Protective efficacy of the Newcastle disease virus genotype VII–matched vaccine in commercial layers

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ABSTRACT Newcastle disease virus (**NDV**) is a major threat to the poultry industry worldwide, with a diversity of genotypes associated with severe economic losses in all poultry sectors. Class II genotype VII NDV are predominant in the Middle East and Asia, despite intensive vaccination programs using conventional live and inactivated NDV vaccines. In Egypt, the disease is continuously spreading, causing severe economical losses in the poultry industry. In this study; the protective efficacy of a commercial, inactivated recombinant genotype VII NDV–matched vaccine (KBNP-C4152R2L strain) against challenge with the velogenic NDV strain (Chicken/USC/Egypt/2015) was evaluated in commercial layers. Two vaccination regimes were used; live NDV genotype II (LaSota) vaccine on days 10, 18, and 120, with either the inactivated NDV genotype II regime or inactivated NDV genotype VII–matched vaccine regime on days 14, 42, and 120. The 2 regimes were challenged at the peak of egg production on week 26. Protection by the 2 regimes was evaluated after experimental infection, based on mortality rate, clinical signs, gross lesions, virus shedding, seroconversion, and egg production schedule. The results show that these 2 vaccination regimes protected commercial layer chickens against mortality, but some birds showed mild clinical signs and reduced egg production temporarily. However, the combination of live NDV genotype II and recombinant inactivated genotype VII vaccines provided better protection against virus shedding (20% and 0% vs. 60% and 40%) as assessed in tracheal swabs and (20% and 0% vs. 20% and 20%) in cloacal swabs collected at 3 and 5 D post challenge (**dpc**), respectively. In addition, egg production levels in birds receiving the inactivated NDV genotype VII–matched vaccine regime and in those given inactivated genotype II vaccines were 76.6, 79, 82, and 87.4% and 77.7, 72.5, 69, and 82.5% at 7, 14, 21, and 28 dpc, respectively. The results of this study indicate that recombinant genotype-matched inactivated vaccine along with a live attenuated vaccine can reduce virus shedding and improve egg production in commercial layers challenged with a velogenic genotype VII virus under field conditions. This regime may ensure a proper control strategy in layers.

Key words: Newcastle disease virus, genotype-matched vaccine, genotype VII, layer

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INTRODUCTION

Newcastle disease virus (**NDV**) is *Avian avulavirus type-1* that belongs to the genus *Avulavirus* of the family Paramyxoviridae (Swayne and Glisson, 2013). Newcastle disease virus isolates vary in their pathogenicity to chickens and may include at least 3 pathotypes; lentogenic (low virulence), mesogenic (moderate virulence), and velogenic (high virulence). Velogenic NDV remain a major threat to the poultry industry because of high mortality and reduced egg production in laying hens (Cho et al., 2008). Velogenic NDV are further divided into viscerotropic velogenic and neurotropic velogenic pathotypes (Cattoli et al., 2011). Based on the sequence of the fusion protein gene (**F gene**) of NDV, isolates may be classified genetically into 2 classes, class I (a single genotype) and class II (21 genotypes based on phylogenetic analysis of the "F gene") (Shittu et al., 2016). Most

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velogenic NDV belonged to class II, and the majority of recently isolated strains associated with NDV outbreaks worldwide belong to genotype V, genotype VI, genotype VII (**GVII**), and genotype IX of class II. Newcastle disease virus GVII viruses were initially divided into 2 subgenotypes: NDV GVIIa, representing viruses that emerged in the 1990s in the Far East and spread to Europe and Asia, and NDV GVIIb, representing viruses that emerged in the Far East and spread to South Africa (Aldous et al., 2010). Later on, with the recognition of more recent viruses worldwide, the 2 subgenotypes were further classified into several subgenotypes, including subtype VIIc, subtype d, subtype e, subtype h, and subtype i, representing isolates from China, Kazakhstan, and South Africa. On the other hand, subtype VIIf, subtype g, subtype I, and subtype h presented the African isolates (Wang et al., 2006; Bogoyavlenskiy et al., 2012; Snoeck et al., 2013). In Egypt, the subgenotype VIId is predominant and caused several ND outbreaks (Shunlin et al., 2009). It was noticed that GVII strains cause more severe lesions in the lymphoid organs than genotype I strain Australia (Kristeen-Teo et al., 2017). In addition, a previous study has indicated that GVII strains of different host origin can induce higher lesion severity in the spleen than other velogenic genotypes, such as Hertts/33strain (genotype IV) (Wang et al., 2012). Moreover, Ecco et al., 2011 have shown that the GVII strain ZJ1 and genotype V strain CAO2 produced more severe clinical and pathological signs in the spleen than other virulent strains "Australia, Turkey ND, and Texas GB." This finding suggests that neither the fusion protein (F-protein) cleavage site sequence nor the intracerebral pathogenicity index is a sufficient tool to fully predict the clinicopathological outcomes associated with the virulent viruses. The aforementioned studies have consistently proposed that viruses belonging to these genotypes can produce severe damage in the different lymphoid organs compared with other virulent strains. More importantly, Qin et al., (2008) and Yu et al., (2001) performed cross-protection studies that showed that the LaSota vaccine provides poor protection against some isolates belonging to NDV GVII. In addition, some studies have shown that homolog vaccines can provide good protection and inhibit virus shedding after challenge with GVII NDV (Shunlin et al., 2009). These findings indicate that NDV GVII can disseminate efficiently in birds vaccinated with conventional non–genotype-matched vaccines, with a potential to induce lesions and injuries in the lymphoid tissues and impair the immune response.

