



Molecular characterization of full genome sequences of Newcastle disease viruses circulating among vaccinated chickens in Egypt during 2011–2013

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ABSTRACT. Although intensive vaccination programs have been implemented, Newcastle disease (ND) outbreaks, accompanied by severe economic losses, are still reported in Egypt. The genetic characterization of ND virus (NDV) strains isolated from ND-vaccinated chicken flocks provides essential information for improving ND control strategies. Therefore, here, 38 NDV strains were isolated and identified from outbreaks among vaccinated flocks of broiler chickens located in the provinces of Qena, Luxor, and Aswan of Upper Egypt during 2011–2013. The investigated broiler chicken flocks (aged 28 to 40 days) had high mortality rates of up to 80%. All NDV isolates were genetically analyzed using next-generation DNA sequencing. From these isolates, 10 representative NDV strains were selected for further genetic analyses. Phylogenetic analysis of full-length coding genes revealed that the Egyptian NDV isolates belonged to a single sub-genotype, VII.1.1. These isolates were phylogenetically distant from the vaccine strains, including La Sota or Clone 30 (genotype II), which have been commonly used to vaccinate chicken flocks. Amino acid substitution K78R was observed in the neutralizing epitopes of the F proteins; whereas several mutations were found in the neutralizing epitopes of the hemagglutinin-neuraminidase proteins, notably, E347K. Overall, our results suggested that the occurrence of neutralizing epitope variants may be one of potential reasons for ND outbreaks. Further studies are needed to determine the protective effect of current vaccines against circulating virulent NDV strains.

KEY WORDS: Egypt, genotyping, Newcastle disease virus, vaccinated chicken, VII.1.1

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Newcastle disease (ND) is one of the most devastating viral diseases in poultry, and has resulted in heavy economic losses to the poultry industry worldwide [1]. ND is caused by *Avian orthoavulavirus 1* (formerly designated as ND virus (NDV) which has been commonly used and is also used here), which belongs to the genus *Orthoavulavirus* in the family *Paramyxoviridae* under

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order *Mononegavirales* [8]. It is an enveloped, non-segmented, and negative-sense single-stranded RNA virus. Its genome is at least of three sizes; 15,186, 15,192, and 15,198 nucleotides in length and contains six genes that encode nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large polymerase protein (L). In addition, NDV encodes two more proteins, named V and W, which are synthesized by the mRNA editing of the P gene [34].

Based on pathogenicity, NDVs are categorized into 3 pathotypes in increasing order of virulence, namely lentogenic, mesogenic or velogenic. In general, velogenic strains cause high mortality rates (up to 100%) in chickens with pronounced intestinal and/or neurological clinical signs [1]. Additionally, the amino acid (aa) sequences of the F protein cleavage site have been used to distinguish between virulent and avirulent NDV strains. According to this approach, virulent strains have the aa sequence motif $^{112}\text{R/G/K-R-Q/K-K/R-R-F}^{117}$ at the F cleavage site. In contrast, the motif in avirulent NDV strains is $^{112}\text{G-R/K-Q-G-R-L}^{117}$ [3, 41]. Although all NDV strains belong to a single serotype, significant genetic diversity has been observed among different NDV isolates [7]. NDV strains have been classified into two major classes with different genotypes based on their F gene sequences. Class I consists of only genotype I and contains mostly avirulent viruses isolated from wild birds. Class II includes both avirulent and virulent isolates from wild birds and domestic poultry. Class II viruses are divided into 21 genotypes (I–XXI). Among the genotypes circulating worldwide, genotype VII is one of the most prevalent groups comprising virulent NDVs. Regarding the updated classification and nomenclature of NDV, previously identified NDV VII genotype were divided into sub-genotypes VII.1.1 and VII.1.2. The sub-genotype VII.1.1 combines the former sub-genotypes VIIb, VIId, VIIe, VIIj, and VIII. The former sub-genotype VIIf was classified as sub-genotype VII.1.2. Sub-genotypes VIIa, VIIh, VIIi and VIIk were merged into a single sub-genotype, VII.2 [8].

To control ND, a worldwide poultry vaccination program has been implemented. Currently, the most commonly used vaccine strains, Hitchner B1, La Sota, and Clone 30, belong to genotype II. At the genetic level, these strains had a percentage nucleotide sequence of 18.3–26.6% dissimilar to the virulent NDV strains globally circulating among chickens [9].

In Egypt, ND remains one of the primary poultry diseases in both commercial and backyard chickens since recorded in 1948, despite vaccination. As previously reported, genotype VII has been responsible for ND outbreaks in the commercial chickens; whereas genotype VI has been isolated from diseased pigeons since early 1981 in Egypt [23, 32]. Continuous active surveillance and genetic analysis of NDVs isolated from vaccinated flocks will provide insights into the current situation. The current study demonstrate the molecular characterization and phylogenetic analysis of whole genome sequences of NDVs isolated from vaccinated broiler chicken flocks located in Upper Egypt during 2011–2013. The analysis of genetic variation between the NDVs isolates and the commercial vaccine strains would be helpful in improvement of ND vaccination strategy.

MATERIALS AND METHODS

Ethical approval

This research does not contain studies involving human participants that have been performed by any of the authors. Sample collections were conducted by South Valley University under a research project agreement with prior permission from the poultry farm owners.

