

Evaluation of Two Inactivated Newcastle Disease Virus Vaccines (Genotype II and VII) Against Challenge of Newcastle Disease Genotype VII Infection in Chicken

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Abstract: Despite massive Newcastle Disease (ND) vaccination programs in Egypt several ND outbreaks were recorded since mid-2010 causing high mortalities (20-80%). The ND genotype VII Virus (vNDV) was the causative agent highlighting the need for evaluation of the efficacy of currently available and/or newly developed ND vaccines. In this study, the effectiveness of two inactivated NDV vaccines “a virulent LaSota-like (NDV/CK/Egypt/11478AF/11) (genotype II) and vNDV, (NDV/CK/Egypt/567F/12) (genotype VII) strains were evaluated. The two NDV inactivated vaccines were prepped using 350 HAU/ dose and adjuvanted with Montanide ISA 70®. Post-Vaccination (PV) antibody response was monitored on weekly basis using Hemagglutination Inhibition (HI) test against both vaccinal antigens and the vaccine potency was tested at 4 weeks PV using 10⁶ EID₅₀/bird of vNDV (genotype VII) strain via oculonasal route. The vNDV inactivated vaccine induced significantly higher and earlier antibody titer than the LaSota-like strain inactivated vaccine by 2 weeks PV. The LaSota-like vaccinated birds showed mild clinical signs by 2 Days Post Challenge (DPC) and 20% mortality rate. In contrast, the vNDV vaccinated birds did not show any clinical signs or mortalities. Primarily, the vNDV strain vaccine reduced virus shedding compared to the LaSota-like strain vaccinated birds that showed virus replication with relatively high titers up to 6 DPC. In conclusion, superior protection against vNDV can be achieved using the closest vaccine seed to the circulating field isolates considering the induction of higher and earlier immune response, protection against morbidity and mortality and reduction of the challenge virus shedding.

Key words: Chicken, inactivated vaccine, NDV Newcastle Disease Virus, DPC, ND

INTRODUCTION

Newcastle Disease (ND) causes serious problems and high economic losses (mortalities may reach 100%) for poultry production and increase trade restraints worldwide. ND was first described in 1926 in Java, Indonesia and in Newcastle-Upon Tyne, UK, though there have been some suggestions that there may have

been earlier outbreaks and was characterized by rapid spread during ND epizootics in susceptible poultry (Swayne and Halvorson, 2008).

Newcastle Disease Virus (NDV) is an Avian Paramyxovirus serotype 1 (APMV-1) belongs to the genus Avulavirus, subfamily Paramyxovirinae, family Paramyxoviridae (Lamb *et al.*, 2005). NDV viral genome is approximately 15 kb is composed of 6 genes encoding 6

structural proteins; Fusion (F), Nucleoprotein (NP), Matrix (M), Phosphoprotein (P), RNA polymerase (L) and Haemagglutinin-Neuraminidase (HN). The cleavability of protein F is the main determinant for viral virulence but other proteins such as HN, V and L proteins are also believed to influence pathogenicity (De Leeuw *et al.*, 2003; Mebatsion *et al.*, 2002). NDV isolates are classified into two classes and several genotypes based on restriction site mapping, genome length and F gene sequences (Ballagi-Pordany *et al.*, 1996; Czegledi *et al.*, 2006).

NDV is further classified into several pathotypes (asymptomatic enteric, lentogenic, mesogenic, viscerotropic or neurotropic velogenic) (Kattenbelt *et al.*, 2006). The OIE definition for virulent NDV depends on following criteria; the presence of multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117 at the N-terminus of the F1 protein or the virus has an Intracerebral Pathogenicity Index (ICPI) in day-old chicks of ≥ 0.7 (Kattenbelt *et al.*, 2006).

In Egypt, NDV was identified for the first time in 1948 (Daubney and Mansi, 1947). Since then, Egypt became an endemic country and subsequently an intensive mass vaccination program against NDV has been practiced in all poultry production sectors. During last 3 years, severe ND outbreaks and ND vaccine failure were recorded in Egypt. These outbreaks were attributed to circulation of newly discovered genotypes associated with increased virulence or expanded host range (Wan *et al.*, 2002; Zanetti *et al.*, 2008). Based on several reported field outbreaks, regardless of intensive vaccination, the concern of ND vaccination failure and capabilities of the commonly used commercial ND vaccines to achieve the accepted protection levels and to limit virus shedding against the different genotypes become an issue that require deep investigation (Miller *et al.*, 2013).