Therefore, farm vaccination policies constitute one of the most important preventive measures against ND; now commercially available NDV vaccines, live attenuated or killed, are considered effective tools to protect poultry from virulent NDV infection. However, despite the routine vaccinations programs, ND outbreaks caused by GVII NDV in poultry have been reported in South America (Diel et al., 2012) and the Far East (Cho et al., 2007). In recent years, such vaccination failures have been reported among poultry farms in Egypt (El Naggar et al., 2018). This raised the question that commercial conventional NDV vaccines might not effectively protect poultry against the virulent GVII NDV owing to their antigenic differences (Shunlin et al., 2009). This may also be attributed to improper biosecurity measures, vaccination programs, and elimination of poultry manure. Recently, recombinant GVII-matched chimeric vaccines, such as Himmvac Dalguban N (Plus) Oil Vaccine (KBNP, Inc., Gyeonggi, Korea), have been developed by the reverse genetics method and showed significant protection in specific pathogen-free chicken against the homologous genotype NDV (Miller et al., 2007). Vaccines based on a recombinant LaSota strain backbone were also developed (KBNP-C4152R2L), where both antigenic genes, F and hemagglutinin-neuraminidase (**HN**) genes, were replaced by other genes from genotype VIId virus, KBNP-4152 (Cho et al., 2008; Jeon et al., 2008). However, before replacement, a mutation was induced to attenuate the recombinant strain at the F cleavage motif by changes from (112) RRQKR (116) to (112) GRQAR (116). To maintain this attenuation and reduce the pathogenic instability, a single-point mutation was placed by insertion at codon 115. In addition, a 6-nucleotide sequence was inserted at the intergenic region between the matrix protein and F gene for attenuation without breaking the "rule of six." The HN protein length was increased from 571 to 577 as a marker for later differentiation (Cho, et al., 2008). NDV belonging to genotype VIId was associated with severe problems in oviducts, which leads to the production of soft-shelled, shell-less eggs and even decreased or loss of egg production, whereas the virus infection was associated with severe infiltration of lymphocytes (Li et al., 2016). In addition to follicular degeneration effects, the qualities of the egg shell and egg albumen in velogenic Newcastle disease virus (**VNDV**)-infected birds were low owing to inadequate production of steroid hormones and yolk resorption (Al-Garib, et al., 2007). For a long time, it was widely accepted that commercial conventional NDV vaccines were effective in controlling ND, but they could not prevent infection and virus shedding completely owing to differences between vaccine genotypes and the circulating ones (Miller, et al., 2007; Miller, et al., 2013; Liu et al., 2017; Shahar et al., 2018). Recent reports claim that genotype-matched vaccines provide better protection against challenge with GVII field virus. They also significantly reduce virus shedding when compared with genotype II (**GII**) NDV vaccines (Miller et al., 2007; Kapczynski et al., 2013; El Naggar et al., 2018; Ji et al., 2018; Shahar et al., 2018; Ayoub et al., 2019). When live and inactivated ND vaccine genotypes were antigenically matched, especially in F and HN genes, they produce a higher specific immune response to the challenge viruses than the non–genotype-matched vaccines, reflected in the form of significant protection and reduced virus shedding levels (Miller et al., 2007, 2009). More recently, recombinant ND vaccines with the F gene and HN gene (homologous to the challenge virus)

placed on a LaSota backbone were shown to induce higher levels of specific antibodies for GVII virus, with reduced viral shedding after challenge, than commercial genotype I/GII vaccines (Cardenas-Garcia et al., 2015). Teams from Korea (Cho et al., 2008), China (Liu et al., 2015), and Indonesia (Xiao et al., 2012) reported similar findings in terms of reduction of viral shedding. However, other authors reported no improvement with homologous recombinant NDV vaccines (Dortmans et al., 2012). In Egypt, a vigorous vaccination program is applied, including several live and killed boosts, during a breeder's life to provide high maternal antibodies and protect against egg production losses and clinical disease. In addition, hatched chicks receive dual vaccination (live/killed) on day 7, followed by 2 live vaccine doses (on days 17 and 24). Nonetheless, field outbreaks occur in vaccinated broilers and breeders or commercial layers, leading to high mortalities and egg production losses. This may be due to the observation of the vast majority of studies conducted on NDV vaccines, which were mainly obtained from NDV antibody–free chicken within negative-pressure rooms that do not reflect the full protective efficacy under field situations. On the other hand, commercial poultry is vaccinated with live attenuated and/or inactivated vaccines several times during lifetime. But some recent findings suggested that the use of homologous NDV vaccines under commercial field conditions showed improvements in clinical protection in suboptimally vaccinated birds, pointing an advantage for the use of close antigenically matched vaccine seeds from the circulating viruses (Dimitrov et al., 2017). In this study, 2 combination regimes of live attenuated genotype II vaccine with either inactivated recombinant GVII–matched vaccine or inactivated conventional genotype II ND vaccine were used for conferring protection against challenge with the velogenic GVII in commercial layers in Egypt.

MATERIALS AND METHODS

Ethical Statement

Animal studies were approved by the Animal Welfare and Research Ethics Committee of the University of Sadat City (approval ID: 20120632), and all procedures were conducted strictly in accordance with the Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize animal suffering.

Challenge Virus and Vaccines

Previously identified and characterized NDV GVII virus, shown in Figure 2 (NDV Chicken/USC/Egypt/El Gharbia/2015), accession number MG029120, was used as a challenge virus in this study (Hassan, 2018). Three different commercial NDV vaccines were used for layer vaccination in the study. They included a GII NDV (LaSota strain) live vaccine (LaSota GoldND, Boehringer Ingelheim, Germany) and a GII NDV-inactivated oil emulsion vaccine (LaSota strain/NDV Genotype II, EID_{50}: 8.2 log_{10}; Volvac B.E.S.T AI ND, Boehringer Ingelheim, Germany), which was kindly supplied by Vetmedica, Cairo, Egypt, and inactivated oil emulsion recombinant GVII NDV vaccine (Himmvac Oil Vaccine/NDV Genotype VIId "KBNP-C4152R2L strain" EID_{50} = 8.2 log_{10}/batch no.: CMR 1115, KBNP Inc., Gyeonggi, Korea), which was kindly supplied by Egyptian Company for Trade and Distribution (Egy-Co), Cairo, Egypt.

Experimental Design

Two hundred one-day-old commercial Lohman Brown layer chicks with maternal NDV antibodies were supplied from a local hatchery. They were housed in separated pens with food and water provided *ad libitum* at a biosecured experimental facility. The chicks were randomly divided into 4 groups (G1 to G4) of 50 birds each. Of these, 3 groups (G1, G2, and G4) were vaccinated 3 times with the live GII NDV vaccine on days 10, 18, and 120 via the oculonasal route and 3 doses of inactivated GII NDV (for G1 birds) or recombinant GVII NDV (for G2 birds) vaccines on days 14, 42, and 120 subcutaneously. This was in accordance with a conventional vaccination program used in Egypt, as shown in Figure 1. The other group (G3) of birds received no vaccination during the experiment. Nine week after the last vaccination (on week 26), the G1, G2, and G3 birds were challenged with the virulent NDV, USC2015 strain, ($10^{5.5}EID_{50}$ per bird) via the intramuscular route. Meanwhile, G4 birds were sham challenged with phosphate-buffered saline solution via the same route. Tracheal and cloacal swabs were collected from all groups before the challenge and were checked by real-time reverse transcriptase polymerase chain reaction (RT-PCR) for egg production–threatening pathogens (avian influenza H5 and H9 viruses, infectious bronchitis viruses, and NDV) using primer sets, as shown in Table 1. All experimental birds were monitored and recorded for clinical signs and egg production for 4 wk after the NDV challenge. Bloods samples were collected from all experimental groups at an interval of 1 wk after the NDV challenge. Tracheal and cloacal swabs were collected at 0, 3, 7, and 10 D post challenge (**dpc**) for monitoring virus shedding (Table 3).

Serology

Newcastle disease serum antibodies were quantified by hemagglutination inhibition (HI) assay in U-bottomed 96-well microtiter plates as per the procedure recommended in the OIE Manual (OIE, 2012). The test was performed using 4 hemagglutination units of the NDV LaSota antigen. The end point was determined as the reciprocal of the highest serum dilution that exhibits complete inhibition of hemagglutination. Hemagglutination inhibition titers were expressed in terms of log_2, and samples showing values ≤ 3 were considered negative (OIE, 2012).