Sample collection and virus isolation

The 38 isolates reported in the study were obtained from different outbreaks among vaccinated broiler chicken flocks located in 3 Egyptian provinces along the Nile River (Qena, Luxor, and Aswan) during 2011–2013. Each spleen, trachea, cecal tonsils, and intestine homogenated or tracheal swab sample was propagated by inoculation in 10 day-old specific-pathogen-free embryonated eggs via the allantoic route, according to OIE recommendations [26]. Subsequently, the harvested allantoic fluids were tested hemagglutination (HA) activity and serologically by HA inhibition (HI) tests for the presence of NDV. Similarly, at the molecular level, NDV-positive samples were detected using real-time reverse transcription polymerase chain reaction (real-time RT-PCR) [43]. The allantoic fluid samples were then transferred to Obihiro University of Agriculture and Veterinary Medicine (Obihiro, Japan), according to the research project agreement with South Valley University (Qena, Egypt). The allantoic fluids were passaged once in embryonated eggs, then tested by HA and HI tests using 0.5% chicken red blood cells according to the OIE manual of standards for diagnostic tests and vaccines [26]. These NDV-positive allantoic fluid samples were used for further studies.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from NDV-positive allantoic fluids using the ISOGEN II (NIPPON GENE, Tokyo, Japan) reagent according to the manufacturer's instructions. The reverse transcription reaction was performed using random primers (Invitrogen, Carlsbad, CA, USA) and M-MLV Reverse Transcriptase (Invitrogen) under the following conditions: 25°C for 10 min, 37°C for 60 min, and 65°C for 10 min.

Nucleotide sequencing and phylogenetic analyses

The nucleotide sequence of the full-length viral genome was generated by next-generation sequencing (NGS) as previously reported [37]. In brief, sequencing libraries were constructed from RNA using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. After determining the library quantity on a Qubit 4.0 Fluorometer (Invitrogen), DNA was loaded into the reagent cartridge using a MiSeq Reagent Kit v3 (150 cycles) (Illumina, San Diego, CA, USA) and a deep-sequence was performed using a MiSeq bench-top sequencer (Illumina).

FASTQ formatted sequence data files were created using MiSeq Reporter program (Illumina). The trimmed sequences were generated from 51 nucleotide length of raw paired-end reads to trim low-quality sequence reads using Trim Sequences command with default parameter in NGS Core tools in CLC Genomics Workbench. Then, contigs were generated from trimmed sequence reads with default parameter setting using *de novo* assembly command with Map reads back to contigs option.

The nucleotide and deduced aa sequences of each protein were aligned and analyzed using the ClustalW multiple alignment tool in BioEdit Ver. 7.2 software [38]. The nucleotide and aa sequences were analyzed and compared with other sequences available in the GenBank identified by a BLASTn search (<http://www.ncbi.nlm.nih.gov/>) using GENETYX Ver.10 software (GENETYX Corp., Tokyo, Japan). Phylogenetic trees were constructed by the maximum-likelihood method using the General Time Reversible model with 1,000 bootstrap replicates in the MEGA6 software [36].

Recombination events, evolutionary distances, and selection profiles of NDV gene sequences

Putative recombinations involving NDV genes were identified using a recombination detection program (RDP) version Beta 4.97 and SimPlot program (version 3.5.1) [17, 20]. Evolutionary distances were estimated using the MEGA6 software by employing the Maximum Composite Likelihood model [35, 36]. Elaboration of evolutionary selection profiles was performed using Datamonkey (<http://www.datamonkey.org/>) by following the Fast Unconstrained Bayesian AppRoximation (FUBAR) method [24].

Nucleotide sequence accession numbers

The complete genome sequences of 33 NDV strains were deposited in the GenBank database with the following accession numbers: MK495878–MK495910. The incomplete genome sequences of the other 5 NDV strains were not deposited in the GenBank database.

RESULTS

Analysis of predicted aa sequences encoded by F and HN genes

A total of 38 NDV strains were isolated from outbreaks in vaccinated broiler chicken flocks in the Qena, Luxor, and Aswan provinces of Upper Egypt in which NDV outbreaks occurred (Fig. 1) during 2011–2013. The investigated broiler chicken flocks (aged 28 to 40 days) suffered from high mortality of up to 80%. All samples showing HA activity were tested for the presence of NDV using real-time RT-PCR detection of the NDV M gene. Using NGS, coding genes sequences of 33 and 5 other incomplete genome of NDV strains were obtained. Among the complete genome of the 33 obtained NDV strains, 10 representative NDV strains were selected for phylogenetic and genetic analyses based on either geography or the year of isolation. The results of the molecular analyses are shown in Table 1. The common F protein aa cleavage site motif for all of the NDV isolates was ¹¹²R–R–Q/K–K–R–F¹¹⁷. Only one strain (NDV/Egypt/Luxor/2012/5) had the aa sequence motif ¹¹²R–R–K–K–R–F¹¹⁷.

The analysis of the aa sequences encoded by the complete F gene of the 10 representative strains also showed that the six potential N-glycosylation sites and 12 cysteine residues previously described [40] were conserved in all strains. The aa residues D72, E74, A75, K78, A79, L343, and the stretch of aa residues 157–171, involved in the formation of neutralizing epitopes [16, 47], were found to be conserved in all 10 strains, with the exception of position 78, which exhibited the substitution of K by R (Table 1 and Supplementary Fig. 1). Additionally, when compared with vaccine strains (La Sota and Clone 30), the 10 strains had aa substitutions in the functional domains of the F proteins (Supplementary Table 1). They had thirteen in the signal peptide, 3 in the fusion peptide, and 4 in the transmembrane domain substitutions. Three heptal repeat regions (HRs) contained a total of 10 substitutions in HRa, HRb, and HRc in all 10 strains. Some aa substitutions were also found in other locations of the F proteins of the Egyptian strains (refer to each accession number in GenBank).

