IZO, Italy with Batch n.0463M), a freeze-dried live attenuated strain, contain at least 10^6 EID_{50}/dose of live NDV B1 strain. The vaccine was administrated via intraocular route.

2. Nobilis Clone30 (MSD, Kenilworth, NJ, USA with Batch n. A320AJ0) a freeze dried live attenuatedstrain; contain at least 10^6 EID_{50} of live NDV Clone 30 virus particles /dose. The vaccine was administrated via intraocular route.

3. Himmvac Dalguban N (Plus) Live Vaccine (KBNPC4152R2L strain), KBNP Inc., Gyeonggi, Korea with Lot number CMRL1218, a live freeze-dried attenuated strain, contain at least 10^6 EID_{50}/dose of live NDV KBNP-C4152R2L strain (genotype VII). The vaccine was administrated via intraocular route.

4. Volvac® ND KV (Boehringer Ingelheim, Germany), oil emulsion inactivated vaccine, contains LaSota NDV with lot number 19030104. The vaccine was injected at a dose of 0.5 ml per bird, subcutaneously in the middle third part of the back of the neck.

5. Himmvac Dalguban N (Plus) Oil Vaccine (KBNP, Inc., Gyeonggi, Korea oil emulsion inactivated recombinant vaccine, contains KPNB-C4152R2L NDV strain, with Lot number CMR0319. The vaccine was injected at a dose of 0.5 ml per bird, subcutaneously in the middle third part of the back of the neck.

Virus

A reference genotype VII vNDV strain termed as (NDV/chicken/Egypt/1/2015), with accession number KX231852 was used as the challenge virus. In 9-day-old embryonated chicken eggs (ECEs), the virus was propagated and titrated. The infective dose was adjusted to contain $10^{6.5}$ embryo infective dose 50 (EID_{50})/ 0.1 ml and administrated via oculonasal route.

Experimental design

One hundred and seventeen Arbor Acres broiler chicks obtained from EL-Abrar Company for poultry production at Ismailia Governorate were divided into 9 groups, each of 13 birds (10 challenged and 3 in contact added post challenge). From group one to seven, several NDV vaccination regimens were administered and two groups were kept non-vaccinated as control groups. The vaccination programs applied for the different groups are described clearly in Table (1).

All the groups except (group 9) were challenged with $10^{6.5}$/EID_{50}/100 uL per bird of vNDV genotype VII strain (NDV/chicken/ Egypt/1/2015) at 31st day old via oculonasal route. Three sentinel birds to each group were inserted between the vaccinated birds forty-eight hours post challenge. Blood samples were collected at first day old for evaluation of maternal-dervied NDV immunity and weekly from the wing vein according to the approval of (ZU-IACUC/ 2/ F/ 187/2019). Tracheal and cloacal swabs were taken from five challenged birds and three contact sentenile birds from each group and were collected into sterile phosphate buffer saline containing antibiotics at 3 and 5 post challenge from challenged birds and at 3, 5 & 7 days post contact from sentenile birds. Challenged birds were examined daily for clinical signs and mortality. The clinical indicies were calculated as previously described by Grund et al (24)

Table 1: Experimental design of different NDV vaccination regimes in chicken Arbor Acres broiler broilers

Number of groups	Number of chicks		1 day old	10 days old	18 days old	31 days old
	Challenged	Contact				
I	10	3	HitchnerB1		Clone 30[1]	Challenged[5]
II	10	3	HitchnerB1	LaSota Inactivated[2]		Challenged[5]
III	10	3	HitchnerB1	LaSota Inactivated[2]	Clone 30[1]	Challenged[5]
IV	10	3	HitchnerB1		Dalguban Live[3]	Challenged[5]
V	10	3	HitchnerB1	Dalguban Inactivated[4]		Challenged[5]
VI	10	3	HitchnerB1	Dalguban Inactivated[4]	Dalguban Live[3]	Challenged[5]
VII	10	3	HitchnerB1	Dalguban Inactivated[4]	Clone 30[1]	Challenged[5]
VIII	10	3	-			Challenged[5]
IX	10	3	-			-

1& 3 were applied via intraocular route with at least 10^6 EID_{50}/dose. 2 &4 were injected at a dose of 0.5 ml per bird, subcutaneously. 5 was applied oculonasal with $10^{6.5}$/EID_{50}/100 uL per bird.

Table 2: Primers and Probe used to detect the amount of vNDV in tracheal/ cloacal swabs after challenge

Gene	Primer/ probe sequence 5'-3'
NDV Genotype VII (F)	F-Egy-289-FW CGSARGATMCAAGGGTCT
	F-Egy-378-Rev CTACACTGCCAATAACRGC
	F-Egy-361-Probe [FAM] AGGAGACRAAAACGYTTTATAGGTCG[TAMRA]

The primers and probes in this study were received as a gift from prof Dr Christian Grund, FLI (Friedrich Loeffler institute; The Federal institute for Animal Health of Germany 2016 (unpublished)

Evaluation of humoral NDV immunity

Blood samples were taken from chicks at various ages, including 1 day, 7, 14, 21, 28, 35, and 38 days. Serum samples were separated and haemagglutination inhibition (β procedure) assay was performed for measuring the NDV antibody titer. Serum was initially diluted into 1/2 and a two-fold dilution series of each tested serum sample was prepared according to OIE (5). Four HA units of the NDV LaSota antigen were added to the serially diluted serum samples, and 1% chicken red blood cells were utilised as an indicator.

