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Original Research Article

GENOTYPING AND GENETIC EVOLUTION ANALYSIS OF NEWLY ISOLATED NEWCASTLE DISEASE VIRUS IN EGYPT

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Abstract: In spite of enormous vaccination programs that were implemented in the Egyptian poultry farms, Newcastle Disease Virus (NDV) remains one of the major concerns to the poultry industry. Therefore, molecular analysis for the circulating NDV strains is crucial to monitor their genetic evolution. Twenty-three tracheal samples were collected from vaccinated broiler, layer and breeder flocks suffered from respiratory, nervous symptoms and drop in egg production between October 2019 and December 2020. Only ten samples (10/23; 43.5%) showed HA activity after propagation into ECE while only six samples were positive for Avian avulavirus 1 based on real time RT-PCR. Nucleotide sequences for both F and HN genes showed high similarity to recently reported Egyptian NDV isolates. Sequencing for the F protein cleavage site showed the typical sequence of velogenic NDV strains (^{112}R-R/K-Q-K-R↓F^{117}). Deduced amino acid analyses for the cleavage site, fusion peptide, glycosylation sites, heptad repeat region and transmembrane domain of F protein were conducted along with the transmembrane domain of HN protein that showed different substitutions in comparison with the commonly used vaccine strains. Phylogenetic analyses for the reported isolates in this study based on the full-length F gene revealed their clustering within sub-genotype VII.1.1. 3D protein structural modelling suggested that amino acids substitutions within the fusion peptide sequences result in conformational changes in the F protein structure. In conclusion, NDV continues to evolve and further in vivo studies are strongly recommended to define the precise efficacy of applied NDV vaccines against the circulating strains in Egypt.

Key words: poultry; Newcastle Disease; fusion protein; genotype VII; velogenic; 3D protein structure

Introduction

Newcastle Disease (ND) is one of the most important viral diseases affecting poultry, which caused by Avian avulavirus 1 (APMV-1) (1). A wide range of birds are considered as a natural hosts of Newcastle Disease virus (NDV) including chickens, turkeys, ducks, geese, pigeons, quail, pheasants, guinea fowl, ostriches, and many wild birds' species (2). ND is a highly contagious acute notifiable viral disease affects domestic poultry regardless of bird's age or sex (3, 4) causing great economic losses to the poultry industry worldwide and especially in developing countries (5). OIE ranked NDV as the second-highest endemic disease in many countries (6). Poultry flocks with mixed viral infections beside NDV infection like infectious bronchitis virus (IBV), low pathogenic avian influenza viruses (LPAIV), high pathogenic avian influenza viruses (HPAIV) or avian reovirus, usually have greater losses (19, 20–21). In the past years, many NDV outbreaks were reported in many countries, as Japan (8), Brazil (9), China (10), South America (11) and Malaysia (12). NDV

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was reported in Egypt in 1942 (13), and there have been multiple reports since then (14, 15, 16). Recently, many vaccinated or unvaccinated Egyptian poultry flocks have shown outbreaks of NDV (17, 18). Improper NDV vaccination or altered immunity are considered the main causes for ND outbreaks in vaccinated flocks (42). NDV infection can be manifested, as respiratory, intestinal, and/or neurological form with mortality rate can reach up to 100% depending on the virus strain and its pathogenicity (22). NDV strains are classified into five pathotypes based on their pathogenicity; asymptomatic enteric, lentogenic, mesogenic, neurotropic velogenic, and viscerotropic velogenic (23). NDV is an enveloped virus; has a single-stranded linear negative sense RNA genome (~15.2 kb); belongs to family Paramyxoviridae, order Mononegavirales (24- 26). NDV genome encodes six structural proteins; including RNA-dependent RNA polymerase (L), hemagglutinin-neuraminidase protein (HN), fusion protein (F), matrix protein (M), phosphoprotein (P), and nucleoprotein (N) (27). Also, it encodes for two nonstructural proteins (V) and (W) proteins via P protein mRNA editing (28). Both F and HN proteins are membrane glycoproteins, and involved in the antigenic and pathogenic determination of NDV strains (27). The F protein and its cleavage site sequence is the most important key in determining the pathogenicity of NDV (16, 29). HN has a neuraminidase (NA) activity which mediates the virus attachment to sialic acid-containing receptors (39). Sequence analyses for both F and HN membrane glycoproteins are widely used for the molecular identification of NDV strains (22). Brevity, most of NDV vaccination programs currently applied in the Egyptian poultry industry include live and/or inactivated NDV genotype I, genotype II and genetically modified vaccines (19, 40).