Also, the role of genetic and antigenic variability as drivers behind ND vaccine failures were discussed (Maas *et al.*, 2003) and it was suggested that homologous vaccines to the challenge viruses genotype capable of reducing viral shedding should be a critical component of disease control and vaccine evaluation studies (Hu *et al.*, 2011). The current study was planned to evaluate effectiveness of two ND inactivated vaccines prepared from LaSota-like and vNDV (genotype VII) isolates in SPF chickens against challenge with vNDV genotype VII.

MATERIALS AND METHODS

Viruses and vaccines: Two NDV viruses were selected to represent the recent circulating avirulent NDV and

velogenic NDV (vNDV) in Egypt in 2011 and 2012, respectively. The strain (NDV/CK/Egypt/11478AF/11) is avirulent LaSota-like NDV strain and the other isolate was velogenic (NDV/CK/Egypt/567F/12) (vNDV) strain. Both viruses were isolated at the Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Animal Health Research Institute, Dokki, Giza, Egypt. The identity and purity of both viruses were approved by RQLP (data not shown). Genetic characterization of both viruses was conducted by RT-PCR using specific oligonucleotide primers for the F gene (Radwan *et al.*, 2013). Phylogenetic and molecular evolutionary analyses were conducted through a bootstrap trial of 1000 using MEGA version 6 (Tamura *et al.*, 2013). Cross-HI test using both strains and corresponding antisera was carried out simultaneously. Antigenic relationship were calculated using Archetti and Horsfall formula (Archetti and Horsfall, 1950).

Vaccines formulation: Three different vaccine batches were prepared from each strain at Middle East for Veterinary Vaccine (ME VAC) Company. The two NDV viruses were propagated using 10 days Specific Pathogen Free (SPF) Embryonated Chicken Eggs (ECE). The harvested allantoic fluid was inactivated after sterility testing using 0.02% formalin (Sigma, St. Louis, MO) at 37°C for 18 h. The inactivation completion was then confirmed by 3 successive passage in 10 days old Specific Pathogen Free (SPF) embryonated chicken eggs via allantoic sac inoculation. The inactivated allantoic fluids were then mixed with MONTANIDE™ ISA 70 VG adjuvant (SEPPIC® SA, France) in a ratio of 70:30 of adjuvant: aqueous antigen and prepared as an emulsion. Both NDV and vNDV contain 10^8 /mL EID₅₀ titers/dose. The three different vaccine batches were designated as follow; Vaccine A-inactivated NDV LaSota-like vaccine batches (A1, A2 and A3). Vaccine B-Inactivated vNDV vaccine batches (B1, B2 and B3).

Animal experiment: One hundred twenty SPF chickens of 2 weeks old were allotted into 8 groups (15 bird/group). Groups I-negative control unvaccinated 15 SPF chickens, group II-unvaccinated challenge control positive group. The birds in groups III-IV were vaccinated via subcutaneous route with 0.5 mL/dose according to type of vaccine used. Group III (A1, A2, A3) were inoculated with A1, A2 and A3 vaccines respectively. Group IV (B1, B2, B3) were inoculated with B1, B2 and B3 vaccines, respectively. Sera were collected on weekly bases for 4 weeks Post Vaccination (PV).

Hemagglutination Inhibition (HI) test was carried out according to the World Organization for Animal Health manual to monitor the post-vaccination humeral immune response for each vaccine batches using the homologous ND antigen. Testing with heterologous ND antigen was conducted on the sera collected from the groups selected for challenge (A3 and B3). Briefly, two-fold serial dilutions of sera were mixed with 4 Hemagglutination (HA) units of each virus. The HI reactivity was determined using a 1% suspension of chicken red blood cells.

Challenge experiment: The challenge experiment was conducted in biosafety level III chicken isolators at the laboratory animal facility at Middle East Company for Veterinary Vaccine (MEVAC). The experiment was conducted according to the MEVAC guidelines on research ethics in animals. Fifteen SPF chickens of the groups II, III (A3) and IV (B3) were challenged at 4 weeks PV with 10^6 EID₅₀/0.1 mL of virulent NDV/CK/Egypt/567F/12 via ocular-nasal route. Other 15 SPF un-vaccinated chicken (group I) were used as control negative non-challenged. All 3 challenged and non-challenged birds were monitored on daily basis for 14 Days Post Challenge (DPC). The vNDV specific clinical signs, morbidity, mortality, the number of birds shedding the vNDV virus and virus titers (EID₅₀/mL) via the respiratory tract were monitored.