Measurement of viral shedding using qRT-PCR:

Tracheal and cloacal swabs were randomly taken from 8 birds in each group (5 infected + 3 sentinel birds). Viral RNA samples were extracted using an RNeasyPlus Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Specific probes and primers were designed based on the F protein gene to detect viral RNA using qRT-PCR (Table 2).

The primers, probes, and PCR conditions for the assay was applied according to the manufacturer's instructions. The reaction was subjected to the cycling conditions of primers and probes using Real time PCR machine (Stepone applied biosystem). The amount of viral shedding was be expressed as the number of viral RNA copies. Absolute quantification of the number of viral RNA copies in the swabs was be based quantification cycle (Cq) values for each sample and the standard curve method for qPCR.

Statistical analysis:

Using a One-Way Analysis of Variance (ANOVA) and SPSS (ver. 21.0; IBM, USA), statistically significant variations in the serological analysis data between various groups was assessed.

Results

Serological responses following vaccination with different NDV vaccination regimes

Prior to challenge, antibody responses from zero day and weekly after different NDV vaccination regimes were evaluated (Figure 1 and Table 3). The birds had high maternal antibody arithmatic mean titers against NDV of (7.5±1.50) $\log_2$, which gradually decreased to < 4 log2 at 14 days old. The results showed no significant differences between the different vaccinated groups at 14 days old, regardless of the genotype of the used inactivated or live vaccine. At 21 days old, the antibody titers in all vaccinated groups began to increase gradually. One week later, groups (3, 6, 7) that received a combination of live and inactivated vaccines either of genotype II or VII origin provided the highest pre challenge HI- NDV Ab titers (6±0.00, 5.6±0.24 & 5.8±0.20)$\log_2$, respectively. The NDV-HI-Ab titers in the aforementioned groups were statistically significant at ($P< 0.05$) when compared with the other vaccinated groups. Vaccination with inactivated ND vaccine only of either genotype II (Gp2) (5.2±0.20^{bc}) or VII (Gp5) (5±0.00^{c}) led to NDV- higher and statistically significant HI-Ab titers when compared with vaccination with live vaccine only (Gp1 & Gp4) (4.2±0.24^{d} & 4±0.00^{d}), respectively.

Table 3: Statistical analysis of HI result following vaccination with different NDV regimes

Age of birds	Arithmatic mean titers of differentially vaccinated NDV vaccinated groups								
	Gp1	**Gp2**	**Gp3**	**Gp4**	**Gp5**	**Gp6**	**Gp7**	**Gp8**	**Gp9**
1st day	7.5±1.5	7.5±1.5	7.5±1.5	7.5±1.5	7.5±1.5	7.5±1.5	7.5±1.5	7.5±1.5	7.5±1.5
7th day	4.5±0.50	4.5±0.50	4.5±0.50	4.5±0.50	4.5±0.50	4.5±0.50	4.5±0.50	4.5±0.50	4±0.00
14th day	2.3±0.29	2.4±0.37	2.7±0.42	2.6±0.37	2.4±0.37	2.6±0.43	2.6±0.20	2±0.44	2±0.44
21st day	2.8 ± 0.20^{b}	3.4 ± 0.24^{ab}	4 ± 0.00^{a}	2.8 ± 0.20^{b}	3.2 ± 0.20^{ab}	3.2 ± 0.20^{ab}	3.6 ± 0.24^{ab}	1 ± 0.32^{c}	1 ± 0.32^{c}
28th day	4.2 ± 0.20^{d}	5.2 ± 0.20^{bc}	6 ± 0.00^{a}	4 ± 0.00^{d}	5 ± 0.00^{c}	5.6 ± 0.24^{ab}	5.8 ± 0.20^{a}	0.4 ± 0.24^{e}	0.4 ± 0.24^{e}
35th day	2.4 ± 0.24^{c}	3.4 ± 0.24^{ab}	4 ± 0.00^{a}	2.4 ± 0.24^{c}	3.2 ± 0.24^{b}	3.4 ± 0.24^{ab}	3.6 ± 0.24^{ab}	3.6 ± 0.24^{ab}	0.0 ± 0.00^{e}
38th day	9 ± 0.00^{a}	9 ± 0.00^{a}	8.8 ± 0.20^{a}	8.6 ± 0.24^{ab}	8.8 ± 0.20^{a}	8 ± 0.00^{ab}	8 ± 0.00^{ab}	2.8 ± 1.71^{c}	0 ± 0.00^{d}

Data are represented as arithmatic Mean titers ±SE; Gp: Group
Means within the same raw with different superscript letters are significantly different (P< 0.05) .

