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Evaluation of protection efficiency of different vaccination programs against velogenic Newcastle disease virus in broiler chickens: Comparative in field and in laboratory studies

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Original Article

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Abstract

This study was conducted to evaluate the protective efficiency of different vaccines and vaccination programs against Newcastle disease virus (NDV) in chickens, in the field and the laboratory. In the field, three commercial chicken farms (LA, LB, and QC) used different types of live vaccines and vaccination programs via drinking water (DW) were serologically estimated by hemagglutination inhibition (HI) assay to detect the level of circulating antibodies against NDV. The laboratory study was carried out to assess whether the types of vaccine and their route of administration influence the HI level and viral shedding. Chicken groups (G1-G5) except for the control were challenged with velogenic NDV genotype VIIj circulating among vaccinated chickens in Egypt. The protection efficiency was evaluated by HI test pre-and post-challenge, and the virus shedding post-challenge was quantitated by real-time RT-PCR. The results of the field study indicated that although LA and QC farms showed clinical signs accompanied with high mortality after the 2nd (HI=$2^{2.1}$) and 3rd (HI=$2^{3.2}$) vaccination doses, respectively, and velogenic NDV destroyed chickens in these farms, the LB farm showed no deaths with apparently healthy birds (HI=$2^{3.3}$). In the laboratory inactivated and alternative vaccinations have the highest protection HI titer with no virus shedding while live vaccination either in DW or eye drops showed low protection and the virus was detected in chickens from the DW live vaccinated group. In conclusion, the currently used in field vaccination programs and their routes of administration should be reconsidered to combat NDV infection.

Keywords: Vaccines, Chickens, NDV, Real time RT-PCR, HI assay

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Introduction

Newcastle disease (ND) represents a seriously hazardous disease to poultry investments worldwide because of its high morbidity and mortality rates (up to 100%) among infected birds, impaired body weight gain and decreased egg production (Alexander 1997; Alexander *et al*., 2012; Hines and Miller 2012). ND is caused by the ND virus (NDV) or *avian paramyxovirus-1* which was recently reclassified as *avian orthoavulavirus-1* (AOAV-1), *Avulovirinae* sub-family, *Paramyxoviridae* family in the order *Mononegvirales* (ICTV 2019). Several genotypes of NDV have been distributed among domesticated and wild birds and genotype VIIj is the most predominant among the vaccinated chickens (Radwan *et al*., 2013; Abdel-Glil *et al*., 2014; Hassan *et al*., 2016; Fuller *et al*., 2017).

Over the last few decades serious outbreaks have been reported among vaccinated chickens all over the world (Kapczynski and King 2005; Fuller *et al*. 2017). In Egypt, despite the application of routine prophylactic measurements via intensive vaccine programs, where the ND is endemic, ND outbreaks frequently occur (Radwan *et al*., 2013; Abdel-Glil *et al*., 2014; Nabila *et al*., 2014; Hassan *et al*., 2016). The question arises as to why a massive vaccination program cannot protect chickens against NDV infection and why the current vaccination regimes do not effectively prevent the clinical disease and virus shedding.

The factors that may impair antibody responses are the immune status of the bird, environmental conditions of the farm and the level of maternal immunity (Wajid *et al*., 2018). In addition to these, the failure of the existing vaccine strains to produce protective antibodies due to the genetic divergence between the circulating strain and the vaccine strain and the possibility of lower virus titer in live vaccines due to heat instability (Yi *et al*. 2011), retarded biosecurity and vaccination strategy and their routes of administration (Martinez *et al*., 2018). Vaccination is still the most effective method to combat ND distribution among chickens.

The current research study was conducted to compare the protective efficiency of the applied vaccines and the vaccination strategies under field and experimental conditions. Also, to elucidate the possible factors that may affect the vaccination protection for chickens against NDV.

Materials and Methods

The field study: Three commercial broiler farms located in Luxor and Qena governorates (designated as LA, LB, and QC) were investigated for the immunological status of the vaccinated chickens against NDV. The farm information included location, capacity, bird strain, type of vaccine used, schedules and routes of vaccination are shown in Table (1). The representative serum samples (n=50) were collected from each farm based on the schedules of vaccine administration and the HI titer was determined against the NDV La Sota strain antigen according to OIE (2018). The reference antiserum for NDV (GD lab., Holland) was used as a positive control for the HI test.