Virus titration: Tracheal swabs were collected from all survival challenged birds in 1 mL of phosphate buffered saline at 3, 6 and 10 DPC to monitor virus shedding. The collected swab samples were processed at the same day of collection. Swabs were vortexed and then centrifuged at 2000 rpm for 10 min at 4°C to pellet the debris. The supernatants were then used for virus titration in 10 days SPF embryonated chicken eggs and EID₅₀/mL were calculated (Reed and Muench, 1938).

Statistical analysis: To estimate differences among HI antibody titers and virus titers, one-way ANOVA with Tukey's post-test was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS AND DISCUSSION

Phylogenetic analysis of the two NDV strains: The phylogenetic analysis of partial sequences of the *F* gene showed that the strain NDV/CK/Egypt/11478AF/11 is avirulent LaSota-like NDV belongs to genotype II that represent the vaccine seed strains. However, the other

strain NDV/CK/Egypt/567F/12 is a vNDV strain belongs to genotype VII (Fig. 1). The amino acid sequences of the F0 protein proteolytic cleavage site motifs (residues 112-117) of the NDV strains *F* gene were compared. Results showed the strain NDV/CK/Egypt/11478AF/11 shared the cleavage site motif ¹¹²GRQGRL¹¹⁷, characteristic for Lentogenic NDV strains; however the strain NDV/CK/Egypt/567F/12 shared the motif ¹¹²RRQKRF¹¹⁷ of the velogenic NDV strains (Fig. 2 and 3). The cross HI of the two strains showed about 2 log₂ difference when using heterologous antigen. Both strains showed 85% antigenic relatedness based on cross HI results.

Post-Vaccination (PV) antibody response: The post-vaccination antibody response showed detectable HI antibody titers by 1 week PV; the mean HI antibody titer induced by the vNDV vaccinated chickens was relatively higher than the LaSota-like vaccinated chickens (2.8±1.9 and 1.3±1.6, respectively). By 2 weeks PV the immunized chickens with the vNDV vaccine showed significantly higher HI antibody titers ($p < 0.01$) as compared to the LaSota-like vaccine. However, by 4 weeks PV, the LaSota-like vaccine showed significantly higher antibody titers than the vNDV vaccine (9.1±0.74 and 7.9±0.9, respectively). The same trend was observed in the 3 batches of vaccines produced from either NDV or vNDV strain with no significant differences between batches of each NDV strain (Table 1).

Results of challenge against vNDV

Morbidity and mortality: The control nonchallenged birds (group I) shows no clinical signs during the experiment, while all birds in the challenge control birds (group II) showed typical vNDV clinical signs such as severe greenish diarrhea, respiratory rales and conjunctivitis and nervous manifestation were observed by 3DPC with 100% morbidity and 100% mortality by 4 DPC. The postmortem lesions were tracheitis and petechial hemorrhage on proventriculus in all dead birds. The LaSota like vaccinated challenged birds (group III), showed 90% morbidity with mild clinical signs such as greenish diarrhea, respiratory rales and watery eye and late nervous manifestations were observed in 2 chickens at 8 and 9 DPC. However, the vNDV vaccinated challenged bird (group IV) did not show any clinical signs. The total mortality in group III was 20%, however there was no mortality in case of group IV. The protection percent (against mortality) were 0, 80 and 100% in case of groups II, III and IV, respectively (Table 2).

Challenge virus shedding: The highest level of virus shedding (4.2 log₁₀ EID₅₀/mL) and percent of shedder