Protective efficacy following vNDV challenge

As shown in Figure 2, the mortality of sham-vaccinated birds challenged with virulent NDV genotype VII (Gp8) began on day 4 and ended on day 6 post challenge, with 20% protection%. The two groups that received either live genotype II (Gp1) or VII (Gp4) vaccine demonstrated 80% protection%, with mortality occurring on days 5 and 6 following challenge. Both groups of birds that received either inactivated genotype II (Gp2) or VII (Gp5) vaccine demonstrated 90% protection, with mortality occurring only 6 days post challenge in both cases. The three groups that received a combination of live and inactivated vaccines from genotype II or VII origin (Gp3, Gp6, and Gp7) demonstrated 100% mortality protection. The negative control group (Gp9) exhibited neither mortality nor clinical signs throughout the experimental period.

Clinical indices following virulent NDV challenge

As shown in Figure3, following challenge with $10^{6.5}/EID_{50}/100$ uL per bird of vNDV genotype VII strain (NDV/chicken/ Egypt/1/2015) at 31 days old via oculonasal route, the clinical index was lowest (00.08) in birds received a combination of live and inactivated vaccines originated from NDV genotype VII (Gp6), indicating longer time for clinical disease to manifest. Furthermore, birds received a combination of inactivated NDV genotype VII vaccine and Clone 30 (Gp7) had clinical index of 00.10. However, birds given

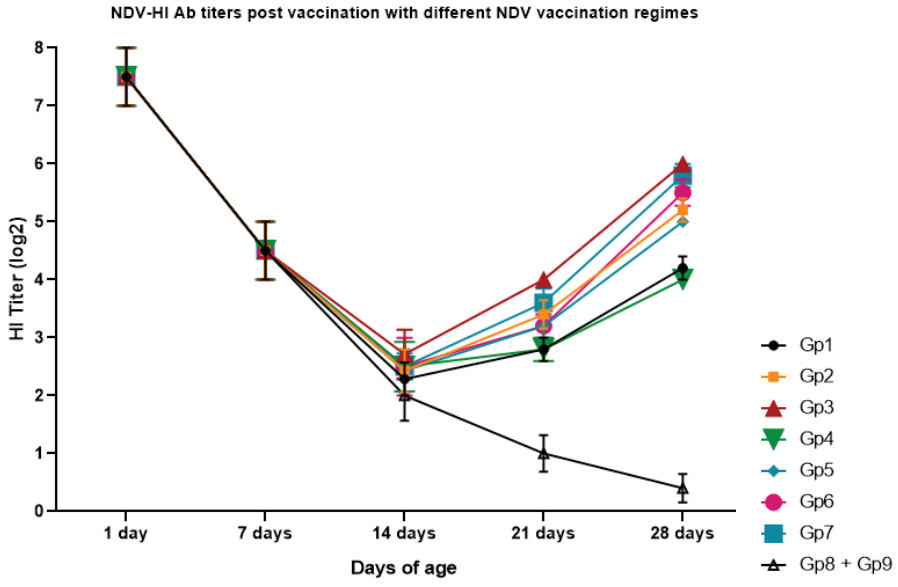

Figure 1: Arithmetic mean HI titers results: pre-challenge serological response of broiler chickens after vaccination with different types of vaccines, serum samples were collected weekly and tested against LaSota to measure the level of specific antibodies (mean HI titer ±SE) in vaccinated and non-vaccinated birds

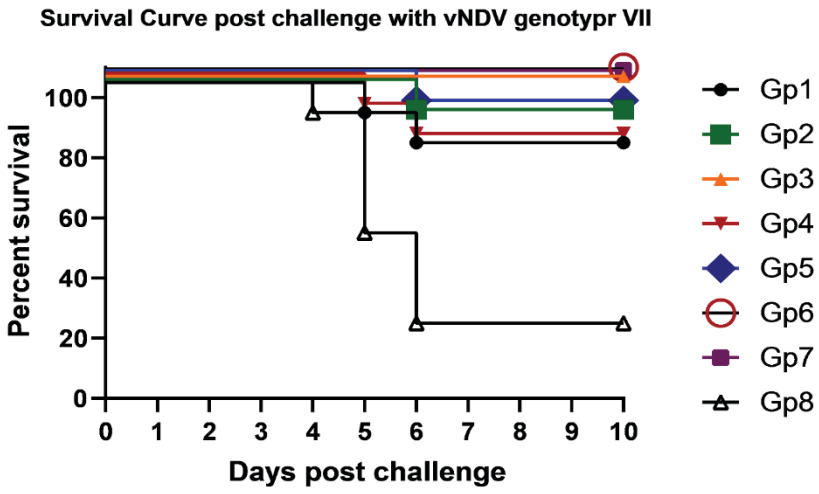

Figure 2: Survival curve: duration of protection following challenge of the vaccinated groups with $10^{6.5}$/EID_{50}/100 ul per bird of vNDV genotype VII strain (NDV/chicken/ Egypt/1/2015) at 31 days old via oculonasal route. The percent survival versus day post challenge was monitored for 10 days. Gp9 survival was 100% as it is non-infected group

Table 4: Clinical observations of the differentially NDV- vaccinated groups 10 days post challenge