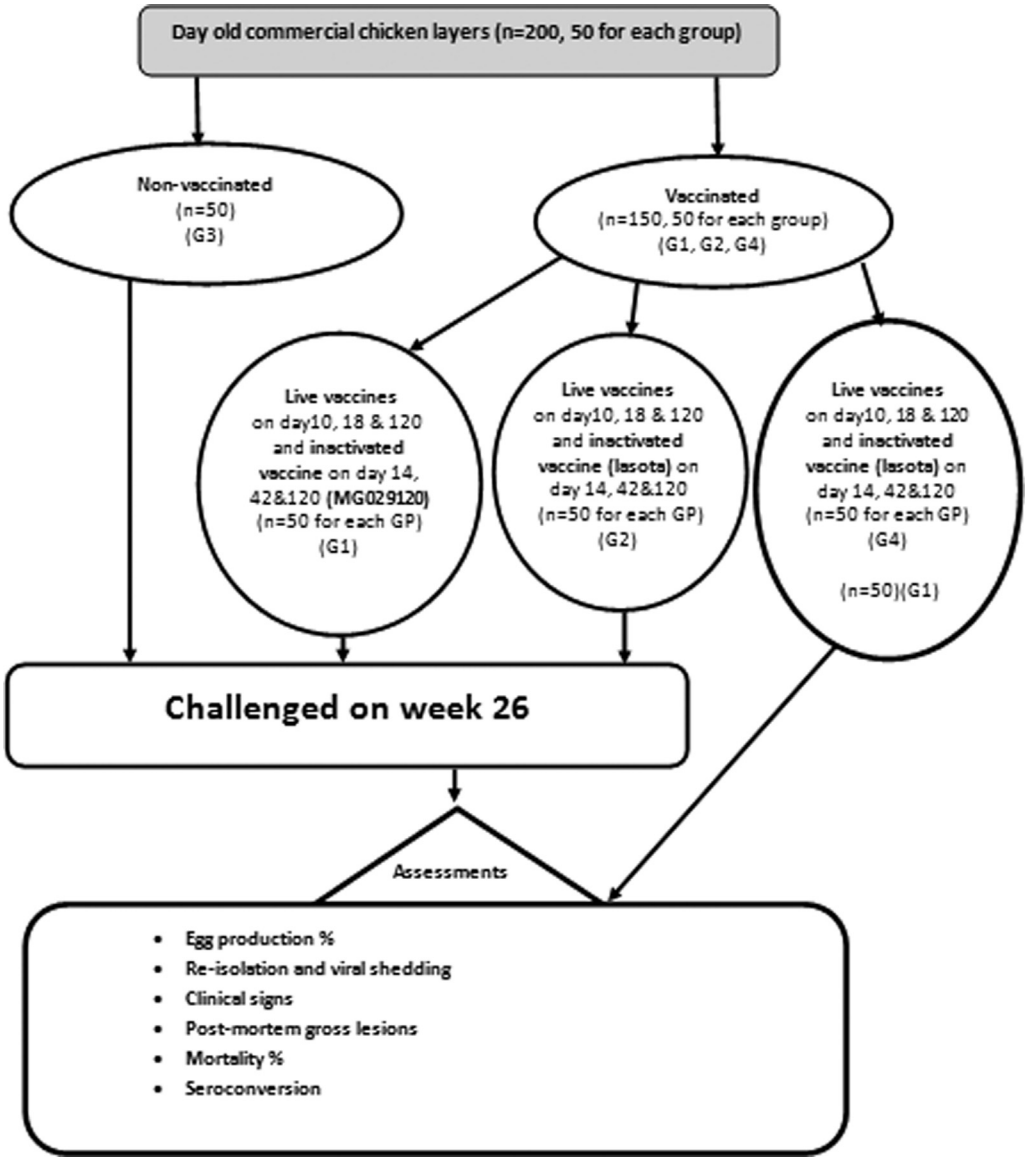

Figure 1. Experimental plan for assessments of NDV vaccine effectiveness against NDV USC2014 strain challenge in commercial chicken layers. G1: genotype II live/genotype VII inactivated and challenged; G2: genotype II live/genotype II inactivated and challenged; G3: unvaccinated and challenged; G4: genotype II live/genotype VII inactivated and unchallenged; NDV, Newcastle disease virus.

Virus Shedding

Tracheal and cloacal swabs collected at 0, 3, 5, 7, and 10 dpc were immersed in 400 µL of Dulbecco's modified Eagle medium with an antibiotic solution. Viral RNA was extracted directly using the TRIzol reagent (Gibco, Invitrogen, Carlsbad, CA), as per the manufacturer's instructions, and suspended in diethyl pyrocarbonate water. Standard RT-PCR was performed using a One-Step RT-PCR kit (QIAGEN, Valencia, CA), with specific primers and probe sets as shown in Table 1. In brief, RT-PCR assay was performed in a final volume of 25 µL containing 12.5 µL of the QuantiTect RT-PCR Master Mix, 0.25 µL of the QuantiTect RT Mix, 0.25 µL of each primer (50 pmol concentration), 0.125 µL of each probe (30 pmol concentration), 3.625 µL of PCR-grade water, and 7 µL of RNA template. Reverse transcription reactions were set up at 50°C for 30 min, followed by a primary denaturation step at 94°C for 15 min, then 40 cycles of denaturation at 94°C for 15 s, annealing at 54°C for 30 s, and extension at 72°C for 10 s. The reaction was carried out using an MX3005P real-time PCR machine (Stratagene, La Jolla, CA). Real time reverse transcription (**RRT**)-PCR titers were converted into $\log_{10}$ EID_{50}/mL as described previously (Nolan et al., 2006). In brief, a triplicate of 6 10-fold dilutions of challenge NDV (10^6 EID_{50}/mL) were used to generate a standard curve using stock virus dilutions from 10^{-1} to 10^{-6}. Because Ct is defined as the point at which the curve crosses the horizontal threshold line, virus $\log_{10}$ titers of a specimen were plotted against the Ct value, and the best-fit line was constructed. The linear range of the assay ranged from 1 to 10^6 EID_{50}/mL, with a correlation coefficient of 0.99. The system detection limit was 0.5 EID_{50}/mL, as has been standardized and described previously (Sultan et al., 2019). Newcastle disease virus quantity