Table 1 Data from the vaccinated broiler chicken farms

Farm name [a]	LA	LB	QC
Farm capacity	5000	4000	5000
Bird strain	Sasso	Balady	Sasso
Type of flock	Broiler	Broiler	Broiler
Type of vaccine	Live	Live	Live
Schedule of vaccines [b]	8th (Hitchiner B1) 19th (La Sota N-79) 29th (La Sota) [c]	7th (La Sota) 17th (La Sota) 27th (La Sota)	7th (Hitchiner B1) 19th (La Sota) 29th (La Sota) 33rd (La Sota)
Administration route	DW [d]	DW	DW
Location	Luxor	Luxor	Qena

[a] LA and LB stand for Luxor farms A and B; QC stands for Qena farm C
[b] Serum samples were collected before vaccine administration
[c] Not applied outbreak increased with mortality rate from 10% -30%.
[d] DW = drinking water

The laboratory study

The birds: Before purchasing the chicks for the experimental study, poultry houses were cleaned and disinfected by adopting standard disinfection protocols. A total of 180 one-day-old broiler chicks (Sasso-hybrid) were purchased from known sources (Ahem Ghnnnam Breeder Company) and random cloacal swabs and droplets were collected for examination for NDV, and avian influenza virus (AIV) before starting the study. Later on, the chicks were grouped randomly into 6 groups (G). G1-G4 received different vaccination programs against ND (Sultan *et al*. 2016; Hassan *et al*. 2019), while G5 and the control (G6) were unvaccinated groups, as shown in Table (2). All bird groups were kept isolated and strict biosecurity measures were provided. The groups were littering reared and supplied with feed and water ad-libitun during the experiment. The study lasted for a period of seven weeks.

Challenged virus and Vaccines: The challenged NDV (EG/CK/NDV/Luxor/2012) was isolated in 2012 from an outbreak in a vaccinated broiler farm in Luxor, Egypt, and the nucleotide sequences of F and HN

genes were registered in GenBank under the accession numbers MN381174 and MN381175, respectively. The virus was propagated and titrated in 9-11 day old embryonating chicken eggs that were free from maternal antibodies against ND and stored at -70°C until use. The virus challenge dose (10^5EID50/0.2ml/bird) was calculated based on the method described by Reed and Muench (1938). All vaccinated groups as well as G5 were challenged by the EG/CK/NDV/Luxor/2012 via ocular and oral routes at a dose of 0.1 mL/route.

The different NDV live vaccines used in field farms are illustrated in Table (1). Also, different live and inactivated NDV vaccines used throughout the experiment were purchased from local dispensers Table (2) and supplementary table (S1) contain all vaccines details (name, type, batch no., titer, dose, route, manufacturer). The vaccines were stored and diluted according to the manufactures' instructions. Live vaccines were administered via drinking water (DW) or eye drop instillation, while the inactivated vaccines were administered via intramuscular injection in the breast muscle.

Table 2 Time course of the experimental vaccination study

Chicken groups [a]	Vaccination timetable				Program type	Virus challenge
	7th	10th	17th	28th		35th
G1 (n=40)	La Sota N-63 (Live)		Clone 30 (Inactivated)	(La Sota N-79) (Live)	Alternative vaccination	Chickens were challenged by velogenic NDV VIIj (EG/CK/NDV/Luxor/168/2012)
G2 (n=40)	La Sota N-63 (Live)	Clone 30 (inactivated)	La Sota (inactivated)	Ulster (inactivated)	Inactivated vaccination	
G3 (n=30)	La Sota N-63 (Live)		Clone 30 (Live)	(La Sota N-79) (Live)	Live (eye drop) vaccination	
G4 (n=30)	La Sota N-63 (Live)		Clone 30 (Live)	(La Sota N-79) (Live)	Live (DW)[b] vaccination	
G5 (n=20)	**Non-vaccinated chicks (control positive)**					
G6 (n=20)	**Non-vaccinated non challenged chicks (control negative)**					

[a] Live vaccines via eye drop in G1-G3 and inactivated vaccine via intramuscular injection.
[b] DW = drinking water

Table S1 Supplementary table showing details of vaccines used in the laboratory study

Name	Type	Batch no.	Titer	Dose	Route	manufacturer
La Sota N-63	live	94020045	$10^{6.5}$ EID50[a]	1000 dose	DW[c] or eye drop	Intervet Inc., (MSD) USA
La Sota N-79	live	94040053	$10^{6.5}$ EID50	1000 dose	DW or eye drop	Intervet Inc., (MSD) USA
Clone 30	live	0568H	10^{6} EID50	1000 dose	DW or eye drop	Izovac, Bresqa-Italy
La Sota (OL-VAC)	inactivated	311972	100 PD50[b]	0.5 ml/bird	I/M[d]	Fatro, Ozzano Emilia (BO) Italy
Ulster	inactivated	400594	50 PD50	0.3 ml/bird	I/M	Merial Lyon-France
Clone 30	inactivated	1332301	30≥50 PD50	0.1 ml/bird	I/M	Intervet Inc. Boxmeer-Holland

[a] EID50 = egg infectious dose 50
[b] PD50 = protection dose 50
[c] DW = drinking water
[d] I/M = intramuscular

Serum sample collection for the HI assay: Representative serum samples (n=10) were obtained from birds in each group before vaccination at 1 and 7 days old to assess the maternal antibody level by HI assay (OIE 2018). After that, the scheduled timetable was followed to collect serum samples (n=7) from each group weekly until 35 days old before performing the challenge test. Then sera were collected daily post-challenge for the period of a week.