Groups	Conjunctivitis	Swollen Eye lid	Greenish Diarrhea	Nasal discharge	Respiratory sign	Nervous signs	Depression
GP1	4/10	2/10	4/10	3/10	4/10	1/10	4/10
GP2	3/10	1/10	2/10	2/10	3/10	0/10	2/10
GP3	2/10	1/10	1/10	1/10	3/10	0/10	2/10
GP4	3/10	2/10	3/10	2/10	4/10	0/10	4/10
GP5	2/10	1/10	2/10	1/10	1/10	0/10	2/10
GP6	1/10	0/10	1/10	0/10	1/10	0/10	2/10
GP7	1/10	0/10	2/10	0/10	0/10	0/10	2/10
GP8	10/10	8/10	10/10	4/10	6/10	3/10	10/10
GP9	0/10	0/10	0/10	0/10	0/10	0/10	0/10

a combination of inactivated LaSota and live Clone30 (Gp3) had a clinical index of 00.12, while birds given only inactivated LaSota or genotype VII had a clinical index of 00.23 or 00.20, respectively. Higher clinical indices were observed in birds received live vaccines only either of genotype II (Gp1) or genotype VII origin (Gp4) with clinical index of ≥ 00.40. It is worth noting that the clinical index of the non-vaccinated group (Gp8) was 1.14. Regarding to the control negative group (Gp9) the clinical index was 00.00. The recorded clinical signs and the number of sick birds for the differentially vaccinated groups are represented in Table (4).

Virus shedding after challenge with vNDV genotype VII

Three days post virus challenge, virus replicated and was shed efficiently in the upper respiratory (Figure 4A) and intestinal tract (Figure 4C) of control positive chickens (GP8) with arithmetic mean titers of approximately $10^{5.23 \text{ and } 3.7}$ EID_{50}/ml, respectively. Virus shedding in upper respiratory and intestinal continued at high levels at 5th day post challenge with mean titers of approximately $10^{5.67 \text{ and } 4.61}$ EID_{50}/ml, respectively (Figure 4B & D). Surprisingly, three days post challenge, birds that vaccinated with genotype II vaccines (genetic mismatched vaccine) either live, inactivated or a combination of both failed to significantly reduce viral tracheal shedding when compared to control birds. As a result, the tracheal mean virus titers in any of genotype II vaccinated groups (1, 2 & 3) were ($10^{4.96, 4.66 \text{ \& } 3.47}$) EID_{50}/ml, respectively and all the tested birds were 100% shedders (5\5) in (Gps1 & 2). Whereas 80% of birds that received both inactivated LaSota and Clone 30 vaccines (Gp3) were shedders (4\5). On the fifth day post challenge, the tracheal mean virus titers significantly reduced in Gp1, Gp2, and Gp3 and were ($10^{1.64, 1.37 \text{ and } 1.36}$) and 40% of birds were positive shedders (2\5) in each group. Regarding the intestinal shedding, 3 days post challenge, only