Many classification systems were used to classify the NDV strains based on sequencing and phylogenetic analyses (28, 3132, – 36). The first unified NDV classification system was suggested in 2012 (37) where NDV strains were divided into class I and class II. Class I was subdivided into only one genotype, whereas class II were subdivided into at least eighteen genotypes (38). Recently, an updated and unified classification system relied on the phylogenetic analysis of full F gene with a revised nomenclature for Newcastle disease viruses (NDVs) were generated (32) where class II genotypes were supposed to be grouped into about twenty-one genotypes, with some subgenotypes. NDV strains of genotype VII are accused of being responsible for the fourth NDV panzootic that has spread between Asian, African, and European countries (41). In the current study, the full nucleotides sequence analyses for both F and HN genes were carried out for recently isolated and characterized NDV strains collected from different Egyptian poultry flocks during the period from Oct 2019 to Dec 2020.

Materials and methods

Ethical approval

Institutional, national and international guidelines for animal care have been followed.

Sampling and samples history

Tracheal swabs were collected from affected flocks, a total of twenty-three samples were collected from different poultry flocks (broilers (n=15), breeder (n=3), and layers (n=5) from nine Egyptian governorates (Table 5).

Virus Isolation

The collected tracheal swabs were immersed in Phosphate-Buffered Saline (PBS) supplemented with gentamycin (50 µg/ml) and Mycostatin (1000 units/mL).

Initially, samples were named numerically in order manner; for example, sample of flock 1 (F1), sample of flock 23 (F23). Virus clarification was done via centrifugation of each sample at 5000 rpm for 15 minutes. Ten-day-old specific pathogen free embryonated chicken eggs (SPF-ECEs) were inoculated with 200 µl of supernatant fluid from each sample through the allantoic cavity and incubated for 3- 5 days with daily inspection. The allantoic fluid from each egg was collected and tested for hemagglutination (HA) activity based on slide HA test Table (5). Collectively, HA positive samples were named numerically as Sample 1 (S1), Sample 2 (S2) etc.

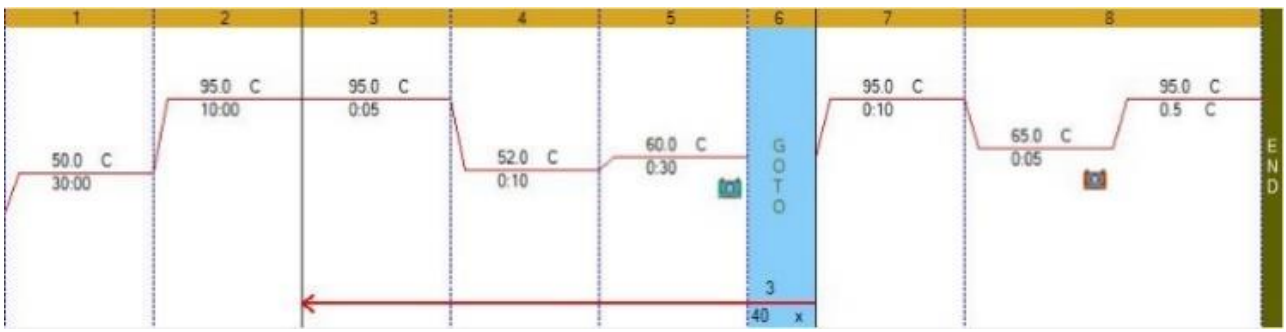

Figure 1: Thermal conditions applied at RT-qPCR and for the melting curve

Bio-Rad CFX manager 3.1 software was used for the calculation of melting temperature (Tm) of melting curves and Cp values (22)

Table 1: Primers used for full F and HN proteins genes amplification

Primer name	Sequence (5′ to 3′)	Position *	Product size bp
7S	GTGAAYTTTGTCTCCTTGAC	3806-3825	981
7A	GAGGCATGTGCRAAAGC	4779-4763	
8S	TTGAYGGCAGGCCTCTTG	4638-4655	912
8A	GTGATAGAAGARCTTGACACCTC	5550-5528	
9S	ATAATATGCGTGCCACCTA	5430-5452	905
9A	ATAYACGGGTAGAACGGT	6334-6317	
10S	TGGCTTGGGAAYAATACCCT	6155-6174	986
10A	TGCAGTGTGAGTGCAACT	7140-7123	
11S	CGGACATCTGCAACAGG	7045-7061	952
11A	GCAGCATACACAACATC	7997-7981	
12S	TTCGGGACRATGCTTGAT	7873-7890	1013
12A	GCATTCTGGTTTCACTCAA	8882-8864	

S: the sense primer, A: the anti-sense primer

* Positions are based on the full-length sequence of LaSota strain of NDV (GenBank accession number: AF077761) (45)

Table 2: Showing gene bank Accession numbers (n =12) of F and HN proteins genes sequences of the study isolates (n=6)