The HN protein of all the Egyptian strains is composed of 571 aa. Alignment of the predicted aa sequences of the complete HN proteins indicated that all the 10 strains possessed the same conserved aa stretches known to form hemagglutinin receptor-binding sites and five N-glycosylation sites [4]. Compared to the vaccine strains (La Sota and Clone 30), nine aa substitutions were found in the transmembrane domain (positions 25–45) of the HN proteins of the 10 strains (Supplementary Table 2). The results also revealed that several aa substitutions occurred in residues associated with the neutralizing epitopes of the HN proteins, such as N263K, E347K, G491D, and I514V [13, 15, 18] (Table 1 and Supplementary Fig. 2).

Phylogenetic characterization

Phylogenetic analyses of the 10 representative NDV strains using full-length F genes indicated that all strains clustered together with genotype VII class II, sub-genotype VII.1.1 strains, and clearly separated from the vaccine strains (genotype II) (Fig. 2 and Supplementary Fig. 3). Also, phylogenetic analyses of the complete open reading frames of NP, P, M, HN, and L genes were performed to confirm the robustness of the genetic grouping and the topology of the complete F-gene based phylogenetic tree (Supplementary Fig. 4). In addition, the results indicated that the 10 strains formed clusters with some previously reported Egyptian NDV strains isolated during 2012 to 2016 (Fig. 2 and Supplementary Fig. 3). Also, compared to the 10 strains in this study, these strains shared identity ranging between 97.1–99.3% and 96.9–100% based on nucleotide and aa levels of F gene sequences, respectively (Supplementary Table 3). Furthermore, the aa residues K and V at the positions 101 and 121, respectively, were observed in the F protein of each strain.



Fig. 1. Map of Egypt showing the provinces from where Newcastle disease virus samples were collected during 2011–2013; Qena (26° 09' 60.00" N 32° 42' 59.99" E), Luxor (25° 41' 14.075" N 32° 38' 22.689" E) and Aswan (24° 5' 20.177" N 32° 53' 59.385" E).

Table 1. Characteristics of Egyptian Newcastle disease virus isolates analyzed in this study

Virus strains	Year	Vaccine status	GenBank accession no.	aa substitutions compared to vaccine strains ^{a)}					
				F-protein cleavage site	Neutralizing epitopes				
					F protein	HN protein			
				¹¹² GRQGR ¹¹⁷	K ⁷⁸	N ²⁶³	E ³⁴⁷	G ⁴⁹¹	I ⁵¹⁴
NDV/Egypt/Luxor/2011/1	2011	La Sota	MK495878	RRQKRF	R	K	K	D	V
NDV/Egypt/Luxor/2011/2	2011	Clone 30	MK495879	RRQKRF	R	K	K	D	V
NDV/Egypt/Luxor/2012/2	2012	Clone 30	MK495883	RRQKRF	R	K	K	D	V
NDV/Egypt/Luxor/2012/3	2012	La Sota	MK495884	RRQKRF	R	K	K	D	V
NDV/Egypt/Luxor/2012/4	2012	La Sota	MK495885	RRQKRF	R	K	K	D	V
NDV/Egypt/Luxor/2012/5	2012	La Sota	MK495886	RRKKRF	R	K	K	D	V
NDV/Egypt/Luxor/2012/7	2012	La Sota	MK495887	RRQKRF	R	K	K	D	V
NDV/Egypt/Qena/2012/2	2012	La Sota	MK495906	RRQKRF	R	K	K	D	V
NDV/Egypt/Luxor/2013/1	2013	Clone 30	MK495910	RRQKRF	R	K	K	D	V
NDV/Egypt/Aswan/2013/1	2013	La Sota	MK495909	RRQKRF	R	K	K	D	V

a) La Sota and Clone 30 strains.

Recombination events, evolutionary distances, and selection profiles among Egyptian NDV sequences

Recombination analyses performed for all coding genes of the 33 Egyptian NDV isolates belonging to the sub-genotype VII.1.1 did not indicate any putative recombination events among these strains or the previous Egyptian sequences downloaded from GenBank. According to the evolutionary distances of the full-length F gene sequences between the Egyptian strains and vaccine strains, the 10 strains selected here were divided into 5 groups based on the location and the collection year, and showed a maximum of 0.19 genetic divergence from the vaccine strains (Table 2). The results also indicated that these strains were closely related to each other. In particular, the evolutionary distances among Egyptian strains isolated from Luxor during 2011–2013 were very short; in addition, short genetic distances were observed in strains isolated from Qena in 2012 and Aswan in 2013 (Table 2). Furthermore, 18 sites in the F genes of all Egyptian strains were found to be under negative selection (with posterior probability of negative selection at a site equal to or more than 0.9) (Table 3).