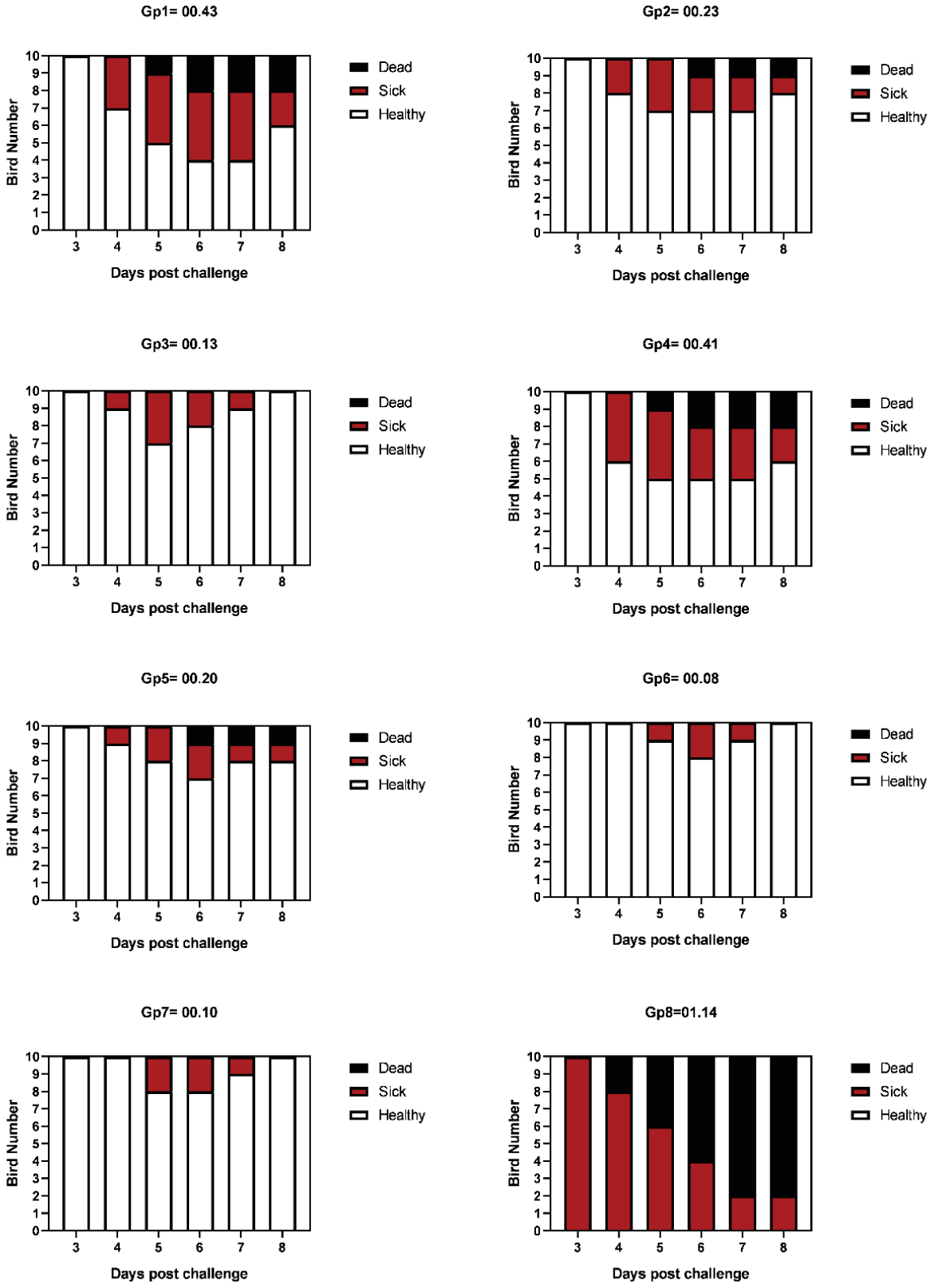

Figure 3: Course of NDV: broiler chickens vaccinated with different vaccine programs were challenged with $10^{6.5}$/ EID_{50}/100 uL per bird of vNDV genotype VII strain (NDV/chicken/ Egypt/1/2015) at 31 days old via oculonasal route. The birds were monitored for clinical signs and the numbers indicate the clinical indices that were calculated as previously described by Grund et al (24). Group 9 was non-infected group and all the birds remain in normal conditions with no clinical signs all over the experimental period

Gp1 that received live genetic mismatched vaccine had mean cloacal titers >10^2 EID_{50}/ml and the shedding was 100% (5\5). However, in Gp2 & 3 the mean titer of viral cloacal shedding were < 10^2 EID_{50}/ml and 40% (2\5) of the birds were shedders in those groups (Figure 4C). Those groups (Gp1, 2 & 3) demonstrated reduced levels or no shedding by day 5 pc with mean cloacal titers of < 10^2 EID_{50}/ml and the shedding were 40% (2\5), 40% (2\5) and 0% (0\5), respectively. In contrast, virus shedding was significantly reduced following using homologous vaccines (genotype VII) to the challenge virus when compared to control group and the genotype II vaccinated groups. The tracheal mean virus titers, 3 days post challenge, in Gp4, Gp5, Gp6 and Gp7 were ($10^{4.40, 2.97, 1.43 \& 1.36}$) EID_{50}/ml, respectively and the shedding were 100% (5\5) in Gp4, 80% (4\5) in Gp5, 20% (1\5) in both group (Gp6 and Gp7). On fifth day post challenge, the tracheal mean virus titers were decreased in Gp4 and Gp5 ($10^{1.39 \& 1.23}$) and 40% of birds were shedders (2\5) in both groups and birds of Gp6 and Gp7 had no detectable tracheal shedding (Figure 4A & B). Regarding the intestinal shedding, group of birds that received live genetic-matched vaccine only (Gp4) had mean cloacal titers of < 10^2 EID_{50}/ml on day 3 & 5 post challenge and 40% of the birds (2\5). Birds immunized with inactivated genetic-matched vaccine to the challenge virus (Gp5, Gp6 and Gp7) had no detectable cloacal viral shedding on day 3 & 5 post challenge (Figure 4C & D).