Isolates	Nomenclature	F protein gene bank Accession No.	HN protein gene bank Accession No.
Isolate 1	CK/Egypt/FAY-ZU/Dec-2019/	MZ826277	MZ826283
Isolate 2	CK/Egypt/GIZ-ZU/Feb-2020/	MZ826278	MZ826284
Isolate 3	CK/Egypt/FAY-ZU/Apr-2020/	MZ826279	MZ826285
Isolate 4	CK/Egypt/BENI-ZU/Oct-2020/	MZ826280	MZ826286
Isolate 5	CK/Egypt/MON-ZU/Nov-2020/	MZ826281	MZ826287
Isolate 6	CK/Egypt/FAY-ZU/Nov-2020/	MZ826282	MZ826288

Viral RNA extraction and Real-time reverse transcription-polymerase chain reaction (RT-qPCR)

Pure Link® (Invitrogen, USA) RNA Mini Kit was used for viral RNA extraction from HA positive allantoic fluids according to its manual instructions. TOPreal™ One-step - SYBR Green with low ROX - RT qPCR Kit (Enzynomics, Korea) was used for RT-qPCR of APMV-1 detection using CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories, USA).

Pair of specific primers for a conserved region of the matrix (M) gene of APMV-1were used as described earlier (44) (Figure 1)

F and HN genes amplification

Positive samples based on RT-qPCR was subjected to one-step RT-PCR using six sets of primes (Table 1) (45) using SuperScript™ III One-Step RT-PCR System according to the manufacturer's instructions using ProFlex PCR thermal cycler (Applied biosystem, USA).

The PCR products were analyzed by agarose gel electrophoresis (1%) and then purified using the

QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions.

Sequencing and Genetic analysis

The purified RT-PCR products were sequenced using Bigdye Terminator V3.1sequencing kit (Perkin Elmer, Foster City, CA) within the Applied Biosystems 3130 Gene Analyzer (ABI, USA). Bioedit software version 7.0.4.1 (46) was used to check the quality, assemble and edit the obtained sequences then the online BankIt tool of the GenBank (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank was used for sequences submission to GenBank and the accession numbers were mentioned in Table 2.

Phylogenetic analysis

The preliminary phylogenetic analysis was based on a pilot tree as previously described (32) to be utilized for rapid genotyping and identification of the new isolates.

Using the neighbor-joining method; bootstrapping at 1000 replicates and the tree was constructed by within Mega 6 software version 7.0.26 (47).

Results

Hemagglutination (HA) assay

After three passages, only ten samples showed positive HA and named as follows; S1-S10 Table (5).

RT-qPCR for detection of NDV

Only six samples out of ten (6/10) positive HA samples were positive for Avian avulavirus 1 by RT-qPCR and named as follows; Isolate 1, 2, till isolate 6 Figures (2, 3 and 4).