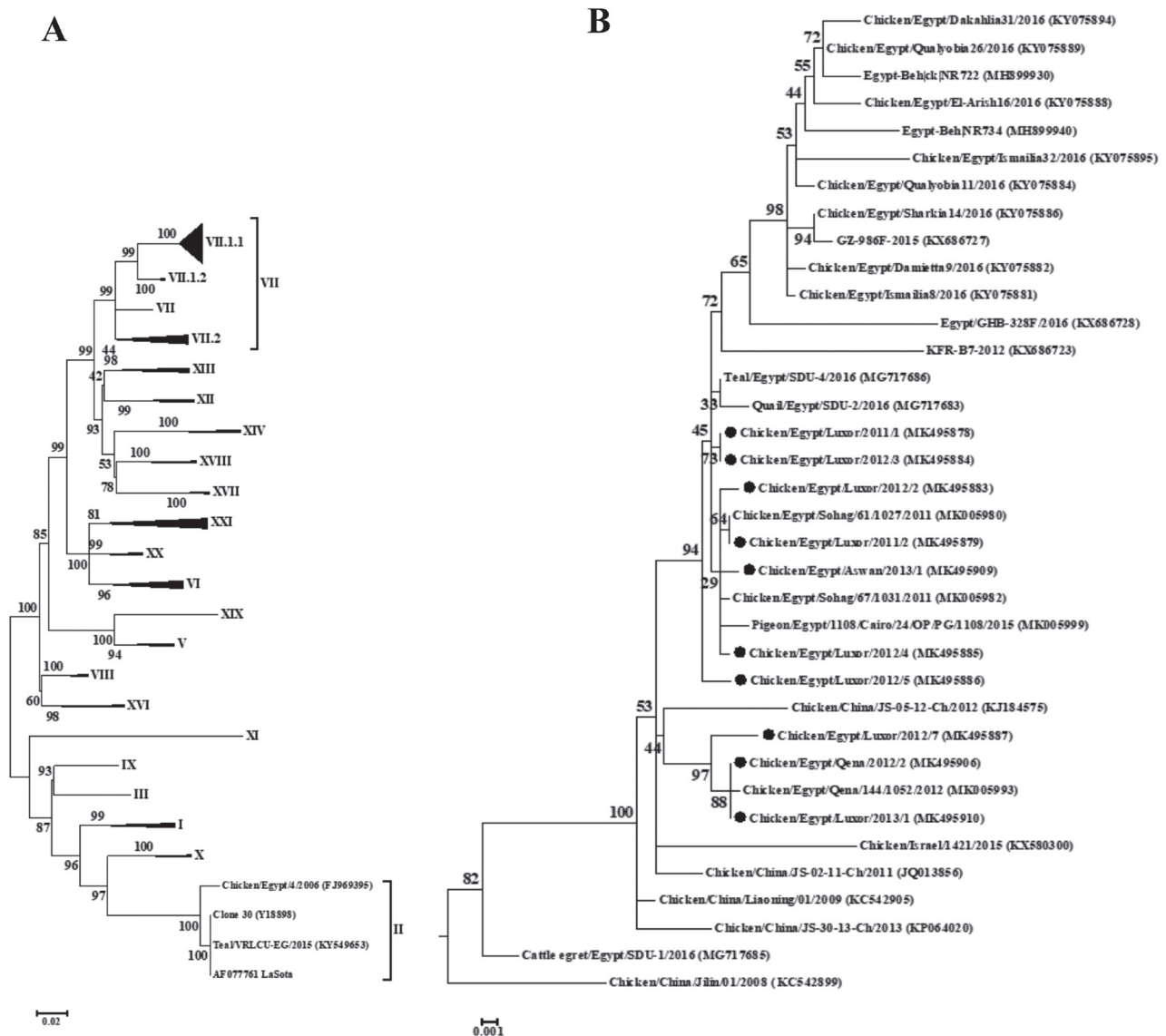


Fig. 2. Phylogenetic trees comparing the complete coding regions of the F genes of Egyptian NDV strains to sequences from GenBank (A) and subgenotype VII.1.1 (B). The tree was constructed using the maximum-likelihood method (1,000 bootstrap replicates) in the MEGA6 software. Bootstrap values are shown at the nodes. The sequences used in this study are labeled with a black dot.

DISCUSSION

Currently, ND is still a serious threat to the poultry industry, even though intensive vaccination programs have been applied. Recently, some studies have reported ND outbreaks among vaccinated commercial flocks, causing up to 76.8–84.4% mortality in India [19], 70–80% mortality in Indonesia [45], 60–70% mortality in Japan [40], and 5–50% mortality in Pakistan [29]. In Egypt, ND was first reported in 1948 [6], and it was classified as an endemic disease by the beginning of the 1960s [10]. To control NDV infection, avirulent and inactivated vaccines, containing NDVs belonging to genotype II (La Sota, Clone 30, and Hitchner B1) have been administered to Egyptian poultry. However, as reported, virulent NDVs were isolated from vaccinated flocks in this country [27]. Another study indicated that vaccinated broiler flocks suffered from a variable mortality of between 15–20% due to virulent NDVs from 2014–2016 [31]. The source of the virulent variants remains unclear.

Results of the present study indicated continuous circulation of the NDV single sub-genotype VII.1.1, class II among broiler chicken flocks in certain provinces of Egypt during 2011–2013. It is noteworthy that all chicken flocks were vaccinated using La Sota or Clone 30 strains but still suffered outbreaks of ND with mortalities between 35% and 80%. However, detailed vaccination procedures have not been obtained. The molecular characterization of NDV strains isolated from vaccinated flocks could give us insights into strategies for improvement of ND control measures.