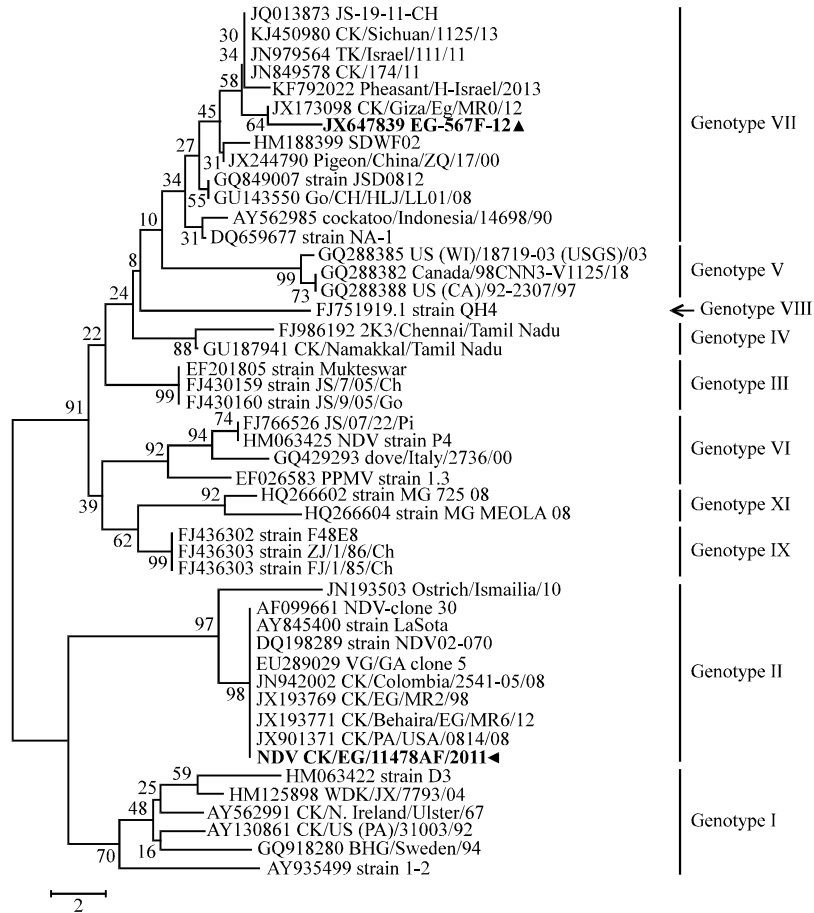


Fig. 1: Phylogenetic analyses of the nucleotide sequences of NDV strains based on partial the F gene. LaSota-like NDV strain (Bold and ◀) and vNDV strain (Bold and ▲) are indicated. Abbreviations: EG, Egypt; CK, chicken; TK, turkey. Phylogenetic relationships through a bootstrap trial of 1000 were determined with the MEGA Version 6 using the Clustal W alignment algorithm and neighbor-joining method for tree construction (Tamura *et al.*, 2013)

	Genotype I	Genotype II	Genotype III	Genotype VI	Genotype XI	Genotype IX
Consensus	SGGRRQKRF	GAIGSVSLG	VATAAQITAA	AALIQAKQNA	ANILRLKESI	AATNE
NDV/CK/Egypt/11478AF/11*	..G..G.L.	G....				
NDV/CK/Behaira/Egypt/MR6/12	..G..G.L.	G....				
NDV/CK/Egypt/MR2/98	..G..G.L.	G....				
NDV/CK/Egypt567F/12**		..V.....	A.			
NDV/CK/Giza/Egypt/MR0/12		..V.....				
NDV-23-NLQP/CH/Sh/EG/04	..K.....		S....			
NDV-37-NLQP/CH/Meno/EG/97	..K.....		S....			

Fig. 2: Comparison of amino acid residues showing the F0 protein proteolytic cleavage site motifs (residues 112-117) of the LaSota-like (*) and vNDV (**) strains used in this study

(100%) were recorded in the control challenge group II. In case of LaSota like vaccinated birds (group III) 73.3% of challenged birds were shedding virus. The vNDV vaccine challenged birds (group IV) only 20% of birds were shedding the virus in low titers (1.4-1.7 $\log_{10}$ EID₅₀/mL) (Table 3). Although, there were no significance difference between amount of virus shedding between group III and IV (1.9 and 1.7 $\log_{10}$ EID₅₀/mL), respectively at 3DPC but

relatively different titers were observed at 6 DPC (3.2 and 1.4 $\log_{10}$ EID₅₀/mL), respectively (Table 3). In general, the two vaccines we able to reduce the level of virus shedding from 4.2 $\log_{10}$ EID₅₀/mL in case of control positive (group II) to 3.2 and 1.7 $\log_{10}$ EID₅₀/mL in group III and IV, respectively.