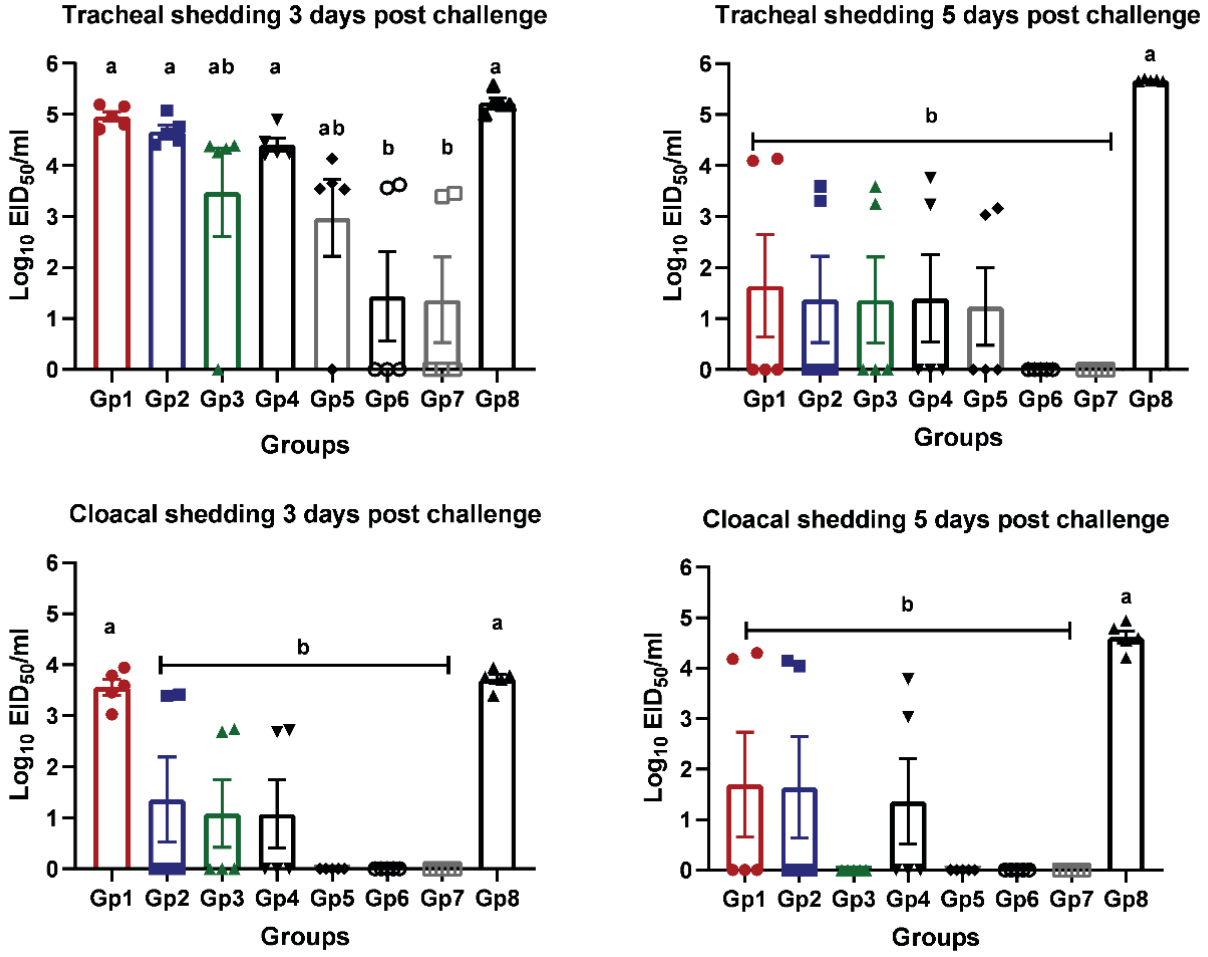

Figure 4: NDV viral shedding of experimentally infected birds with $10^{6.5}$/EID_{50}/100 uLper bird of vNDV genotype VII strain (NDV/chicken/ Egypt/1/2015) at the 31st day of age via oculonasal route. (A) Scatter blot represents mean viral titers of tracheal shedding 3 days post challenge and number of shedders. (B) Scatter blot represents mean viral titers of tracheal shedding 5 days post challenge and number of shedders. (C) Scatter blot represents mean viral titers of cloacal shedding 3 days post challenge and number of shedders. (D) Scatter blot represents mean viral titers of cloacal shedding 5 days post challenge and number of shedders. Titers were calculated as $\log_{10}$ EID_{50}/1 mL (±SE). Group 9 was non-infected control group and showed negative shedding from both tracheal and cloacal routes during all the measuring time points

Virus shedding and mortality of sentinel birds

Three sentinel birds were placed in each group 48 hours after the vNDV genotype VII virus challenge. Virus tracheal shedding was not observed in any of the sentinel birds three days after contact and five days after post challenge. The highest NDV tracheal shedding was observed in contacted sentinel birds with Gp1. The mean NDV virus titers were $10^{4.80\ \&\ 5.99}$ EID_{50}/ml on day 5 & 7 post contact, respectively and 100% of the birds (3/3) were shedders at the two time points. Groups 6 and 7 contacted birds, which received inactivated genotype VII vaccine in addition to either live genotype VII or genotype II vaccine, respectively, did not experience illness or viral shedding. Vaccination with a single dose of live or inactivated genotype II or VII vaccines did not prevent virus transmission, and tracheal virus shedding was (2/3) birds (Figure 5A & B).

Mortalities in sentinel birds were highest (2/3) in birds placed in contact to birds of Gp1, 2 and 3. Only one sentinel bird died in contact to birds of Gp4. No mortality in sentinel birds was observed in sentinel birds in contact to birds of Gp5, 6 and 7.