Here, 10 representative NDV strains were classified as virulent based on the aa sequence motif ¹¹²R/G/K–R–Q/K–K/R–R–F¹¹⁷

Table 2. Evolutionary divergence of full-length F gene sequences between the vaccine strains and Egyptian Newcastle disease virus isolates analyzed in this study

Strain group	Divergence between each pair of group						
	Luxor/11	Luxor/12	Qena/12	Luxor/13	Aswan/13	La Sota	Clone 30
Luxor/11		(0.00)	(0.00)	(0.00)	(0.00)	(0.04)	(0.04)
Luxor/12	0.00		(0.00)	(0.00)	(0.00)	(0.04)	(0.04)
Qena/12	0.01	0.01		(0.00)	(0.00)	(0.04)	(0.04)
Luxor/13	0.00	0.00	0.01		(0.00)	(0.04)	(0.04)
Aswan/13	0.01	0.01	0.00	0.01		(0.04)	(0.04)
La Sota	<u>0.19</u>	<u>0.19</u>	<u>0.19</u>	<u>0.19</u>	<u>0.19</u>		(0.00)
Clone 30	<u>0.19</u>	<u>0.19</u>	<u>0.19</u>	<u>0.19</u>	<u>0.19</u>	0.00	

The number of base substitutions per site is shown by averaging overall sequence pairs between different groups. Standard error estimate is shown above the diagonal (in parentheses) and was obtained by a bootstrap procedure (1,000 replicates). Divergences between the vaccine strains and isolates are underlined. The analysis involved 12 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + non-coding. All positions containing gaps and missing data were eliminated. There were a total of 1,662 positions in the final data. Evolutionary analyses were conducted in MEGA6.

Table 3. Substituted nucleotide positions as negative selection in the F gene sequences of the Egyptian field strains

Nucleotide position	α	β	$\beta-\alpha$	Prob[$\alpha>\beta$]	Prob[$\alpha<\beta$]
19	21.00	1.50	-19.50	0.90	0.00
24	25.00	1.60	-23.40	0.90	0.00
28	33.30	1.90	-31.30	0.90	0.00
94	21.30	1.30	-19.90	0.90	0.00
144	20.55	1.39	-19.16	0.90	0.00
148	20.14	1.38	-18.75	0.90	0.00
160	24.43	1.41	-23.02	0.92	0.05
204	25.40	2.24	-23.16	0.91	0.06
281	24.54	2.23	-22.31	0.90	0.06
286	24.54	2.23	-22.31	0.90	0.06
340	24.17	1.79	-22.37	0.91	0.06
342	21.72	1.74	-19.97	0.90	0.07
364	24.44	1.67	-19.76	0.90	0.07
412	21.45	1.78	-19.67	0.90	0.07
484	21.86	1.66	-20.20	0.90	0.06
514	21.08	1.40	-19.67	0.90	0.06
517	21.90	1.37	-20.53	0.91	0.06
543	21.07	1.43	-19.63	0.90	0.07

α : indicates posterior synonymous substitution rate at a site; β : indicates posterior non-synonymous substitution rate at a site; $\alpha>\beta$: negative selection; $\alpha<\beta$: positive selection; $\alpha=\beta$: neutral selection; Prob[$\alpha>\beta$] \geq 0.9: posterior probability of negative selection at a site; Prob[$\alpha<\beta$] \geq 0.9: posterior probability of positive selection at a site.

at the F protein cleavage site [3, 41]. The presence of several basic aa residues and the phenylalanine at position 117 were found to be characteristic to highly virulent strains [5]. In addition, the length of the HN gene product (571 aa) was also found to be characteristic of virulent NDV strains [30]. These features were observed in all 10 strains assessed (refer to each accession number in GenBank and Table 1).

To understand the genetic properties of NDV affecting poultry flocks despite vaccination, the deduced aa sequences of F and HN proteins, with special emphasis on the functional domains and neutralizing epitopes, were evaluated. Compared with the vaccine strains (La Sota and Clone 30), the 10 strains assessed herein showed several substitutions involving the functional domains of the F and HN proteins (Supplementary Tables 1 and 2). Previous reports indicated that aa substitutions in the fusion peptide and HR regions of F protein may affect the fusion activity of NDV [22, 33]. Another study also reported that variations in the signal peptide and fusion peptide motifs may hinder the viral envelope-cell membrane fusion activity of the F protein [42]. Some aa substitutions were observed in the transmembrane domain of the HN protein, and it has been reported that mutations in this domain may affect virus attachment and neuraminidase activity [21].

Furthermore, the 10 strains had specific aa substitutions in the neutralizing epitopes of the F and HN proteins, which affected the targets of neutralizing antibodies (Table 1). The aa substitution at residue 78 in the F protein and its location in the helix sheet near the cleavage site motif raised the question of whether this substitution influenced the activity of neutralizing antibodies. Previous studies suggested that F protein aa substitutions at residues 78 and 79 may alter the antigenicity [14, 28, 46]. In particular, aa residue 347 in the HN protein may represent a potentially useful marker for the antigenic variation of NDV [2]. Another study

found that the titers of neutralizing antibodies against NDV strains with this substitution showed 2- to 3-fold reductions when compared to those of controls [39]. Alternatively, the current study clearly indicated that Egyptian isolates were phylogenetically distant from the vaccine strains (genotype II). This finding is consistent with that of a previous report that showed that current ND vaccine strains (genotypes I and II) are genetically distant from the virulent NDV strains (18.3–26.6% nucleotide distance) [9]. As regards ND control strategies, commercial vaccines in combination with biosecurity systems in the farm are still believed to have a good protection against NDV infections. Also, there are multiple factors influenced the effectiveness of ND vaccine. It was supposed that the efficacy of vaccine depended on numerous factors within a host (immunosuppressive condition), improper vaccination and the occurrence of pathogenic virus [25]. Besides, the attenuated genotype VII-matched vaccines may provide better protection in term of reducing virus shedding than vaccines using common NDV strain belonging to genotype II [12, 44]. However, the possible involvement of genetic distance in case of NDV infection in vaccinated chicken flocks is still unknown. Therefore, there is an urgent need to address how ND outbreaks can occur in vaccinated chicken flocks.