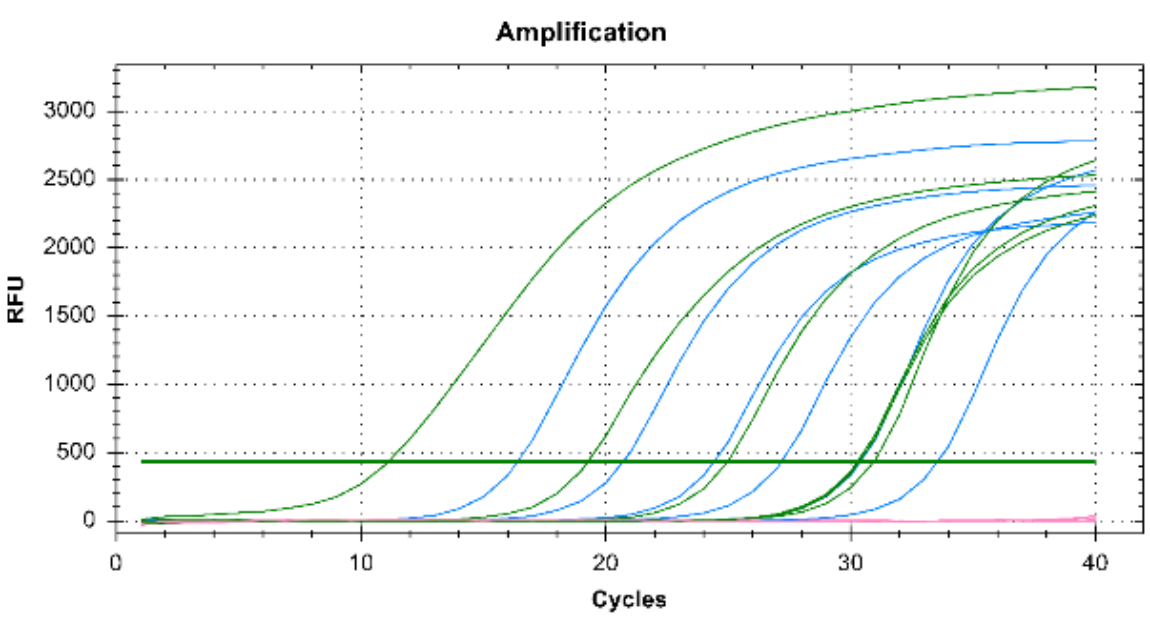

Figure 2: Threshold cycles of tested samples, blue lines (n=6) represent positive control samples (standard curve samples), the green lines (n=6) represent positive for Avian avulavirus samples, and pink lines (n=4) represent the negative for Avian avulavirus samples

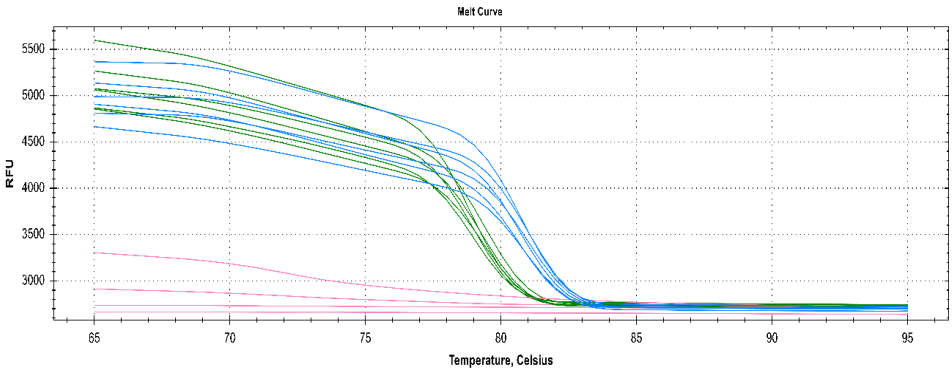

Figure 3: Melting curve of tested samples, blue lines (n=6) represent positive control samples (standard curve samples), the green lines (n=6) represent positive for Avian avulavirus samples, and pink lines (n=4) represent the negative for Avian avulavirus samples

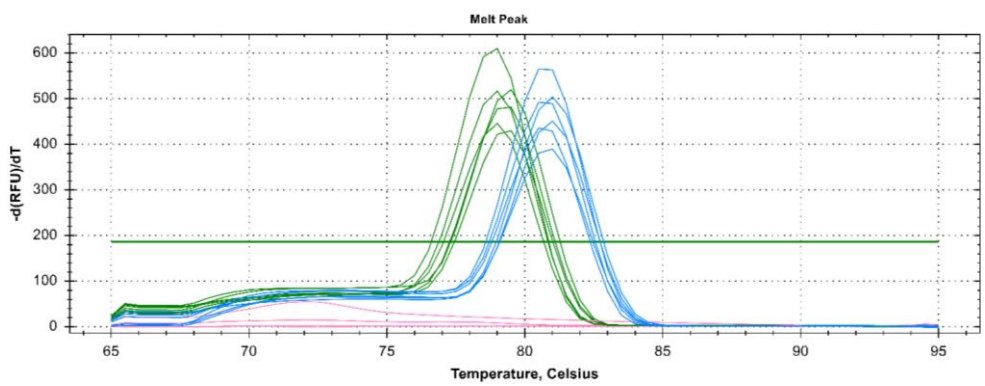

Figure 4: Melting peak of tested samples, blue lines (n=6) represent positive control samples (standard curve samples), the green lines (n=6) represent positive for Avian avulavirus samples showed a different melting peak, and pink lines (n=4) represent the negative for Avian avulavirus samples.

F gene Genetic analysis

Blasting the obtained full-length F gene sequences for isolates 1, 2, 3, 4, and 6 showed high similarities with old Egyptian isolates ranged from 98.74 % to 99.76 % while Isolate 5 showed less similarity between 96.95 % and 97.17 %.

Evolutionary distance analyses for our isolates (n=6) reported in this study compared to other Egyptian isolates, and commonly used vaccine strains. Showed in supplementary table 1.

All the six isolates showed multiple basic amino acid residues at the cleavage site (F0) of the F protein ^{112}R-R-Q-K-R^{116} sequence, except the CK/Egypt/FAY-ZU/Dec-2019 isolate (Isolate 1) that has 12R-K-Q-K-R116 sequence with an interesting R113K substitution. In addition, all isolates have a phenylalanine residue at position 117 (116↓F^{117}) (Table 7). The fusion peptide sequence (aa117– 141) within the six isolates exhibited the same substitutions; L117F, I121V, and G124S.