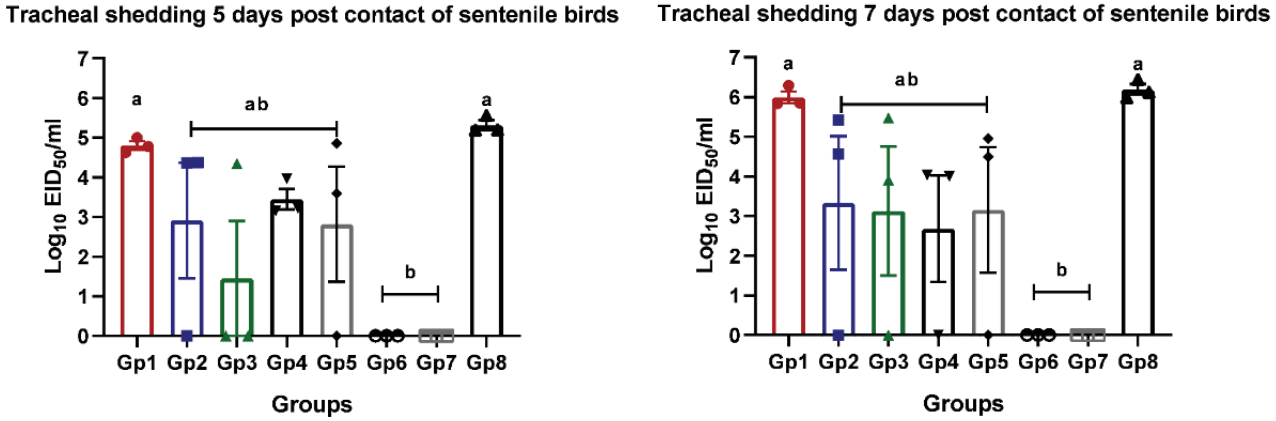

Figure 5: NDV viral shedding of sentinel birds in-contact with experimentally infected birds with 106.5/ EID50/100 uL per bird of virulent NDV genotype VII strain (NDV/chicken/ Egypt/1/2015) at 31 days old via oculonasal route. (A) Scatter blot represents mean tracheal viral titers at 5 days post contact to infected birds and numbers of shedders. (B) Scatter blot represents mean tracheal viral titers at 7 days post contact to infected birds and numbers of shedders. Titers were calculated as log_{10} EID_{50}/1 mL (±SE).

Discussion

Newcastle disease (ND) is one of the most devastating diseases that considerably cripple the global poultry industry, because of its enormous socioeconomic importance and potential to rapidly spread to nattive birds in the vicinity. Newcastle disease is included among the list A of avian diseases that must be notified to the OIE immediately upon recognition (25).

Prevention of the disease includes, in addition to biosecurity, the immunization of birds using different type of commercial NDV vaccines either lentogenic or mesogenic in either live or inactivated form. The right parameters for evaluation of the NDV vaccines are the ability to decrease or eliminate clinical disease as well as bird's mortality, decrease the amount of virulent virus shed and increase the infectious dose of the challenge virus (26). In the past, the inactivated or live NDV genotype II-based vaccines had provided only adequate protection against clinical disease and mortality (26). Although, they can reduce vNDV shedding but the virus shedding still cannot be completely inhibited in the vaccinated birds (13). In the last ten years, an increased number of NDV outbreaks were reported in the vaccinated birds which caused by genotype VII (27, 28). The later suggested that the well-established inactivated or live genotype II-based vaccines, LaSota strain as example, may not produce enough clinical protection against the newly emerged genotype (10, 26, 27, 29). Therefore, in the past few years antigenically matched engineered vaccines to vNDV genotype VII strains were constructed using reverse genetics in either live or inactivated form (26). One such vaccine based on the use of the

LaSota vaccine backbone with the replacement of the fusion and hemagglutinin neuraminidase genes, which replaced with their correspondence in genotype VII virus. Furthermore, the cleavage site of the F gene was modified to be identical to the cleavage site of the LaSota vaccine strain (30). There has not yet been a report that is clear and highlights an appropriate vaccination schedule for using these promising vaccines in the most beneficial way to reduce NDV. Therefore, in this study we designed several vaccination programs using either the genetic-matched or mismatched NDV vaccines as well as combination of them to combat the recently isolated vNDV genotype VII challenge (17), in comprehensive experimental trials. The different vaccination regimes were evaluated from different prospective. Accordingly, the birds were bled weekly to monitor the vaccine serological responses. Serological monitoring of vaccinated birds is one of the important parameters for evaluation of the protective level afforded by applied vaccine (31). The experimental birds had high maternal derived immunity of (7.5±1.5) $\log_2$, which decreased gradually to < 4 $\log_2$ at 14 days old in non-vaccinated birds (32). At the fourth week of age, significant immune responses were peaked in all vaccinated groups with protective antibodies titers ranged from 4-6 $\log_2$ (33). In fact, only when at least 75-80% of the flocks have antibody titers that are equivalent to or higher than 4 $\log_2$ does the herd immunity against NDV exist (33). In the present work, regardless of the vaccine's genetic background, the type of vaccination used had a significant impact on the level of NDV vaccine-induced Ab. Hence, the highest antibodies titers were recorded in the three groups that were vaccinated with combination of live and inactivated NDV vaccines, followed by the two groups vaccinated with inactivated vaccine only. The application of live vaccine only had the lowest seroconversion results regardless of its genetic type. However, the seroconversion results in the present study did not distinguish between the responses of genetic-matched and mismatched vaccines. The previous mainly due to the presence of the both genotypes II and VII in one serotype and the using of LaSota strain as HI antigen.