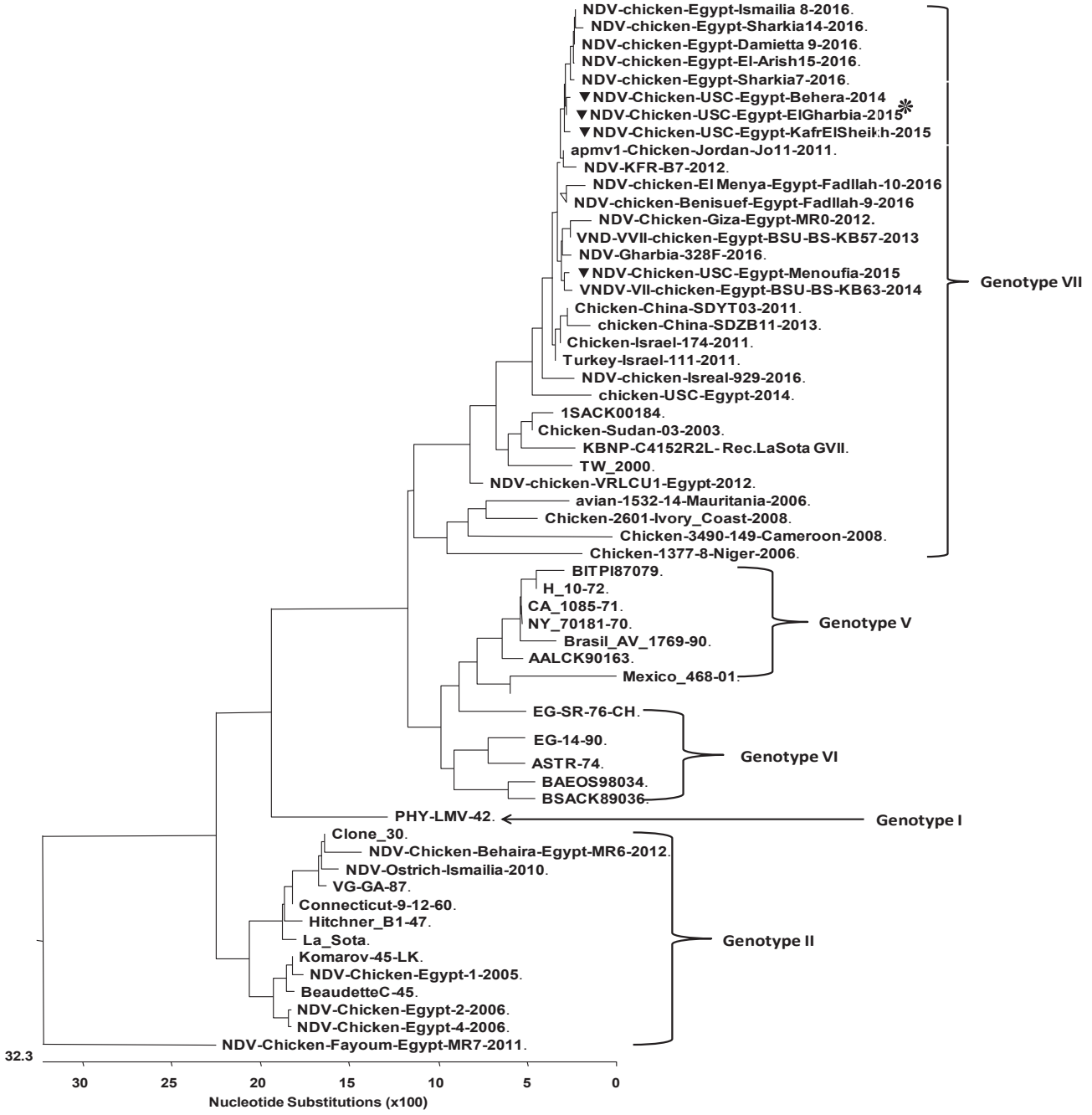

Figure 2. Phylogenetic relationship between the isolates obtained in this study and other isolates previously isolated in Egypt with some reference strains retrieved from the GenBank, using the maximum likelihood method. *Challenge virus (NDV Chicken/USC/Egypt/El Gharbia/2015) with the accession number MG029120. NDV, Newcastle disease virus.

in unknown samples was derived by plotting the Ct of an unknown samples against the standard curve and expressed in terms of $\log_{10}$ EID_{50}/mL equivalents.

Statistical Analysis

Whenever necessary, data were analyzed using the Student t test or ANOVA, followed by application of Duncan's new multiple range test to determine the significance of differences between individual treatments and corresponding controls (Steel and Torrie, 1960).

RESULTS

Clinical Signs

Clinical signs were monitored for 4 wk after the challenge with NDV USC2015 strain. In the groups G1 and G2 (vaccinated and challenged), clinical signs were observed in some birds. In the group G1 (recombinant GVII–vaccinated and challenged), 20% (10/50) of the vaccinated birds showed mild or moderate clinical signs in the first week pc; then, most of the affected birds recovered (80% clinical protection), although 2% of the

Table 1. Oligonucleotide primers used for the amplification of the NDV F-protein gene, avian influenza virus (H5N1-H9N2) HA gene, and IBV S1 gene.

Gene	Primer	Primer sequence (5′–3′)	Reference
NDV F gene	Forward	5′-TCCGGAGGATACAAGGGTCT-3′	Al-Habeeb et al., 2013
	Reverse	5'-AGCTGTTGCAACCCCAAG-3′	
	Probe	5′-FAM-AAGCGTTTCTGTCTCCTTCCTCCA-BHQ-1–3′	
AIV H5	H5-LH1	5′-ACATATGACTACCCACARTATTCA-3′	Slomka et al., 2007
	H5-RH1	5′-AGACCAGCTAYCATGATTGC-3′	
	Probe	5-FAM-TCWACAGTGGCGTTCCCTAGCA-Tamra-3	
AIV H9N2	H9-F	5′-ATGGGGTTTGCTGCC-3′	Monne et al., 2008
	H9-R	5′-TTATATACAAATGTTGCAC(T)CTG-3′	
	Probe	5-FAM-TTCTGGGCCATGTCCAATGG-Tamra-3	
IBV S1 gene	Forward	5′-GCTTTTGAGCCTAGCGTT-3′	Callison et al., 2006
	Reverse	5′-GCCATGTTGTCACTGTCTATTG-3′	
	Probe	5-FAM-CACCACCAGAACCTGTCACCTC-BHQ1-3	

Abbreviations: AIV, Avian Influenza virus; F gene, fusion protein gene; HA gene, Hemagglutinin gene; IBV, Infectious bronchitis virus; NDV, Newcastle disease virus.

birds (1/49) showed nervous signs (twisted neck) at the recovery stage. The mortality rate was 2% (1/50 at 7 dpc) during the experimental period, as shown in Table 2. In the group G2 (GII-vaccinated and challenged), 26% (13/50) of the vaccinated birds showed mild or moderate clinical signs in the first week pc, and then, most of the affected birds recovered (76% clinical protection), whereas 4.1% (2/49) showed nervous signs (twisted neck) at the recovery stage. The mortality rate was 2% (1/50 at 5 dpc), as shown in Table 2. The group G3 (unvaccinated and challenged) showed severe clinical signs typical of ND in all birds, and the mortality was 85% (43/50) at 7 dpc (Table 2). All survivors in the G4 group (n = 7) showed nervous signs at 2 wk (pc), as shown in Table 2.