In comparison with the commonly applied NDV vaccines, all our isolates showed amino acid substitution at the 2nd glycosylation site (aa 191-193) as follows; K192N.

Also, isolates 2 (CK/Egypt/GIZ-ZU/Feb-2020), 3 (CK/Egypt/FAY-ZU/Apr-2020), and 4 (CK/Egypt/BENI-ZU/Oct-2020), showed the same substitution at the 4th glycosylation site (aa 471-473) as follows; S473F.

All the six isolates showed the same substitutions at the heptad repeats (HR) regions [HRa (aa 143–185), HRb (aa 268–299), and HRc (aa 471–500)] and at the transmembrane region (aa from 501-521) of F protein.

Only K78R substitution was noticed in all six isolates from all of the predicted F protein antigenic epitopes.All F protein gene substitutions presented in Table 3

Phylogenetic tree based on of the full-length F gene showed that all our six isolates are closely related to genotype VIIVII.1.1 (Figure 5)

Table 3: Summarizing the amino acids substitutions at Cleavage site, fusion peptide, glycosylation sites, heptad repeat region and transmembrane domain of the F protein of the study isolates in comparison to the same amino acids sequences of the most commonly applied NDv vaccinal strains

	Cleavage site (112-116)	Fusion peptide (117– 141)	Glycosylation sites					Heptad repeat (HR) regions			Transmembrane domain (501–521)
								HRa (143–185)	HRb (268–299)	HRc (471–500)	
JF950510.1_LaSota *	G-R-Q-G-R	LIGAIIGGVALG VATAAQITAAAAL	85N-R-T87	191N-K T193	366N-T-S368	471N-N-S473	541N-N-T543	QAKQNAANILRLKESIAATNEA VHEVTDGLSQLAVAVGKMQQF	LITGNPILYDSQTQLL GIQVTLPSVGNLNNMR	NNSISNALNKLEESNR KLDKVNVKLTSTSA	LITYIVLTIISLVFGILSLIL
KM056356.1_Avinew	R113K	A139S	===	===	===	===	===	K145N, D170N	===	N479D, R486S	I509V, F514C, I520V
Y18898.1_Clone_30	===	===	===	===	===	===	===	===	===	===	===
AF309418.1_HB1	===	===	===	===	===	===	===	===	===	===	===
AY935499.2_I-2	G112R, R113K	A139S	===	===	===	===	===	K145N	T288I	N479D, R486S, T496A	I509V, V513I, F514C, I520V
CK/Egypt/FAY-ZU/Dec-2019	G112R, R113K, G115K	L117F, I121V, G124S	===	K192N	===	===	===	A176S	N272Y, Q279H, T288N	N479D, K480R, E482A, R486S, D489E, K494R	I509V, V513I, I516A, I520A
CK/Egypt/GIZ-ZU/Feb-2020	G112R, G115K	L117F, I121V, G124S	===	K192N	===	S473F	===	A176S	L268R, N272Y, Q279H, T288N	S473F, N479D, K480R, E482A, R486S, D489E, K494R	I509V, V513I, I516A, I520A
CK/Egypt/FAY-ZU/Apr-2020	G112R, G115K	L117F, I121V, G124S	===	K192N	===	S473F	===	A176S	N272Y, Q279H, T288N	S473F, N479D, K480R, E482A, R486S, D489E, K494R	I509V, V513I, I516A, I520A
CK/Egypt/BENI-ZU/Oct-2020	G112R, G115K	L117F, I121V, G124S	===	K192N	===	S473F	===	A176S	N272Y, Q279H, T288N	S473F, S475G N479D, K480R, E482A, R486S, D489E, K494R	I509V, V513I, I516A, I520A
CK/Egypt/MON-ZU/Nov-2020	G112R, G115K	L117F, I121V, G124S	===	K192N	===	===	===	A176S	N272Y, Q279H, T288N	N479D, K480R, E482A, R486S, D489E, K494R	I509V, V513I, I516A, I520A
CK/Egypt/FAY-ZU/Nov-2020	G112R, G115K	L117F, I121V, G124S	===	K192N	===	===	===	A176S	N272Y, Q279H, T288N	N479D, K480R, E482A, R486S, D489E, K494R	I509V, V513I, I516A, I520A

*LaSota NDv strain Amino acids sequence was used as the reference sequence according which the substitutions were illustrated

Table 4: Summarizing the amino acids substitutions at transmembrane domain, Head–stalk linker region, Heptad repeats (HR) region, Neutralizing epitopes at C-terminal globular head, predicted linear B-cell epitope, and thermostability residues of the HN protein of the study isolates in comparison to the same amino acids sequences of the most commonly applied NDv vaccinal strains