Following challenge the birds were monitored and scored daily according to their clinical conditions as well as the clinical indices were calculated according to Grund et al (24). The non-vaccinated birds had the highest clinical index (1.14) and clinical signs reflecting the characteristic of velogenic NDV infection and its pneumotropic and viscerotropic properties (7). Hence, the non-vaccinated birds suffered from depression, loss of appetite, feverish, conjunctivitis, swollen eyelids, greenish diarrhea and respiratory signs in form of nasal discharge and rales. On the other hand, the lowest clinical indices were found in the two groups that received combination of genetic-matched inactivated NDV vaccine together with live vaccine of either genetic-matched or mismatched origin with 100% survival. The consistent presence of genetic-matched inactivated vaccine in the two groups induced the superior clinical protection indicate the protective importance of the inactivated vaccine in the designing of NDV vaccination programs. Although vaccination with combination of live and inactivated genetic-mismatched vaccine (Gp3) inhibited vNDV induced-mortality, this certain group had higher clinical index and the clinical signs started one day earlier, compared to that birds vaccinated with genetic-matched inactivated NDV vaccine and live vaccine of either genetic-matched or mismatched origin. Further, in order to maximize protection, it has been recommended that the adjuvant-based ND inactivated vaccine be made from local strains of velogenic NDV (29). Regarding to birds that received live vaccine only (Gp1 & Gp4) had high clinical indices and mortality was approximately 20% in the two groups. These results confirm that whatever the genetic relatedness of the applied live vaccine, it cannot be effective alone for inhibition of NDV infection. Even vaccination with inactivated vaccine without boostering with live vaccine could not provide the birds with complete protection from mortality and clinical signs. It has been found earlier inactivated vaccines based on genotype VII strain could protect birds against clinical disease, especially when used together with live attenuated vaccines (34).

The level of viral shedding in poultry house is also considered an important parameter for evaluation of vaccine and for determination of NDV transmissibility across surrounding poultry flocks (35). The genotype II commercial applied NDV vaccines can protect against clinical disease but not able to prevent virus replication and shedding from infected vaccinated birds to the surrounding environment. The previous could be attributed to the genetic distance between the

vaccine strains and the circulating virus strains (as example; genotype VII) (13, 36, 37). Previous studies showed that using of antigenically matched vaccines homologus with the field virus increases the capacity to prevent or reduce challenge virus shedding in the terms of amount and number of shedders (10, 38). In the present work, the application of live vaccine only of either genotypes II or VII failed to significantly reduce viral tracheal and cloacal shedding 3 days post challenge when compared to control group. However, the duration of virulent virus tracheal shedding was lesser in these groups compared with control groups. In consistent, application of heterologous inactivated vaccine, either alone or boost with heterologous live vaccine, could not prevent viral tracheal shedding of vNDV genotype VII at the third day post challenge. However, it minimize the duration of vNDV shedding from the same route (10). The previous reports stated that vaccination with a combination of live and inactivated LaSota vaccines can protect against clinical disease but not prevent infection and virus shedding (13, 31, 33, 38, 39). On contrary, the two groups vaccinated with inactivated homologues vaccine that boosted with live homologues or heterologous vaccines had minimal or completely diminished viral tracheal shedding at the two evaluated time points (40). From these results we can conclude that when inactivated genotype VII matched vaccines was used, viral shedding was significantly reduced or nearly inhibited (23, 36, 41). Beside the importance of the vaccine genetic homology (27), this result could be attributed to the high humoral immunity specific for NDV genotype VII induced by inactivated vaccine which could prevent virus replication in experimentally infected birds (42). Virus replication in challenged birds was sufficient to transmit to sentinel chickens 3 days post exposure to infected birds

For Newcastle disease control, it is important to take into account the environmental contamination that exists in an outbreak scenario and/or with any population, that has received vaccinations. This is particularly valid for nations where vNDV is endemic. In the present work and inconsistent with the shedding study and the number of shedders results, the virus transmissibility was lowest in the instance of the two groups vaccinated with inactivated homologues vaccine that boosted with live homologues or heterologous vaccines with no recorded mortality. It is thought that for a bird to infected with NDV, between 10^3 and 10^4 EID_{50} of virus must be administered (43) and those two particular groups had lesser amount of virus shedding from both routes. Additionally it is believed that vaccination programs used genotype VII inactivated and live vaccines initiated a high level of monospecific genotype VII antibody titers that neutralize the virus and limit the transmission and virus shedding to the contact birds (42).

In conclusion, the use of NDV vaccination regimens based on heterologous vaccine to the field strain could reduce losses from death, but it did not reduce losses from NDV caused clinical symptoms and transmission among poultry flocks. In order to prevent the transmission and circulation of the NDV genotype VII virulent strain among chicken flocks, a stringent control strategy based on the use of homologous inactivated NDV vaccines with a prime-boost strategy would be helpful.

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