Postmortem Examination

Dead birds in the groups G1, G2, and G3 exhibited gross lesions such as congestion of the trachea, necrotic foci on the spleen, swollen kidney, petechial haemorrhages on the tips of proventriculus glands, and bluish red button-like ulcers in the intestinal mucosa. Dead birds in all the groups were positive for virulent NDV, as assessed by RT-PCR. In the group G4 (vaccinated and unchallenged), all birds showed neither clinical signs nor mortality during the experimental period.

Egg Production

This ranged from 86 to 88% before the NDV challenge. The group G1 showed a drop in egg production to 76.6%, on the first week post challenge (**pc**); then it, started to rise to 79% on the second week pc to reach 82% and 86% on the third and fourth weeks pc, respectively. In the group G2, egg production dropped to 76.6% in the first week pc and continued to decrease to 72.5% and 69% by the second and third weeks pc, respectively. However, production was elevated to 82.6% at the fourth week pc. Egg drop in the group G2 was relatively lower than that in the group G1 during all the monitoring periods (4 wk pc), as shown in Figure 4. Meanwhile, egg production in the control group G3 (nonvaccinated and challenged) was 42.5, 25, 32, and 37.7% vs. 88, 88.6, 89.7, and 90.3% in the groups G3 and G4 at weeks 1, 2, 3, and 4 pc, respectively, as shown in Figure 4. In the group G3, there was a significant downgrading in quality of eggs since the fourth dpc (which resulted in shell-less, soft-shelled, cracked, small-sized, or deformed eggs).

Serological Response

The dynamics of the NDV serological response were examined for 4 wk pc using the HI test. The results were summarized in Figure 3. Before the NDV challenge, the mean HI titers of the vaccinated groups (G1, G2, and

Table 2. Clinical protection of vaccinated commercial layers with live and inactivated vaccines against challenge with genotype VII NDV USC2014 strain.

	Clinical signs at weeks after the challenge[2]												
	Week 1			Week 2			Week 3			Week 4			
Group[1]	RS	DS	NS	RS	DS	NS	RS	DS	NS	RS	DS	NS	Mortality (%)[3]
G1	10/50	8/50	0/50	9/49	5/49	0/50	3/49	1/49	1/49	2/49	0/49	2/49	1/50 (2%)
G2	12/50	10/50	0/50	11/49	9/49	0/50	5/49	4/49	2/49	4/49	0/49	2/49	1/50 (2%)
G3	50/50	40/50	0/50	7/7	5/7	7/7	7/7	3/7	7/7	2/7	1/7	7/7	43/50 (86%)
G4	0/50	0/50	0/50	0/50	0/50	0/50	0/50	0/50	0/50	0/50	0/50	0/50	0/50 (0%)

Abbreviations: DS, digestive sign; NDV, Newcastle disease virus; NS, nervous sign; RS, respiratory sign.

[1]G1: genotype II live/genotype VII inactivated and challenged; G2: genotype II live/genotype II inactivated and challenged; G3: unvaccinated and challenged; G4: genotype II live/genotype VII inactivated and unchallenged.

[2]Clinical signs were observed daily after the challenge with genotype VII NDV (USC2015 strain) at the age of 26 wk.

[3]All chicken deaths occurred during the first week after the challenge.

G4) varied from 8.31 ± 0.61 to 8.63 ± 0.73, whereas the group G3 (unvaccinated birds) was tested negative (mean HI titers ≤3). However, in the groups G1 and G2, the mean HI titers skyrocketed at the first week pc (mean HI titers ≥12) after the NDV challenge. In the group G3, the survived birds showed a seroconversion mean HI titer of 8 at the first week after the NDV challenge, whereas the group G4 (vaccinated and unchallenged birds) did not exhibit a significant increase in HI titers (range = 8.5–9) during the production period.

Virus Shedding

All experimental birds were examined by RT-PCR for virus shedding using tracheal and cloacal swab samples collected at 0, 3, 7, and 10 dpc. The results are summarized in Table 3; Figures 5 and 6. Before the NDV challenge, all the tested birds were negative for NDV, Avian Influenza virus (**AIV**), and Infectious Bronchitis virus (**IBV**), the pathogens associated with drop in egg production. Tracheal swabs showed 20% shedders with 3.3 ± 0.21 EID_{50} and 60% shedders with 4.5 ± 0.51 EID_{50}, in the groups G1 and G2 at 3 dpc, respectively. Cloacal swabs obtained on the same days showed significantly lower shedding titers in the group G1, than in the group G2 (40% shedding with titers of 1.6 ± 0.22 EID_{50} vs. 40% shedders with titers of 2.3 ± 0.41 EID_{50}, respectively). G3 showed 100% shedding at titers of 6.3 ± 0.82 and 3.6 ± 0.33 EID_{50} in the tracheal and cloacal swabs, respectively. At 7 dpc, the tracheal shedding levels were 0% and 20%, at a titer of 2.6 ± 0.26 EID_{50} in the groups G1 and G2, compared with 100% shedding at a titer of 4 ± 0.56 EID_{50} in the group G3. Furthermore, cloacal swabs showed nondetectable shedding vs. 20% shedding at a virus titer of 1.3 ± 0.19 EID_{50} or 100% shedding at a titer of 2.5 ± 0.21 EID_{50} in the groups G1, G2, and G3 at 7 dpc, respectively. At 10 dpc, the groups G1 and G2 showed nondetectable shedding in both tracheal and cloacal swabs, whereas the group G3 showed 100% shedding with a titer of 1.5 ± 0.11 EID_{50} in the cloacal swabs vs. nondetectable shedding in the tracheal swabs. All birds in the group G4 showed nondetectable shedding throughout the monitoring periods, as shown in Table 3; Figures 5 and 6.

DISCUSSION

In the Middle East, Africa, and Asia regions, GVII was associated with a vast majority of recently reported outbreaks (Cho et al., 2007; Khan et al., 2010). However, countries within those regions share common practices, including the use of commercial vaccines (prepared from GII, either live or inactivated) and similar vaccination regimes (number of doses, route of administration, combination of live and inactivated options). This results in the same kind of problem, frequent outbreaks of ND or nonoptimum control strategies, with some flocks being infected and showing virus shedding and economic losses (mortalities, drop in production), regardless of vaccination regimes that actually depend on conventional, old non–genotype-matched vaccines away from the circulating NDV in those regions (Rehmani et al., 2015).