Clinical signs, postmortem lesions and mortality rate: The mortality rate and clinical signs post-challenge were recorded daily and dead birds were subjected to postmortem examination.

Virus shedding estimation by real time reverse transcription polymerase chain reaction (real-time RT-PCR): Cloacal swabs (n=8 swabs) from randomly selected birds in each group were collected daily post-challenge from 36 to 42 days old, 8 and 14 days from last vaccination, for detection and quantification of viral shedding by real time RT-PCR method as previously described by Wise *et al*., (2004) for the identification of virulent NDVs. The swabs were collected in 1.5 ml sterile Eppendorf tubes containing phosphate buffer saline (PBS) and antibiotics and centrifuged at 5000xg / 15mins for virus clarification and the supernatant was stored at – 80°C until subsequent analysis. The RNA was extracted from the samples using a QIAamp viral RNA Min (Qiagen, USA) extraction kit following the manufacturer's instructions. Real-time RT-PCR process was performed using QuantiTect® Probe RT-PCR kit (Qiagen, USA), primers (Forward 5'-TCCGGAGGATACAAGGGTCT-3', Reverse 5'-AGCTGTTGCAACCCCAAG-3'), and probe ([FAM]AAGCGTTTCTGTCTCCTTCC-TCCA[TAMRA]) targeting 101 bp of the F gene (Wise *et al*., 2004). Briefly, the reaction mixture volume was a total of 25µl in a 0.2 ml optical tube (Applied Biosystem) containing the following: 12.5µl master mix, 0.5µl forward primer, 0.5µl reverse primer, 0.125 µl probe, 0.125 µl QuantiTect RT mix, 4.25 µl RNase free water, and 7 µl of extracted RNA template. The

reaction was conducted using a real-time RT-PCR machine (Stratagene MX3005P) with a thermal profile of RT at 50°C/30 mins for one cycle, initial denaturation at 95°C/5mins for one cycle, and 40 cycles of denaturation (95°C/20secs), annealing (52°C/30secs), and extension (72°C/10 secs).

Statistical analysis: The HI titers were expressed as the geometric means of the evaluated serum samples with geometric standard deviations. The variances among the chicken groups were analyzed by two-way ANOVA with Tukey's multiple comparisons test in Graph-Pad Prism version 8.4.2. A P value < 0.05 was considered statistically significant (*P < 0.05, **P < 0.01, and ***P < 0.001) and, a P value > 0.05 indicated no significant (ns) difference.

Ethical approval: All experiment procedures carried out on chickens were approved by the Ethics Committee of the Faculty of Veterinary Medicine, South Valley University, Qena, Egypt and care was taken to minimize the number of animals used. All serum samples were collected from farms carried out after the advance agreement of the owners.

Results

Immune response of vaccinated broiler chicken farms (field study): The geometric means of the HI titers of the vaccinated broiler chickens in the 3 evaluated farms were plotted as shown in Fig. (1). In the LA commercial farm, the HI titer was $2^{5.8}$ at one-day-old and then dropped to $2^{2.1}$ after the 1st vaccination and slightly increased to $2^{3.2}$ after the 2nd vaccination dose. After the 3rd vaccination, a few birds died with unclear PM lesions and then the mortality speedily increased to reach up to 80% of the flock (Fig. (1) and Table (1)). The collected cloacal swabs from the chickens after obvious mortality revealed NDV infection with *Ct* value 31.85 using real-time RT-PCR.