With regard to recombination among NDV, Han *et al.* [11] stated that recombination plays a role in NDV evolution. In particular, live vaccines seem to have played an important role in shaping NDV evolution by homologous recombination with circulating viruses. In the epidemiological investigation of vaccinated chicken flocks, no recombination was found to have occurred in the Egyptian NDV strains isolated from three provinces in 2011–2013. It was, thus, suspected that the recombinant variant was not introduced into Egypt during this time period.

In conclusion, the current investigation has provided valuable data on the genetic characterization and epidemiology of NDV strains circulating among vaccinated chicken flocks in certain provinces of Upper Egypt during 2011–2013. These findings will contribute to the understanding of the genetic diversity and dynamics of NDV infection in vaccinated chickens. Furthermore, to control NDV infection in Egypt more efficiently, it will be necessary to assess the efficacy of current vaccines against predominant NDV strains.

CONFLICTS OF INTEREST. The authors declare no conflicts of interest.

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REFERENCES

- Alexander, D. J. and Senne, D. A. 2008. Newcastle disease, other avian paramyxoviruses, and pneumovirus infections. pp. 75–116. *In: Diseases of Poultry*, 12th ed. (Saif, Y. M., Fadly, A. M., Glisson, J. R., McDougald, L. R., Nolan, L. K. and Swayne, D. E. eds.), Iowa State University Press, Ames.
- Cho, S. H., Kwon, H. J., Kim, T. E., Kim, J. H., Yoo, H. S. and Kim, S. J. 2008. Variation of a newcastle disease virus hemagglutinin-neuraminidase linear epitope. *J. Clin. Microbiol.* **46**: 1541–1544. [Medline] [CrossRef]
- Collins, M. S., Bashiruddin, J. B. and Alexander, D. J. 1993. Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. *Arch. Virol.* **128**: 363–370. [Medline] [CrossRef]
- Connaris, H., Takimoto, T., Russell, R., Crennell, S., Moustafa, I., Portner, A. and Taylor, G. 2002. Probing the sialic acid binding site of the hemagglutinin-neuraminidase of Newcastle disease virus: identification of key amino acids involved in cell binding, catalysis, and fusion. *J. Virol.* **76**: 1816–1824. [Medline] [CrossRef]
- Courtney, S. C., Susta, L., Gomez, D., Hines, N. L., Pedersen, J. C., Brown, C. C., Miller, P. J. and Afonso, C. L. 2013. Highly divergent virulent isolates of Newcastle disease virus from the Dominican Republic are members of a new genotype that may have evolved unnoticed for over 2 decades. *J. Clin. Microbiol.* **51**: 508–517. [Medline] [CrossRef]
- Daubney, R. and Mansy, W. 1948. The occurrence of Newcastle disease in Egypt. *J. Comp. Pathol. Ther.* **58**: 189–200. [Medline] [CrossRef]
- Diel, D. G., da Silva, L. H., Liu, H., Wang, Z., Miller, P. J. and Afonso, C. L. 2012. Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. *Infect. Genet. Evol.* **12**: 1770–1779. [Medline] [CrossRef]
- Dimitrov, K. M., Abolnik, C., Afonso, C. L., Albina, E., Bahl, J., Berg, M., Briand, F. X., Brown, I. H., Choi, K. S., Chvala, I., Diel, D. G., Durr, P. A., Ferreira, H. L., Fusaro, A., Gil, P., Goujgoulova, G. V., Grund, C., Hicks, J. T., Joannis, T. M., Torchetti, M. K., Kolosov, S., Lambrecht, B., Lewis, N. S., Liu, H., Liu, H., McCullough, S., Miller, P. J., Monne, I., Muller, C. P., Munir, M., Reischak, D., Sabra, M., Samal, S. K., Servan de Almeida, R., Shittu, I., Snoeck, C. J., Suarez, D. L., Van Borm, S., Wang, Z. and Wong, F. Y. K. 2019. Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. *Infect. Genet. Evol.* **74**: 103917. [Medline] [CrossRef]
- Dimitrov, K. M., Afonso, C. L., Yu, Q. and Miller, P. J. 2017. Newcastle disease vaccines-A solved problem or a continuous challenge? *Vet. Microbiol.* **206**: 126–136. [Medline] [CrossRef]
- El-Nassary, B. B. and Eskarous, J. K. 1960. The distribution of fowl plague and Newcastle disease in upper Egypt. *Arch. Mikrobiol.* **36**: 147–150. [Medline] [CrossRef]
- Han, G. Z., He, C. Q., Ding, N. Z. and Ma, L. Y. 2008. Identification of a natural multi-recombinant of Newcastle disease virus. *Virology* **371**: 54–60. [Medline] [CrossRef]
- Hu, S., Ma, H., Wu, Y., Liu, W., Wang, X., Liu, Y. and Liu, X. 2009. A vaccine candidate of attenuated genotype VII Newcastle disease virus generated by reverse genetics. *Vaccine* **27**: 904–910. [Medline] [CrossRef]
- Iorio, R. M., Syddall, R. J., Sheehan, J. P., Bratt, M. A., Glickman, R. L. and Riel, A. M. 1991. Neutralization map of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus: domains recognized by monoclonal antibodies that prevent receptor recognition. *J. Virol.* **65**: 4999–5006. [Medline] [CrossRef]
- Ke, G. M., Yu, S. W., Ho, C. H., Chu, P. Y., Ke, L. Y., Lin, K. H., Tsai, Y. C., Liu, H. J. and Lin, M. Y. 2010. Characterization of newly emerging Newcastle disease viruses isolated during 2002–2008 in Taiwan. *Virus Res.* **147**: 247–257. [Medline] [CrossRef]
- Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N. and Sternberg, M. J. 2015. The Phyre2 web portal for protein modeling, prediction and analysis. *Nat. Protoc.* **10**: 845–858. [Medline] [CrossRef]