In this study, protective efficacy of two inactivated vaccines prepared from either LaSota-like and vNDV

genotype VII strains isolated from Egypt were evaluated. The two selected NDV strains used in this study were identified and pathotyped. Molecular pathotyping was conducted based sequence analysis of the multiple basic

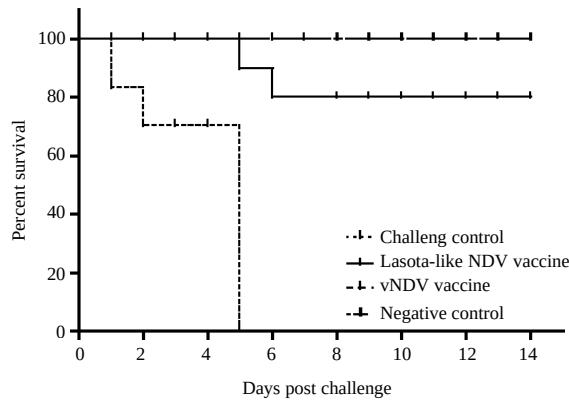


Fig. 3: Percent survival in vaccinated SPF chickens challenged with virulent NDV

Table 1: Weekly post-vaccination antibody titers ($\log_{10} \pm \text{SD}$) in SPF chickens vaccinated with three different LaSota-like and vNDV vaccines batches

Vaccine	Groups	Weeks Post Vaccination (PV)				Heterologous HI titers at 4 weeks PV
		1	2	3	4	
LaSota-like vaccine	A1	0.9±1.3	3.8±0.71	7.1±0.6	9.0±0.5	ND ^a
	A2	1.5±0.9	3.6±0.70	6.4±0.8	8.4±0.5	ND
	A3	1.4±2.3	3.6±0.68	6.5±0.5	9.1±0.7	7.1±0.7
	Mean	1.3±1.6	3.6±0.68 ^a	6.6±0.7	8.8±0.6 ^c	-
vNDV vaccine	B1	2.3±0.7	5.9±1.25	7.0±0.5	8.4±0.7	ND
	B2	1.5±1.1	4.3±0.71	6.0±0.5	7.0±0.9	ND
	B3	1.9±1.1	4.5±0.85	6.7±0.7	8.2±0.4	6.4±0.6
	Mean	2.8±1.9	4.9±1.15 ^b	6.6±0.7	7.9±0.9 ^d	-

^{a-d}Titers followed by different lower case letters in the same column are statistically different ($p < 0.01$); ^aNot Done

amino acids region of the F1 protein. This method is a rapid and reliable method for NDV pathotyping as compared to the mean death time, the intravenous pathogenicity index or intracerebral pathogenicity index tests (De Battisti *et al.*, 2013; Ganar *et al.*, 2014; Gohm *et al.*, 2000).

The results of sequence analysis of the 2 isolates of NDV revealed that the NDV/CK/Egypt/11478AF/11 is avirulent LaSota-like NDV belongs to genotype II of the vaccine strains. However, the other strain NDV/CK/Egypt/567F/12 is a vNDV strain possessing the motif¹²RRQKRF¹¹⁷ of the velogenic NDV strains. The phylogenetic analysis of isolated Egyptian NDV strains including our strain (NDV/CK/Egypt/567F/12) responsible for recent outbreaks showed that they belong to genotype VII (Abdel-Glil *et al.*, 2013; Mohamed *et al.*, 2011; Radwan *et al.*, 2013). Moreover, the recently isolated strains are genetically distant from vaccine strains indicating the potential evolution of virulent NDV in Egyptian poultry (Miller *et al.*, 2013, 2009).

Considering the single dose regime in this study, the formulated vaccines using MONTANIDE™ ISA 70 induced protective antibody response by 2 week PV. The inactivated vNDV vaccines batches induced significantly earlier high antibody titer than those induced by the LaSota-like vaccines batches. Similar differences in antibody responses at days 3, 10 and 21 PV were recorded (Miller *et al.*, 2013) and it was found that high levels of antibodies induced by vaccines formulated with NDV of the same genotype of the challenge viruses can significantly decrease viral replication and shedding.

In addition to the low antigenic relatedness between the two NDV strains, the serology results were also

Table 2: Clinical signs, virus isolation and mortalities in SPF challenged groups

DPC ^a	Negative control		Challenge control		LaSota-like NDV vaccine		vNDV vaccine	
	C. Signs/ lesions	Virus isolation ^b	C. Signs/ lesions ^c	Virus isolation	C. Signs/ lesions ^d	Virus isolation	C. Signs/ lesions ^e	Virus isolation
1	0/15		15/15		0/15		0/15	
2	0/15		12/12		14/15		0/15	
3	0/15	0/15	9/9	15/15	14/15	10/15	0/15	0/15
4	0/15		6/6		14/15		0/15	
5	0/15		NA ^f		13/15		0/15	
6	0/15	0/15			10/14	3/14	0/15	0/15
7	0/15				7/13		0/15	
8	0/15				6/13		0/15	
9	0/15				3/12		0/15	
10	0/15	0/15			2/12	0/12	0/15	0/15
11	0/15				0/12		0/15	
12	0/15				0/12		0/15	
13	0/15				0/12		0/15	
14	0/15	0/15			0/12	0/12	0/15	0/15
Mortality at 14DPI	0%		100		20%		0%	