In the LB commercial farm, the sera collected from the chickens at one-day-old, 16, 26, and 40 days old showed $2^{4.6}$, $2^{2.1}$, $2^{3.2}$, and $2^{3.3}$ antibody titers against NDV before and after vaccination (Fig. (1) and Table (1)). On the other hand, in the QC farm the maternal antibodies were $2^{8.2}$ at 7 days old, 12 days after the 1st vaccination dose with Hitchiner B1, it decreased to 2^{3} and to $2^{2.1}$ a week after administration of 2nd La Sota vaccine dose (Fig. (1) and Table (1)). No serum samples could be obtained after the 3rd dose due to the farm's biosecurity measurements. Interestingly, the HI titer speedily increased 5 days after the 4th vaccination dose up to $2^{9.9}$ (Fig. (1)) and inconsistent with that increased mortality started to destroy up to 85% of chickens with the classical clinical signs (greenish diarrhea, off food, nervous manifestations, facial edema) and PM lesions (petechial hemorrhage on proventriculus, hemorrhage in cecal tonsils, intestinal ulceration) of ND, and confirmed isolation and identification of the velogenic NDV were done from the tissue samples that were collected at PM examination from the dead birds by real time RT-PCR.

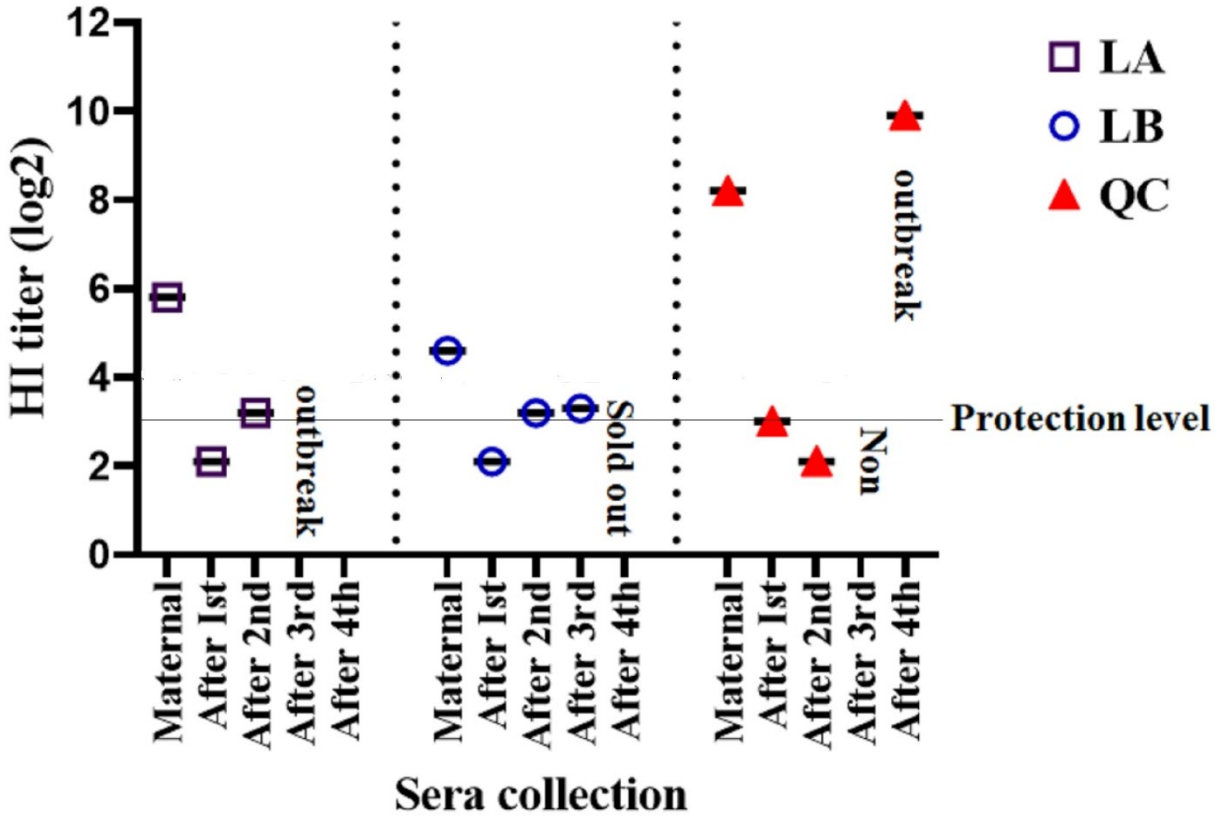

Figure 1 The calculated geometric mean of the HI titers for vaccinated broiler chickens using live vaccines via DW in the investigated farms in Luxor (LA, and LB) and in Qena (QC) shown at one day old (maternal antibodies) and after each vaccine administration. The vaccinated chickens in the LA and QC farms revealed increased mortalities after the 2nd and 3rd vaccination doses, respectively. The protection level (≥ 3log2) is shown as a horizontal line.

Assessment of HI titers of chicken groups (in laboratory) receiving different vaccination programs: The result of antibody titers in different groups are presented in fig (2). The HI antibody titers in all vaccinated groups were highly significant ($P < 0.0001$) in comparison to the control group, except for at 1 and 7 days old (Fig. 3).