16. Liu, X. F., Wan, H. Q., Ni, X. X., Wu, Y. T. and Liu, W. B. 2003. Pathotypical and genotypical characterization of strains of Newcastle disease virus isolated from outbreaks in chicken and goose flocks in some regions of China during 1985–2001. *Arch. Virol.* **148**: 1387–1403. [[Medline](#)] [[CrossRef](#)]
17. Lole, K. S., Bollinger, R. C., Paranjape, R. S., Gadhari, D., Kulkarni, S. S., Novak, N. G., Ingersoll, R., Sheppard, H. W. and Ray, S. C. 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J. Virol.* **73**: 152–160. [[Medline](#)] [[CrossRef](#)]
18. Maminaiina, O. F., Gil, P., Briand, F. X., Albina, E., Keita, D., Andriamanivo, H. R., Chevalier, V., Lancelot, R., Martinez, D., Rakotondravao, R., Rajaonarison, J. J., Koko, M., Andriantsimahavandy, A. A., Jestin, V. and Servan de Almeida, R. 2010. Newcastle disease virus in Madagascar: identification of an original genotype possibly deriving from a died out ancestor of genotype IV. *PLoS One* **5**: e13987. [[Medline](#)] [[CrossRef](#)]
19. Mariappan, A. K., Munusamy, P., Kumar, D., Lathief, S. K., Singh, S. D., Singh, R. and Dhama, K. 2018. Pathological and molecular investigation of velogenic viscerotropic Newcastle disease outbreak in a vaccinated chicken flocks. *Virusdisease* **29**: 180–191. [[Medline](#)] [[CrossRef](#)]
20. Martin, D. P., Murrell, B., Khoosal, A. and Muhire, B. 2017. Detecting and analyzing genetic recombination using RDP4. *Methods Mol. Biol.* **1525**: 433–460. [[Medline](#)] [[CrossRef](#)]
21. McGinnes, L., Sergel, T. and Morrison, T. 1993. Mutations in the transmembrane domain of the HN protein of Newcastle disease virus affect the structure and activity of the protein. *Virology* **196**: 101–110. [[Medline](#)] [[CrossRef](#)]
22. McGinnes, L. W., Sergel, T., Chen, H., Hamo, L., Schwertz, S., Li, D. and Morrison, T. G. 2001. Mutational analysis of the membrane proximal heptad repeat of the newcastle disease virus fusion protein. *Virology* **289**: 343–352. [[Medline](#)] [[CrossRef](#)]
23. Mohamed, M. M., Amal, A. M. E., Walaa, M. and Ola, H. 2018. Genetic characterization of Egyptian Newcastle disease virus strains isolated from flocks vaccinated against Newcastle disease virus, 2014–2015. *Slov. Vet. Res.* **55**: 17–29.
24. Murrell, B., Moola, S., Mabona, A., Weighill, T., Sheward, D., Kosakovsky Pond, S. L. and Scheffler, K. 2013. FUBAR: a fast, unconstrained bayesian approximation for inferring selection. *Mol. Biol. Evol.* **30**: 1196–1205. [[Medline](#)] [[CrossRef](#)]
25. Nakamura, K., Ito, M., Nakamura, T., Yamamoto, Y., Yamada, M., Mase, M. and Imai, K. 2014. Pathogenesis of Newcastle disease in vaccinated chickens: pathogenicity of isolated virus and vaccine effect on challenge of its virus. *J. Vet. Med. Sci.* **76**: 31–36. [[Medline](#)] [[CrossRef](#)]
26. OIE 2018. Newcastle disease (Infection with Newcastle disease virus) (chap 3.3.14). pp. 964–983. *In: OIE Terrestrial Manual 2018: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, World Organisation for Animal Health, Paris.
27. Orabi, A., Hussein, A., Saleh, A. A., El-Magd, M. A. and Munir, M. 2017. Evolutionary insights into the fusion protein of Newcastle disease virus isolated from vaccinated chickens in 2016 in Egypt. *Arch. Virol.* **162**: 3069–3079. [[Medline](#)] [[CrossRef](#)]
28. Qin, Z. M., Tan, L. T., Xu, H. Y., Ma, B. C., Wang, Y. L., Yuan, X. Y. and Liu, W. J. 2008. Pathotypical characterization and molecular epidemiology of Newcastle disease virus isolates from different hosts in China from 1996 to 2005. *J. Clin. Microbiol.* **46**: 601–611. [[Medline](#)] [[CrossRef](#)]
29. Rehmani, S. F., Wajid, A., Bibi, T., Nazir, B., Mukhtar, N., Hussain, A., Lone, N. A., Yaqub, T. and Afonso, C. L. 2015. Presence of virulent Newcastle disease virus in vaccinated chickens in farms in Pakistan. *J. Clin. Microbiol.* **53**: 1715–1718. [[Medline](#)] [[CrossRef](#)]
30. Römer-Oberdörfer, A., Werner, O., Veits, J., Mebatsion, T. and Mettenleiter, T. C. 2003. Contribution of the length of the HN protein and the sequence of the F protein cleavage site to Newcastle disease virus pathogenicity. *J. Gen. Virol.* **84**: 3121–3129. [[Medline](#)] [[CrossRef](#)]