The results obtained in the present study highlight the protection levels after experimental infection with NDV GVII virus, where both vaccination regimes showed no significant difference in mortalities and survival rates (98% protection with 2% mortalities in both the G1 and G2 groups) but differed significantly in the nonvaccinated and challenged group (group G3, 14% protection vs. 86% mortalities). At the level of developing nervous manifestations, the group G1 provided a better protection than the group G2 (2% vs. 4.1% developed nervous manifestations), whereas the nonvaccinated challenged group (G3) showed 100% nervous manifestations among survivors (7/7). The superior nervous manifestation results in the G1 group compared with those in the G2 and G3 groups may be attributed to the specificity of the developed humoral immune responses (circulating antibodies) associated with the use

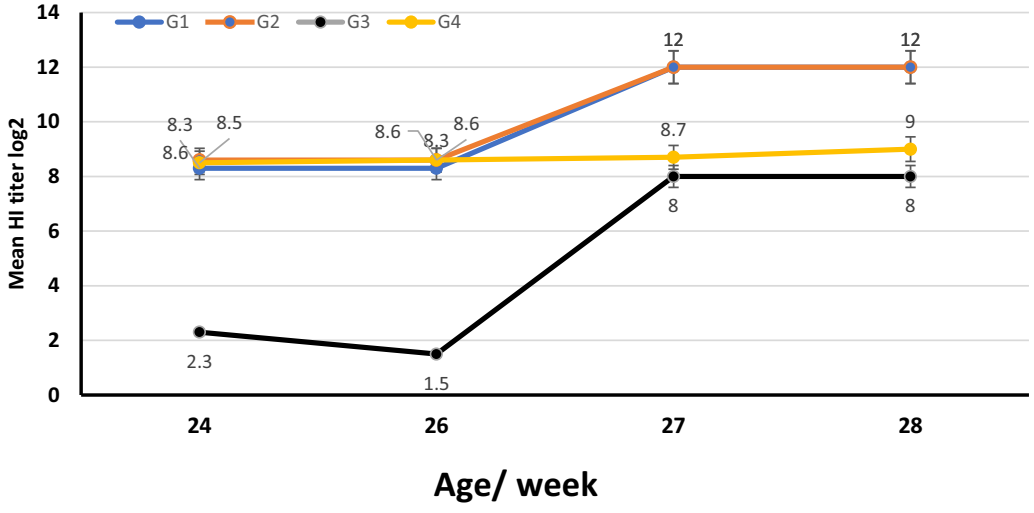

Figure 3. HI titer ± SD in vaccinated and nonvaccinated groups. HI, hemagglutination inhibition.

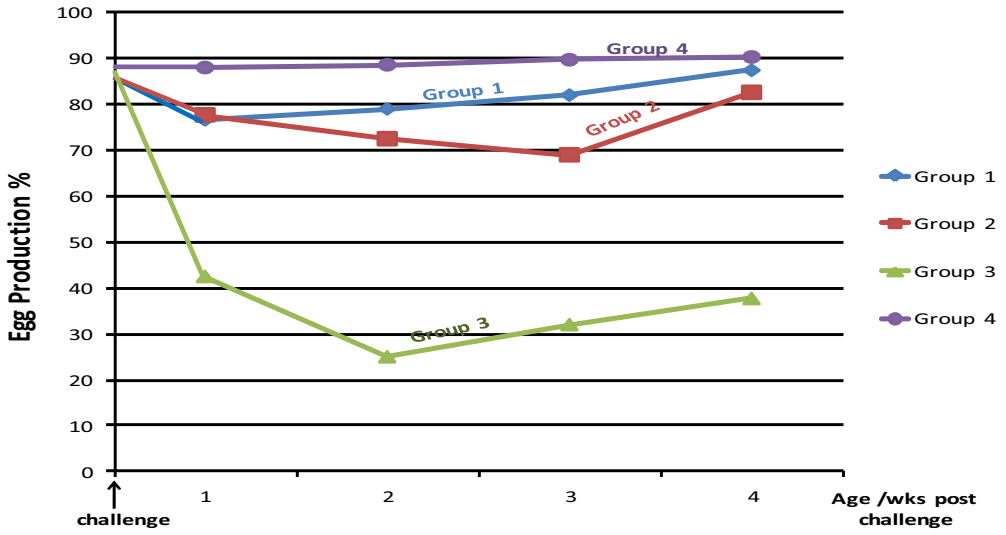

Figure 4. Egg production curve before and after challenge in vaccinated and nonvaccinated groups. Group 1: egg production percentage in birds after vaccination with live attenuated genotype II and inactivated oil emulsion NDV genotype VII vaccines. Group 2: egg production percentage in birds after vaccination with live attenuated genotype II and inactivated oil emulsion NDV genotype II vaccines. Group 3: egg production percentage in nonvaccinated birds challenged with velogenic NDV (genotype VII) "chicken/USC/Egypt/2014" at the age of 185 D. Group 4: egg production percentage in nonchallenged birds vaccinated with live attenuated genotype II and inactivated oil emulsion NDV genotype II vaccines in commercial chicken layers. NDV, Newcastle disease virus.

of genotype-matched vaccines, which also lowered the virus load in the nervous tissues. The obtained results are in partial agreement with a previous report by Nakamura et al. (2014), who showed that vaccination of layer chickens with the NDV vaccine can protect against mortalities and the development of nervous manifestations. The results also agree with those in the study by Shahar et al. (2018), who claimed that overcoming the variation between genotypes of used ND vaccines and circulating viruses (not matching) necessitates higher HI titers obtained from several vaccine doses of the non–genotype-matched vaccine (although homologous ND genotypes require lower doses). Similarly, Sedeik et al. (2018) showed significant higher protection levels against ND clinical signs and mortalities in groups vaccinated with a genotype-matched NDV vaccine (inactivated NDV GVII vaccine), than in groups vaccinated with a non–genotype-matched inactivated NDV GII vaccine challenged with velogenic NDV GVII from Egypt (Mohamed et al., 2016; Sedeik et al., 2018).

Regarding postmortem lesions in the group G3, the dead birds showed typical gross lesions including marked ataxia, resorption, hemorrhage of ovarian follicles, and rupture of the yolk that appeared infiltrated in the abdominal cavities of laying hens infected with NDV GVII virus. While the group G1 only showed signs of intestinal congestion and ulceration of cecal tonsils, the group G2 showed congestion in the oviduct and rupture of yolk. The inactivated vaccine administered in the group G2 did not have F or HN genes that match with

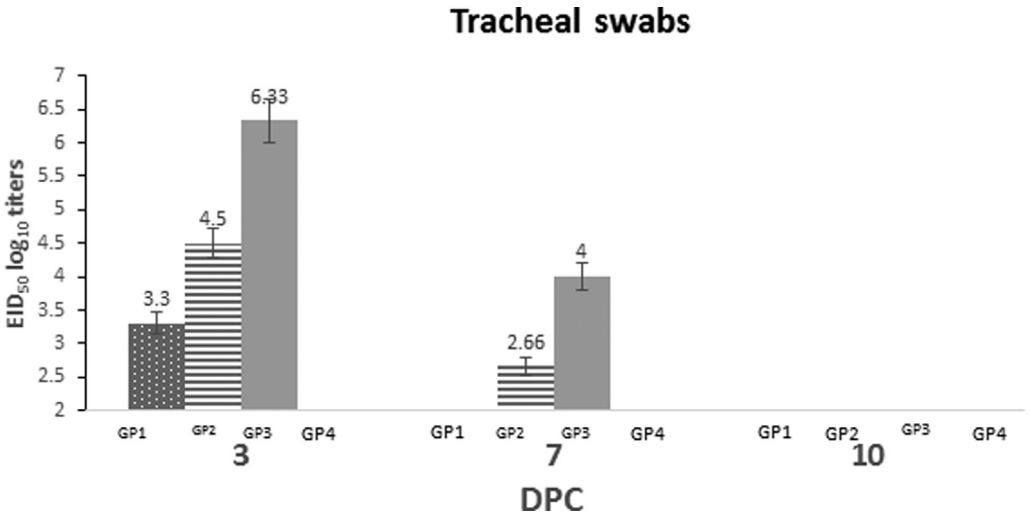

Figure 5. Tracheal shedding titers $\log_{10}$ EID_{50}/1 mL (±SD) in vaccinated and nonvaccinated layer chickens. dpc, d post challenge; EID_{50}, 50% egg infective dose.