By the 17th day, the highest HI geometric mean of antibody titer was observed in G2, followed by G4, while G5 and the control showed the lowest antibody titers. At 28 days old, vaccinated G1 showed the highest antibody titers among different groups as well and the titer level of this group being increased compared to those levels at 17 days old reflecting a higher protection level than G3 and G4 vaccinated with live vaccine only. While in G5 and the control group the antibody titers decreased in comparison to those determined at 17 days old and completely diminished at 28 days old.

G2, receiving inactivated vaccines, a week post last vaccination dose (35 days old), had a highly significant HI titer ($P < 0.0001$) in comparison to other vaccinated groups. Both G2 and G1 showed nearly double the HI titer of G3 and G4 at this period (Fig. (2) and (3)).

Interestingly, G3 showed a highly significant HI titer ($P < 0.0001$) in comparison to G4, although they received the same vaccines but with different administration routes (Fig. (2) and Table (2)).It is worth noting that G1 showed a marked drop in the HI titer 4 days post challenge (dpc) to reach the same level as G3 (Fig. (3)).

The chickens receiving inactivated vaccines (G2) showed a steady HI titer curve, although it was slightly decreased 3 dpc (38 days old). In contrast, the birds that received live vaccines via DW (G4) showed a sudden transient increase in HI titer at the same time period. Also, this group showed a steady increase in HI titer 4 - 7 dpc to overtake birds receiving the same vaccination program via eye drops (G3), (Fig. (3) and Table (2)). Surprisingly, the HI titers of G3 and G4 remained under the level (≤ 4log2) even post-challenge (6 days post challenge for G3), as shown in Fig. (3).

The challenged non-vaccinated birds (G5) showed a prompt increase in the HI titer 3 dpc (Fig. 3), which was accompanied by increased disease severity and fatality. In contrast to this, the non-challenged non-vaccinated chickens (the control group) remained in a sound condition with no HI titer since maternal antibodies had been diminished at 28 days old till the end of the experiment.