	Transmembrane domain	Head–stalk linker region	Heptad repeats (HR) region		Neutralizing epitopes at C-terminal globular head											Predicted linear B-cell epitope	Thermo-stability	
	25–45	115-122	HRa(74-88)	HRb(96-110)	(193-201)	263	287	321	332	333	(345–353)	362	494	(513–521)	569	266	315	369
JF950510.1_LaSota *	FRIAILFLTVV TLAISVASLL	NGAANNSG	LGSNQDV VDRIYKQV	LLKTETT IMNAITSL	LSGCRDHSH	N	D	K	G	K	PDEQDYQIR	G	G	RITRVSSSS	D	V	S	I
KM056356.1_Avinew	F31L, L32S, V41A, S43A, L45A	N120S	==	K98N, T101S	==	==	==	==	==	==	R353Q	==	D	==	==	I	P	V
Y18898.1_Clone_30	==	==	==	K98N, T101S	==	==	==	==	==	==	==	==	==	==	==	==	==	==
AF309418.1_HB1	==	==	==	K98N, T101S	==	==	==	==	==	==	==	==	==	==	==	==	==	==
AY935499.2_I-2	I39V, V41A, S43A, L45A	N120S	==	K98N, T101S	==	==	==	R	==	==	==	==	D	S520G	==	A	P	V
CK/Egypt/FAY-ZU/Dec-2019	I29V, F31L, T33M, V34A, T35M, T36I, V41A, S43A, L45A	A118E, N120S	G75S, N77G, V81I	K98N, T101S, T102V	==	K	==	==	==	==	E347K	A	D	I514V	==	A	P	V
CK/Egypt/GIZ-ZU/Feb-2020	I29V, F31L, T33M, V34A, T35M, T36I, V41A, S43A, L45A	A118E, N120S	G75S, N77G, V81I	K98N, T101S, T102V	==	K	==	==	==	==	E347K	A	D	I514V	==	A	P	V
CK/Egypt/FAY-ZU/Apr-2020	I29V, F31L, T33M, V34A, T35M, T36I, V41A, S43A, L45A	A118E, N120S	G75S, N77G, V81I	K98N, T101S, T102V	==	K	==	==	==	==	E347K	A	D	I514V	==	A	P	V
CK/Egypt/BENI-ZU/Oct-2020	I29V, F31L, T33M, V34A, T35M, T36I, V41A, S43A, L45A	A118E, N120S	G75S, N77G, V81I	K98N, T101S, T102V	==	K	==	==	==	==	E347K	A	D	I514V	==	A	P	V
CK/Egypt/MON-ZU/Nov-2020	I29V, F31L, T33M, V34A, T35M, T36I, V41A, S43A, L45A	A118E, N120S	G75S, N77G, V81I	K98N, T101S, T102V	==	K	==	==	==	==	E347K	A	D	I514V	==	A	P	V
CK/Egypt/FAY-ZU/Nov-2020	I29V, F31L, T33M, V34A, T35M, T36I, V41A, S43A, L45A	A118E, N120S	G75S, N77G, V81I	K98N, T101S, T102V	==	K	==	==	==	==	E347K	A	D	I514V	==	A	P	V

*LaSota NDv strain Amino acids sequence was used as the reference sequence according which the substitutions were illustrated

Table 5: Showing flock history*, HA activity results, RT-qPCR results, and study isolates nomenclature.