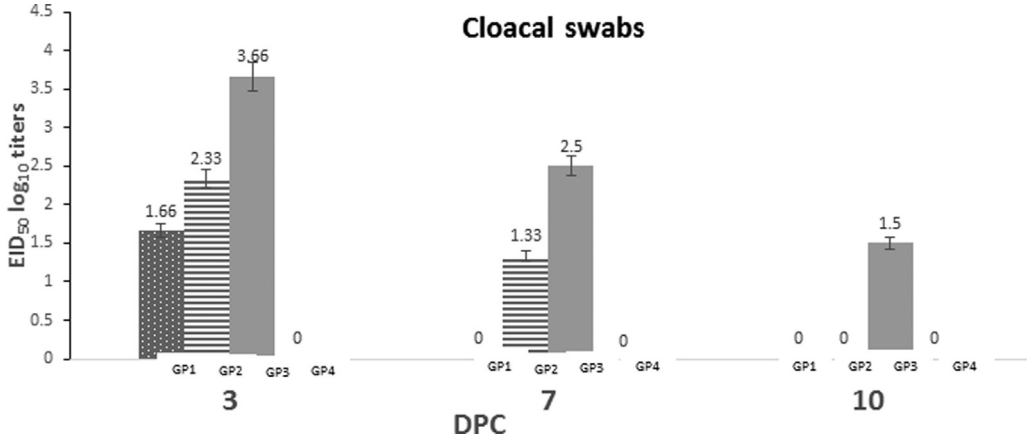

Figure 6. Cloacal shedding titers $\log_{10}$ EID_{50}/1 mL (±SD) in vaccinated and nonvaccinated layer chickens. dpc, d post challenge; EID_{50}, 50% egg infective dose.

those of the challenge virus, thus reducing the specificity of the humoral immune response and allowing more virus particles to reach the reproductive organs, inducing inflammation processes and ruptured egg lesions, which may explain the significant drop in egg production compared with that in the G1 group. As in the G1 group, the ovaries and oviduct appeared to have a lower virus load owing to the high specificity of the inactivated vaccine and its matchability with the challenge virus (Bwala et al., 2011; Absalón et al., 2019).

Serological monitoring of birds before experimental infection emphasized that HI titers in the vaccinated groups using combined multiple doses from live and inactivated NDV vaccines (3 doses of each type) can mount higher humoral immune responses (HI titers = 8.8 ± 0.61 and 8.3 ± 0.73 $\log_2$ in the groups G1 and G2, respectively, as shown in Figure 3). This level has a correlation with protection against mortalities after the challenge by the velogenic NDV, where it provided 98% protection against mortalities in both the groups G1 and G2. These data are in agreement with the previous report of van Boven et al. (2008), who found that herd immunity exists when at least 85% of the flock has specific HI titers equal to or higher than 4 $\log_2$ to NDV (when using 4 hemagglutination units per 50 µL of the antigen). Similar findings were reported by Raghul et al., 2006, who suggested that HI levels of 6 $\log_2$ or higher were typically thought of as being protective against mortalities after the velogenic NDV challenge. However, other authors reported no obvious differences in HI titers between protection against mortalities associated with the use of inactivated NDV GVII or GII vaccines when challenged with the velogenic GVII NDV (Samad et al., 2007; Shunlin et al., 2009; Kapczynski et al., 2013; Yang et al., 2017). With regard to protection against reduced egg production, considerable differences were observed based on the use of genotype-matched vaccines, demonstrating a better protective effect (in the group G1) over the non–genotype-matched group (G2) in relation to the pattern, amount of eggs produced, interval of drop, and required days to return to the normal production level (Figure 4). When the isolated virus affected egg production as in control groups, the drop ranged from 88 to 90%. In the groups G3 (nonvaccinated and challenged) and G4 (nonvaccinated and nonchallenged), drop in egg production was recorded as follows: 42.5, 25, 32, and 37.7% vs. 88, 88.6, 89.7, and 90.3% at 1, 2, 3, and 4 dpc, respectively. There was a significant downgrading in quality of eggs in the group G3 since 4 dpc, which resulted in many shell-less, soft-shelled, cracked, small-sized, and deformed eggs. Such an increased level

Table 3. Virus shedding of vaccinated commercial layers with live and inactivated vaccines against the challenge with genotype VII NDV.

	Viral shedding dpc[2]							
	0		3		7		10	
Group[1]	TR	CL	TR	CL	TR	CL	TR	CL
G1	0/5	0/5	1/5 (3.3 ± 0.21)*	2/5 (1.6 ± 0.22)*	0/5*	0/5*	0/5	0/5
G2	0/5	0/5	3/5 (4.5 ± 0.51)*	2/5 (2.3 ± 0.41)*	2/5 (2.6 ± 0.26)*	2/5 (1.3 ± 0.19)*	0/5	0/5
G3	0/5	0/5	5/5 (6.3 ± 0.82)	5/5 (3.6 ± 0.33)	5/5 (4 ± 0.56)	5/5 (2.5 ± 0.21)	0/5	5/5 (1.5 ± 0.11)
G4	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

*Significantly different from the non vaccinated challenged control at $P \leq 0.05$.

Abbreviations: CL, cloacal swab; dpc, d post challenge; NDV, Newcastle disease virus; RT-PCR, reverse transcriptase polymerase chain reaction; TR, tracheal swab.

[1]G1: genotype II live/genotype VII inactivated and challenged; G2: genotype II live/genotype II inactivated and challenged; G3: unvaccinated and challenged; G4: genotype II live/genotype VII inactivated and unchallenged.