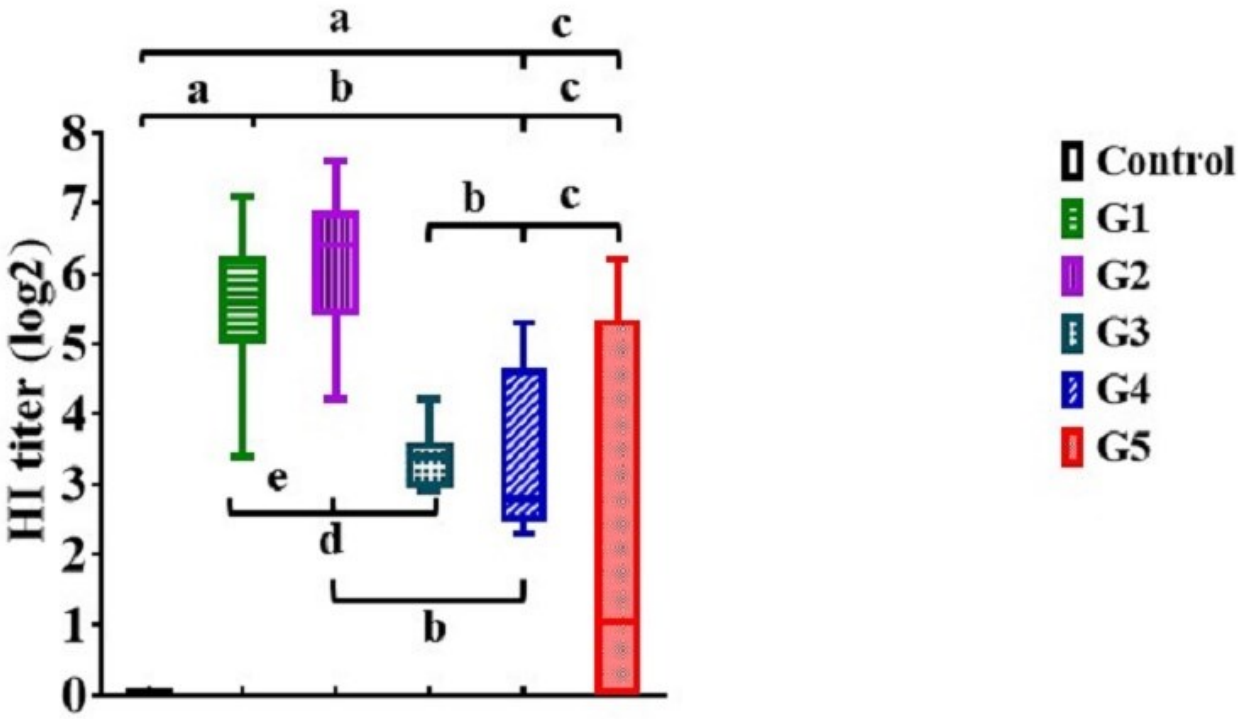

Figure 2 The graph shows the statistical relationship among the chicken groups: G1 (alternative vaccines), G2 (inactivated vaccines), G3 (live vaccines via eye drop), G4 (live vaccine via DW), before and after vaccination and daily post-virus challenge. The G5 non-vaccinated, challenged chickens and the control group (non-vaccinated, non-challenged chickens) regarding HI titers and timing by two-way ANOVA with Tukey's multiple comparisons test in Graph-Pad Prism version 8.4.2. The groups were highly significantly (a, b, c, d and e) different (P ≤ 0.0001) from 17 days old until the end of the experimental period.

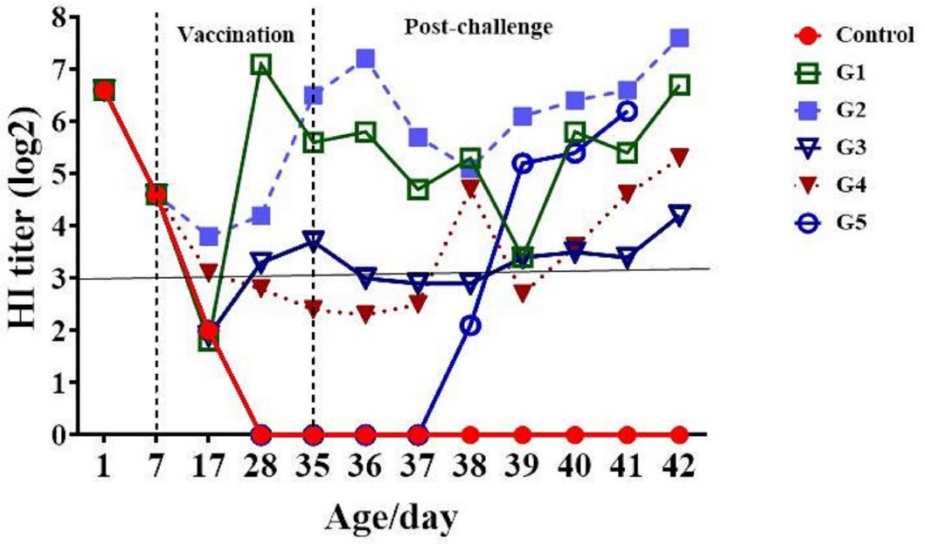

Figure 3 Monitoring of the geometric mean of the HI titers among the chicken groups (G1-G5 and the control): G1 (alternative vaccines), G2 (inactivated vaccines), G3 (live vaccines via eye drop), G4 (live vaccine via DW), before and after vaccination, and daily post-virus challenge. The G5 non-vaccinated, challenged chickens and the control (non-vaccinated, non-challenged chickens) group. The protection level (≥ 3log2) is shown as a horizontal line.

Molecular evaluation of the virus shedding after virus challenge in different groups: The investigation of virus shedding among the vaccinated chicken groups as well as G5 and the control groups was conducted by real-time RT-PCR. All examined cloacal swabs collected from the chicken groups daily after virus challenge showed no viral shedding for G1, G2 and G3 as well as the control group up to 7 dpc (Table (3)). On the other hand, in G5 after detecting HI antibody titer, 3dpc, the *Ct* values were detected, 4dpc (Fig. (3) and Table (3)) where the classical clinical signs and mortality were recorded among birds in this group. Later on, the *Ct* values decreased, indicating a high virus load congruent with the increased severity of clinical signs, mortalities and HI titers (Fig. (3), Table (3)). By 7 dpc, almost all the birds had died in G5 with a mortality rate of 90%, only two birds were alive up to 14 dpc with classical clinical signs of NDV, after that they were euthanized where PM lesions of NDV were obvious. Interestingly, the *Ct* values of 27.27 and 29.7 from an individual bird in G4, receiving live vaccine via DW, were recognized at 6 and 7 dpc with HI titers of $2^{4.6}$ and $2^{5.3}$, respectively, (Table (3), Fig. (3)).

Table 3 Real-time RT-PCR results of the shedding virus from obtained samples from various bird groups at 1 -7 days post-challenge (dpc).