Flock No.	Flock type	Date (Month/ 2020 or 2019)		Governorate	Birds No/Flock	Main symptoms					Age of birds Days (D) or (W)		HA Activity (Positive or Negative)			+Ve HA Samples	RT-qPCR results to NDv (+Ve or -Ve)	+Ve NDv Isolates names	Isolates nomenclature
						Mortality %	Res**	GIT**	Neu**	Egg***			1st passage	2nd passage	3rd passage				
F 1	Broilers [1]	Oct	2019 [1]	Giza [1]	10,000	10 %.	++	----	----	----	32	D	Negative	Positive	S 1		-Ve	----	----
F 2	Breeders [1]	Nov	2019 [2]	Monufia [1]	25,000	----	----	----	----	L	40	W	Negative		----		----	----	----
F 3	Broilers [2]	Nov	2019 [3]	Giza [2]	11,000	10 %	----	----	+	----	22	D	Negative		----		----	----	----
F 4	Broilers [3]	Dec	2019 [4]	Gharbia [1]	4,000	8 %	+	----	+	----	26	D	Negative		----		----	----	----
F 5	Broilers [4]	Dec	2019 [5]	Fayoum [1]	15,000	22.4%.	++	++	----	----	27	D	Positive		S 2		+Ve	Isolate 1	CK/Egypt/FAY-ZU/Dec-2019
F 6	Breeders [2]	Jan	2020 [1]	Beheira [1]	10,000	----	----	----	----	L	60	W	Negative		----		----	----	----
F 7	Layers [1]	Jan	2020 [2]	Beni Suef [1]	7,000	----	----	----	----	L	42	W	Negative		----		----	----	----
F 8	Broilers [5]	Feb	2020 [3]	Giza [3]	4,000	24.3%.	++	++	+		41	D	Positive		S 3		+Ve	Isolate 2	CK/Egypt/GIZ-ZU/Feb-2020
F 9	Layers [2]	April	2020 [4]	Fayoum [2]	7,000	----	----	----	----	L	49	W	Negative	Positive	S 4		+Ve	Isolate 3	CK/Egypt/FAY-ZU/Apr-2020
F 10	Layers [3]	May	2020 [5]	Qalyubia [1]	9,000	----	----	----	----	L	53	W	Negative		----		----	----	----
F 11	Broilers [6]	Jun	2020 [6]	Beheira [2]	12,000	5.7 %	++	----	----	----	25	D	Negative		----		----	----	----
F 12	Broilers [7]	July	2020 [7]	Beni Suef [2]	12,500	6.8 %	++	----	----	----	26	D	Negative		----		----	----	----
F 13	Broilers [8]	Aug	2020 [8]	Beni Suef [3]	12,000	8.4 %	++	----	----	----	23	D	Negative		----		----	----	----
F 14	Broilers [9]	Aug	2020 [9]	Giza [4]	13,000	9.3 %	++	----	----	----	27	D	Negative		----		----	----	----
F 15	Layers [4]	Sep	2020 [10]	Fayoum [3]	10,000	----	----	----	----	L	37	W	Negative		----		----	----	----
F 16	Broilers [10]	Oct	2020 [11]	Beni Suef [4]	17,000	7 %	++	++	----	----	31	D	Positive		S 5		+Ve	Isolate 4	CK/Egypt/BENI-ZU/Oct-2020
F 17	Layers [5]	Nov	2020 [12]	Qalyubia [2]	5,000	----	----	----	----	L	38	W	Negative		----		----	----	----
F 18	Broilers [11]	Nov	2020 [13]	Monufia [2]	13,000	11.7 %	++	++	----	----	29	D	Positive		S 6		+Ve	Isolate 5	CK/Egypt/MON-ZU/Nov-2020
F 19	Broilers [12]	Nov	2020 [14]	Fayoum [4]	12,500	54.8 %	++	++	+	----	39	D	Positive		S 7		+Ve	Isolate 6	CK/Egypt/FAY-ZU/Nov-2020
F 20	Broilers [13]	Dec	2020 [16]	Qena [1]	10,000	60 %	+++	+++	----	----	24	D	Positive		S 8		-Ve	----	----
F 21	Breeders [3]	Dec	2020 [16]	Monufia [3]	25,000	----	----	----	----	L	63	W	Negative		----		----	----	----
F 22	Broilers [14]	Dec	2020 [17]	Minya [1]	9,000	55 %	++	++	----	----	27	D	Positive		S 9		-Ve	----	----
F 23	Broilers [15]	Dec	2020 [18]	Qalyubia [3]	8,000	70 %	+++	----	----	----	25	D	Positive		S 10		-Ve	----	----

*(flock type, sampling date, birds number, birds age, governorate, main symptoms and mortalities %), **Respiratory (Res), Intestinal (GIT), and Neurological (Neu) symptoms, *** L indicating lowered egg production