31. Samar, S. E., Ahmed, A., Sabry, M. T. and Hanafy, M. M. 2017. Molecular characterization of Newcastle disease virus (genotype VII) from broiler chickens in Egypt. *Suef Univ. J. Basic Appl. Sci.* **6**: 232–237.
32. Sabra, M., Dimitrov, K. M., Goraichuk, I. V., Wajid, A., Sharma, P., Williams-Coplin, D., Basharat, A., Rehmani, S. F., Muzyka, D. V., Miller, P. J. and Afonso, C. L. 2017. Phylogenetic assessment reveals continuous evolution and circulation of pigeon-derived virulent avian avulaviruses 1 in Eastern Europe, Asia, and Africa. *BMC Vet. Res.* **13**: 291. [[Medline](#)] [[CrossRef](#)]
33. Sergel-Germano, T., McQuain, C. and Morrison, T. 1994. Mutations in the fusion peptide and heptad repeat regions of the Newcastle disease virus fusion protein block fusion. *J. Virol.* **68**: 7654–7658. [[Medline](#)] [[CrossRef](#)]
34. Steward, M., Vipond, I. B., Millar, N. S. and Emmerson, P. T. 1993. RNA editing in Newcastle disease virus. *J. Gen. Virol.* **74**: 2539–2547. [[Medline](#)] [[CrossRef](#)]
35. Tamura, K., Nei, M. and Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. USA* **101**: 11030–11035. [[Medline](#)] [[CrossRef](#)]
36. Tamura, K., Stecher, G., Peterson, D., Filipowski, A. and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30**: 2725–2729. [[Medline](#)] [[CrossRef](#)]
37. Thampaisarn, R., Bui, V. N., Trinh, D. Q., Nagai, M., Mizutani, T., Omatsu, T., Katayama, Y., Gronsang, D., Le, D. H. T., Ogawa, H. and Imai, K. 2017. Characterization of avian paramyxovirus serotype 14, a novel serotype, isolated from a duck fecal sample in Japan. *Virus Res.* **228**: 46–57. [[Medline](#)] [[CrossRef](#)]
38. Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673–4680. [[Medline](#)] [[CrossRef](#)]
39. Umali, D. V., Ito, H., Shiota, K., Katoh, H. and Ito, T. 2014. Characterization of complete genome sequence of genotype VI and VII velogenic Newcastle disease virus from Japan. *Virus Genes* **49**: 89–99. [[Medline](#)] [[CrossRef](#)]
40. Umali, D. V., Ito, H., Suzuki, T., Shiota, K., Katoh, H. and Ito, T. 2013. Molecular epidemiology of Newcastle disease virus isolates from vaccinated commercial poultry farms in non-epidemic areas of Japan. *Virol. J.* **10**: 330. [[Medline](#)] [[CrossRef](#)]
41. Wang, Y., Yu, W., Huo, N., Wang, W., Guo, Y., Wei, Q., Wang, X., Zhang, S., Yang, Z. and Xiao, S. 2017. Comprehensive analysis of amino acid sequence diversity at the F protein cleavage site of Newcastle disease virus in fusogenic activity. *PLoS One* **12**: e0183923. [[Medline](#)] [[CrossRef](#)]
42. White, J. M., Delos, S. E., Brecher, M. and Schornberg, K. 2008. Structures and mechanisms of viral membrane fusion proteins: multiple variations on a common theme. *Crit. Rev. Biochem. Mol. Biol.* **43**: 189–219. [[Medline](#)] [[CrossRef](#)]
43. Wise, M. G., Suarez, D. L., Seal, B. S., Pedersen, J. C., Senne, D. A., King, D. J., Kapczynski, D. R. and Spackman, E. 2004. Development of a real-time reverse-transcription PCR for detection of newcastle disease virus RNA in clinical samples. *J. Clin. Microbiol.* **42**: 329–338. [[Medline](#)] [[CrossRef](#)]
44. Xiao, S., Nayak, B., Samuel, A., Paldurai, A., Kanabagattebasavarajappa, M., Prajitno, T. Y., Bharoto, E. E., Collins, P. L. and Samal, S. K. 2012. Generation by reverse genetics of an effective, stable, live-attenuated newcastle disease virus vaccine based on a currently circulating, highly virulent Indonesian strain. *PLoS One* **7**: e52751. [[Medline](#)] [[CrossRef](#)]
45. Xiao, S., Paldurai, A., Nayak, B., Samuel, A., Bharoto, E. E., Prajitno, T. Y., Collins, P. L. and Samal, S. K. 2012. Complete genome sequences of Newcastle disease virus strains circulating in chicken populations of Indonesia. *J. Virol.* **86**: 5969–5970. [[Medline](#)] [[CrossRef](#)]
46. Xu, Q., Sun, J., Gao, M., Zhao, S., Liu, H., Zhang, T., Han, Z., Kong, X. and Liu, S. 2017. Genetic, antigenic, and pathogenic characteristics of Newcastle disease viruses isolated from geese in China. *J. Vet. Diagn. Invest.* **29**: 489–498. [[Medline](#)] [[CrossRef](#)]
47. Yusoff, K., Nesbit, M., McCartney, H., Meulemans, G., Alexander, D. J., Collins, M. S., Emmerson, P. T. and Samson, A. C. 1989. Location of neutralizing epitopes on the fusion protein of Newcastle disease virus strain Beaudette C. *J. Gen. Virol.* **70**: 3105–3109. [[Medline](#)] [[CrossRef](#)]