Groups	Positive samples/examined samples (*Ct* value)							Virus shedding
	1dpc	2dpc	3dpc	4dpc	5dpc	6dpc	7dpc	
G1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	ND [a]
G2	0/8	0/8	0/8	0/8	0/8	0/8	0/8	ND
G3	0/8	0/8	0/8	0/8	0/8	0/8	0/8	ND
G4	0/8	0/8	0/8	0/8	0/8	1/8 (27.72) b	1/8 (29.07)	Virus detected
G5	0/8	0/8	0/8	8/8 (32.03)	8/8 (30.62)	8/8 (31.69)	8/8 (28.17)	Virus detected
Control	0/8	0/8	0/8	0/8	0/8	0/8	0/8	ND

[a] ND means not detected
[b] the cycle threshold (*Ct*) value of real time RT-PCR shown in brackets

Discussion

The ND economic losses and costs related to preventive measurements raise the need for evaluating the type of vaccines and the efficacy of vaccination programs used in controlling NDV in Egypt. Therefore, the question arises whether the currently used vaccines under different vaccination schemes in field induce effective immunity in chickens against the virulent NDV, which reflects back into mortality rates, clinical signs, postmortem lesions, level of antibody titer and viral shedding.

In this study, 2 (LA and QC) out of the 3 broiler chicken farms that used live vaccination programs through DW had ND outbreaks, which has been confirmed by molecular and biological identification methods. Also, the level of HI titer reached up to $2^{9.9}$ in the QC farm, which contributed to viral infection. Conversely, to the LA farm, all chickens passed to the market in the LB with HI titer, after the 2nd and 3rd vaccination doses ($2^{3.2}$ and $2^{3.3}$, respectively) similar to HI titer ($2^{3.2}$) of chickens in LA farm. Although the 2 broiler chicken farms, LA and LB, on follow-up, had optimal protection levels of HI titer $2^{3.2}$ and, $2^{3.3}$,

respectively, (Sultan *et al*., 2016), ND outbreaks occurred in LA only, which may be attributed to the presence of immunosuppressive (Njagi *et al*., 2012) factors such as co-infection with other pathogens as AIV H9N2, infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV), fowl adenovirus (FAdV), chicken anemia virus (CAV), infectious laryngotracheitis virus (ILTV), *Escherichia coli* or Mycoplasma (Hassan *et al*., 2016; Sultan *et al*., 2016; Gowthaman *et al*., 2017; Gowthaman *et al*., 2019). In addition to this, the genetic resistance of local chicken breeds plays a role in minimizing and resisting the infection by NDV with or without improving the humoral immune response (Hassan *et al*., 2004; El-Tarabany 2019). This is congruent with the results that although LA and LB farm chickens had the same HI titers but of different bird breeds, only LB farm chickens could remain apparently healthy without NDV mortality. The outbreak occurring in the QC farm has been attributed to sub-optimal HI titer ($2^{2.1}$). The results revealed that commercial attenuated live ND vaccine, especially in DW, cannot provide satisfactory protection against NDV and this agrees with Yu *et al*., (2012). This result is in agreement with Van-Boven *et al*., (2008) and Ashraf and Shah (2014), who revealed that the in-field outbreaks of ND and the re-infection of susceptible birds with NDV have occurred in flocks with lower immunity despite intensive vaccination with various live vaccination schedules. In addition to the immunosuppressive or co-infection agents such as low pathogenic AIV H9N2 which has a detrimental effect on the HI titer and exaggerates the clinical signs in infected birds vaccinated with live NDV vaccines (Ellakany *et al*., 2018), several individual birds showed HI titer less than 2^1, which puts them at risk of NDV infection due to inadequate neutralizing antibody titer. Also, the laboratory study revealed that a live vaccination program via DW did not fully protect the chickens, as individual chickens were infected after virus challenge and virus shedding was detected at 6 and 7 dpc by real time RT-PCR. This result is consistent with previous studies that indicated that live vaccines via DW did not provide sufficient protection to chickens against NDV (Alexander *et al*., 2004; Degefa *et al*., 2004). Meanwhile, infection could not be detected in that group receiving the same live vaccination program through eye drops, which could be because of the specific local antibodies secreted by stimulated plasma cells in the Hadrian gland (Dohms *et al*., 1988; Jayawardane and Spradbrow 1995). The in-field and laboratory studies revealed that the live vaccine application via DW provided a poor HI titer. This is due to the fact that to none of the birds took the optimum amount of vaccine; live vaccines may be affected by environmental conditions such as high temperature in tropical regions, exposure time and the water quality used for vaccine reconstitution, which affects live vaccine viability and stability(Alexander *et al*., 2004).