[2]Virus shedding was tested by RT-PCR.

of egg abnormalities may be associated with a higher virus load in the oviduct, altering the calcium-binding protein D28k (CaBP-D28k), which is considered the main calcium carrier for deposition into egg shells (Li et al., 2017). Both the vaccinated and challenged groups (G1 and G2) showed reduced egg production immediately after the NDV challenge and gradually recovered to near normal at the fourth week pc, unlike the birds in the G4 group (nonvaccinated challenged group, ≤40% drop). After the NDV challenge (Figure 4), egg production dropped in the group G1 to 76.6% at the first week pc, then started to rise again to 79% at the second week pc and to 82% at the third week, and returned to the normal rate 86% (as the rate before the challenge) at the fourth week pc. In the group G2, the egg production dropped to 76.6% at the first week pc, then to 72.5% at the second week pc and to 69% at the third week pc, and then started to rise to 82.6% at the fourth week pc, but it did not reach the normal production record as that before the challenge (86%). This finding may be attributed to the level and amount of humoral immunity (specific for F and HN genes), which is probably supposed to reduce the load of the challenge virus in the reproductive organs of the birds in the group G1 compared with those in the group G2 that received non–genotype-matched "GII" inactivated vaccine. The virus load in ovaries and oviducts may be also associated with the degree of drop in egg production and interval required for tissues to heal and return to the normal production curve. These findings are supported by the previous results of the study by Palya et al. (2014), who concluded that conventional vaccines can protect against mortality caused by field NDV strains, whereas protection against drop in egg production resulting from contemporary field NDV strains has not been fully demonstrated in commercial layer chickens. However, Cho et al. (2008) reported a better protection against drop in egg production in terms of the amount, interval, and required time to return to the normal production when the GVII vaccine seed was used in comparison with the inactivated non–genotype-matched vaccine (GII), given the circulating velogenic NDV GVII. This concurs with the main finding of the study by Miller et al. (2013), who reported a better protection against egg production drop when using a genotype-matched ND vaccine (based on the circulating viruses), in comparison with the non–genotype-matched ND vaccine.

Virus shedding can be an important source of environmental contamination, which in turn acts as a mechanical transmission vector for virus spreading between poultry houses in the farm and/or between farms (Miller et al., 2009). Thus, virus shedding can be considered as an indicator for assessing vaccine efficacy. In vaccinated and challenged birds (in the groups G1 and G2), virus shedding through oral and cloacal routes was reduced significantly based on quality and quantity (number of shedders and amount of the virus shed) and the duration it took, being much shorter than that of the birds in the G3 group (Table 3; Figures 5 and 6). Notably, the genotype-matched inactivated vaccine resulted in significantly lower virus shedding by 3 dpc and the average amount of the virus shed, where the group G1 showed 20% shedding at a titer of 3.3 ± 0.21 EID_{50} vs. the group G2 showed 60% shedding at a titer of 4.5 ± 0.51 EID_{50}. These data support the view that genotype-matched vaccines (carrying F gene and HN gene of GVII or the whole GVII inactivated vaccine) can reduce virus shedding (Miller et al., 2007; Sedeik et al., 2018). Furthermore, at 7 dpc, the birds in the G1 group (vaccinated with genotype-matched vaccine) showed nondetectable virus shedding in both tracheal and cloacal swabs, contrary to the G2 group, which showed 20% shedders with detectable virus titers, which is in agreement with the results of the study by Miller et al. (2007) and Cho et al. (2008), who reported that genotype-matched vaccines for NDV can significantly reduce virus shedding and shorten the shedding interval, augmenting the preventive and control strategies for NDV in endemic areas by reducing the epidemiological load of the virus (Cho et al., 2008; Kapczynski et al., 2013; Miller et al., 2013; Kim and Samal, 2017; Liu et al., 2017).

In this study, the birds in the G1 group (vaccinated with genotype-matched inactivated vaccine) showed better protection at the level of clinical manifestations, postmortem lesions, nervous manifestations, and egg production (amount, quality, and interval to return to the normal level), in addition to the reduction in virus shedding (quantity and the number of shedders and shedding interval). In comparison with the group G2, these differences may be related to the matching of F and HN genes in the vaccine used in the G1 group with the challenge virus, reducing the virus load in the ovary and oviduct, nervous tissues, and systemic organs by virtue of a highly specific humoral immunity after 3 doses. The humoral immune response against non–genotype-matched vaccines (GII) does not have the same specificity for F and HN genes, probably resulting in lower levels of virus neutralization and in turn more severe lesions in birds.

The results of the present study explain and support some of the previous reports that explain, in part, why ND outbreaks occur in commercial poultry farms despite several vaccinations, as claimed by many countries. It is believed that higher HI titers are required to prevent infection. In addition, the closer the degree of homology between the virus outbreak and used vaccine seed (affecting the level of specific humoral immune antibodies against F gene and HN gene of the challenge virus), the better the protection of internal organs after viremia and the lower the virus load in the ovaries, oviduct, and brain (Miller et al., 2007; Mohamed et al., 2016; Liu et al., 2017; Ji et al., 2018; Shahar et al., 2018). However, further studies are needed to confirm the point of the viral load in those organs. Concerning ND protection, the results of this study indicate that use of the homologous genotype that matches with the circulating NDV GVII improves the results and provides a significant reduction in the number of shedders and level of virus shedding (Absalón et al.,

2019). They also reported that antigenic differences are likely to increase the circulation of viruses in farms and the surrounding environment, thus leaving poorly vaccinated or immunocompromised birds susceptible to clinical disease. As antigenic variation takes time and resources to be determined, scientists relied on the analysis of evolutionary relatedness and comparisons between the predicted amino acids in the main surface protein (F and HN genes) of the NDV vaccine seed (Absalón et al., 2019). Furthermore, to achieve significantly enhanced control strategies for NDV in endemic countries, there is a critical need to highlight the value of proper biosecurity measures, in addition to introducing a vaccination strategy based on genotype-matched vaccines rather than using the old conventional non–genotype-matched vaccines, or at least considering vaccines carrying F and HN genes that match with those of the circulating virus. This may ensure significant higher antigenically related antibodies than those in commercial NDV vaccines. Application of this strategy may improve the protection at the level of egg production (drop in number, downgraded quality, and interval required to return to the normal level or close to the normal level), in addition to a significant reduction of virus shedding, which will be translated into lower epidemiological and environmental load of the virus. Proper control of NDV in endemic countries will hopefully reduce opportunistic challenges encountered in poorly vaccinated animals (Rehmani et al., 2015; Cardenas-Garcia et al., 2015; Pandarangga et al., 2016; Dimitrov et al., 2017).

CONCLUSION

The results of this study indicate that the circulating velogenic NDV in Egypt still belongs to GVII and is associated with severe losses, regardless of heterologous conventional genotype II or I vaccines used in commercial egg-laying layers. Use of the genotype-matched inactivated (recombinant GVII) NDV vaccine instead of the genotype-mismatched (GII) NDV can improve egg production performance, shorten time required to restore the egg production to the normal level before infection, induce significant reduction in NDV shedding, and protect against mortalities in commercial egg layers exposed to GVII virulent NDV infection.

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Ethical approval: All animal studies and procedures were carried out in strict accordance with the guidelines and regulations of animal welfare and health. As part of this process, the work was approved by the Ethics Committee of the Faculty of Veterinary Medicine, University of Sadat City, Egypt.

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