^aDPC; days post challenge; ^bVirus isolation by inoculation of individual swab samples into allantoic sac of 9-11 day old SPF embryonated chicken eggs; ^cClinical signs were severe greenish diarrhea, respiratory rales and conjunctivitis. Nervous manifestation observed by the 5th dpc. PM lesions were tracheitis and petechial hemorrhage on proventriculus; ^dMild clinical signs were greenish diarrhea, respiratory rales and watery eye. Nervous manifestation observed after 12 dpc. PM lesions were mild tracheitis; ^eNA; not applicable

Table 3: Virulent NDV respiratory shedding titers in SPF vaccinated chickens challenged with NDV/CK/Egypt/567F/12 (genotype VII) vaccine

Virus titers EID ₅₀ /mL ±SD (No. of positive/total)				
DPC*	Negative control	Challenge control	LaSota-like NDV vaccine	vNDV vaccine
3	0 (0/15)	4.2±0.6 (15/15) ^a	1.9±0.9 (11/15) ^b	1.7±0.5(3/15) ^b
6	0 (0/15)	NA**	3.2±0.7 (4/14)	1.4±0.2 (2/15)
10	0 (0/15)	NA	0 (0/12)	0 (0/15)

*DPC = Days Post Challenge; **NA = Not Applicable; ^{a-b}Virus titers followed by different superscript lower case letters in the same row are statistically different (p<0.01)

consistent with the of the challenge study. Results showed that over 90% of LaSota-like vaccinated birds showed mild clinical signs by 3DPC and continuously over 30% of birds showed clinical signs up to 9DPC. The clinical signs were also accompanied by 20% mortality. In contrast non of the birds vaccinated with vNDV strain vaccine showed any clinical signs and there were no mortality reported. These results further demonstrated that LaSota vaccines are not sufficient to protect chicken flocks and would explain the failure of vaccination programs to prevent vNDV outbreaks (Miller *et al.*, 2013; Yang *et al.*, 1997, 1999).

Though both vaccines were prepared with same dose (virus titers before inactivation was 10⁸ EID₅₀/bird) which has been shown to be sufficient to prevent virus replication, birds vaccinated with the LaSota-like strain (4 out of 14 birds) were shedding the virus at 6DPC with an average virus titer of 3.2±0.7 log₁₀. However, vNDV strain vaccine significantly reduced the virus shedding titers and number of birds shed the virus.

Hence, the earlier immune response induced by vNDV vaccine may give enough time between vaccination and challenge to develop sufficient immunity and to maintain sufficient and uniform flock immunity and subsequently superior protection. Both factors were found to be important in the protection conferred with ND vaccines (Miller *et al.*, 2013; Van Boven *et al.*, 2008).

Outbreaks and spreading of vNDV were attributed to the poor flock immunity due to inadequate vaccination practices rather than antigenic variation (Alexander *et al.*, 1997; Dortmans *et al.*, 2012), however, several studies and our study showed that potentially genetic and antigenic differences may responsible for overcoming vaccination barrier (Panshin *et al.*, 1998, 2002). It should be noted that the challenge was performed at 4 weeks PV in this study when the LaSota-like vaccine showed significantly higher antibody titers than vNDV strain vaccine.

The ineffectiveness of the inactivated LaSota-like vaccine to protect against viral replication as compared to the vNDV inactivated vaccine raises the concern of

continuous circulation of vNDV in Egypt under immune pressure induced by massive heterologous NDV vaccination and subsequently continuous virus evolution and emergence of new genotypes (Ganar *et al.*, 2014; Kapczynski and King, 2005).

CONCLUSION

The current study further indicate that superior protection against vNDV outbreaks through matching of the used vaccines to field isolates considering, higher and earlier immune response and protection level obtained. Additionally, matching vaccine with field viruses is capable of significantly reducing vNDV virus dissemination and subsequently minimizing the risk of NDV virus evolution.

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