Therefore, the basic hypothesis of using different vaccination regimes in the in laboratory study was that the efficacy of live vaccines administered via DW commonly used in the broiler farms in our country could be improved by subsequent administration of inactivated vaccines using different vaccination programs (inactivated and alternative). The commonly used vaccines for controlling NDV based on the lentogenic strains (La Sota, B1, Ulster) and the effective dose of live vaccines (10^4 – 10^5 EID50) that conveys 100% protection under laboratory conditions do not prevent challenge virus infection and replication. However, La Sota derived vaccines of 10^6 EID50 or higher induce efficient HI titer able to prevent or strictly minimize the challenge virus replication (Kapczynski and King 2005; Cornax *et al*., 2012; Cardenas-Garcia *et al*., 2015). The high level of maternal antibodies at the time of vaccination adversely affects the protection derived from a primary or booster vaccination against NDV(Martinez *et al*., 2018). The maternal antibodies can impair the vaccine efficiency though preventing vaccine strain replication and immune response, inhibiting the B cell differentiation into antibody producing plasma cells or masking epitopes, neutralizing and enhancing phagocytosis of virus particles enter blood stream (Hu *et al*. 2020). So, the vaccination programs under laboratory conditions were applied at 7 days old after maternal antibodies had declined and all selected live vaccines in this study were 10^6 EID50 or higher as shown in table (S1).

The birds in all groups were primed with live ND vaccine at 7 days old after the maternal antibody titer dropped to avoid interference with the vaccine, enhance instant protection as well and induce local mucosal immunity (Martinez *et al*., 2018). The decline in antibody titer recorded at 17 days of age in G1, G3, and G4 ($< 2^3$) is explained by the live vaccine being neutralized by remaining maternal antibodies. While in G2, the antibody titer was steadily more stable throughout the experimental course than others, because of the use of inactivated NDV vaccine as a primary or booster vaccination inducing efficient and sustained immunity for a long time and not interfering with maternal immunity as a live vaccine (Chansiripornchai and Sasipreeyajan 2006).

G2 had the highest geometric mean of antibody followed by G1; this indicates a better immune response to NDV. A combination of live and inactivated oil adjuvant ND vaccine is recommended for endemic areas because vaccine combination is known to promote a better immunological protection than administration of only a single live vaccine (Chansiripornchai and Sasipreeyajan 2006). This high antibody response obtained with killed and live vaccine given simultaneously is due to that the live virus replicating quickly in the mucosal membrane of the conjunctiva and nostrils and eliciting a primary immune response. This is followed by a continuous slow release of the killed virus antigen being trapped in the oil medium, thus allowing the killed virus antigen trapped to behave like a booster dose (Chansiripornchai and Sasipreeyajan 2006; Folitse *et al*., 1998).

By the end of the experiment (7 dpc) chickens in G1 and G2 using alternative and inactivated vaccinations showed a geometric mean of HI titer > 2^6. While HI titers for G3 and G4 received only live vaccine by eye drops and DW, respectively, remained lower ($\leq 2^{2.5\text{-}4}$ in G3 and G4). This finding is also in agreement with the FAO (Alexander *et al*., 2004) notification which

concluded that killed ND vaccine induced more effective and long-term immunity than live ND vaccine La Sota.

Concerning viral shedding, all groups were protected against viral shedding except for G4, where it occurred due to the homogenized high antibody titer in G1, G2, and G3 that may have been responsible for non-detectable virus shedding compared with G4. Controversially, Sedeik *et al*., (2019) reported virus shedding and clinical signs in all chicken groups receiving heterologous or homologous inactivated vaccine 2 and 3 weeks post-challenge with HI titer < 2^4 before challenging the vaccinated chicken groups. This is due to the higher maternal antibody ($2^{7.9}$) at time of vaccination and the lack of a booster vaccination dose. The lowest HI titer permitted the virus to replicate and shed within the fecal samples and the birds in this group (G4) were not fully protected against infection (Reynolds and Maraqa 2000; Jalil *et al*., 2009). Also, at 3dpc the HI titer was $2^{4.7}$ in G4 and increased by up to $2^{5.3}$ at 7dpc which can be counted towards the infection of NDV which was confirmed by the detection of *Ct* value of individual birds in this group by real-time RT-PCR at 6 and 7 dpc.

In conclusion, the results of our study point out that the commonly used vaccination programs in-field should be reconsidered in both the type of vaccine and the route of its application among commercial chicken broiler farms. The use of alternative and inactivated vaccine programs induces high HI titers with non-detectable viral shedding. The application of alternative and inactivated vaccine programs in endemic areas has the potential to provide not only good protection but also to reduce dissemination of virulent NDV. Therefore, it is necessary to pay great attention to the types of vaccines, schedules of vaccination and application processes to obtain an efficient protection level for birds against NDV.

Conflict of interest: All the authors declare that they have no conflicts of interest and they did not receive any financial support to do this research.

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