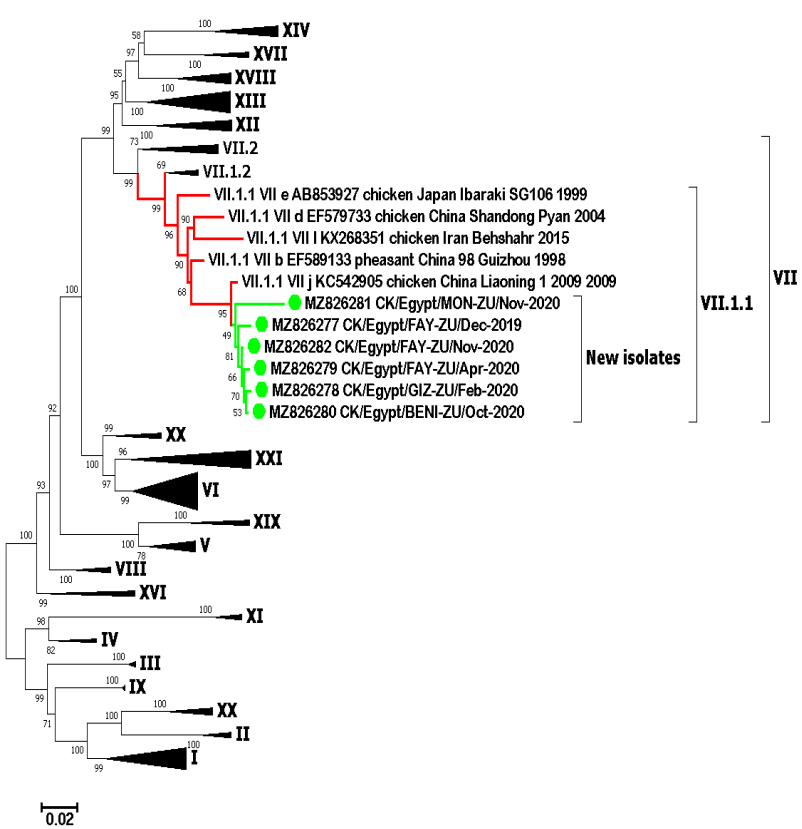

Figure 5: Neighbor-joining phylogenetic tree of the full-length F gene of the study isolates of in comparison with pilot tree suggested by (32) to be used for rapid genotyping and identification of new isolates. Bootstrap values are shown above the branches. New Isolates are indicated by a solid green circle.

HN gene Genetic analysis

Analyzing the sequence results of full length HN gene revealed that our isolates have high similarity with previously reported Egyptian isolates varied from 98.31 % to 99.42 %. The nucleotide identity for our isolates (n=6), other Egyptian isolates (n=17), and commonly used NDV vaccine strains (n=5) was shown in supplementary table 2.

Comparison between the commonly used NDV vaccine strains and our reported isolates revealed nine amino acids substitutions at transmembrane domain while the head–stalk linker region sequence analysis showed the same two substitutions among all isolates. All six isolates showed three substitutions at HRa with additional three substitutions at HRb of heptad repeats (HR) region of HN protein sequence.

All of the study isolates showed the same five a.a. substitutions at different neutralizing epitopes at C-terminal globular head of HN protein.

All isolates showed thermostable substitutions at residues of the HN protein among the studied isolates.

All HN protein gene substitutions publicized in Table 4

Discussion

The poultry industry experienced severe economic losses as a result of the continuous and recurrent NDV outbreaks in spite of the mass vaccination programs either in Egypt (18) or worldwide (41).

Many reports proposed that one of the noticeable causes of NDV outbreaks is replying on high genetic differences between field strains and the applied vaccines leading to non-protective immune (48).

In the current study, six (6/23) NDV isolates are classified as genotype VII.1.1 (or VII j) (22, 41, 49)

Mixed infection with other hemagglutinating viruses as influenza (H9 or H5) may be the cause behind the negative RT-qPCR results [four positive HA samples; S1, S8, S9, and S10 Table (5).

There are pathogenicity and virulence indicators (post mortem lesions) are well known for the identification for any NDV outbreak, however the molecular and phylogenetic analysis of F gene sequence with focus on cleavage site sequence can be used for initial characterization (15, 50, 51).

Our results revealed that F protein cleavage site sequences for five (5/6) isolates has polybasic sequence of velogenic NDV strains ^{112}R-R-Q-K-R^{116} (51). While, one isolate (1/6) showed an interesting R113K substitution R113K, which is characteristic substitution for NDV genotype I and genotype XX (32) (Table 3). These results highlight the questionable role of live vaccines on virulent NDV evolution.

Our six isolates reported in this study have a phenylalanine residue at position 117, which could explain the neurological symptoms recorded at broiler flocks infected with similar isolates (52).

Five potential glycosylation sites with the following motif Asn-X-Ser/Thr (N-X-S/T, where X can be any residue except aspartic acid [D], and proline [P]) in the F protein were reported in our isolates in the current study similar to previous studies (53). These sites include aa 85-87, aa 191-193, aa 366-368, aa 471-473 and aa 541-543, and they are highly conserved in majority of NDV genotypes.

HN gene sequence length for all six isolates was 1716 bp nucleotides length encodes for 571 residues, which is a common feature for most of virulent NDV strains (53, 54, 55).

Compared to the most applied vaccine strains, all our six isolates have A176S substitution at the HRa, which thought to be a potential antigenic epitope (56, 57, 58) which also may affect the fusion activity of the F protein (28,52,53,54).

In addition, they showed only a K78R substitution in the F protein antigenic epitopes (K87) among the predicted F protein antigenic epitopes (D72, E74, A75, R78, A79, S157, T161, D170, G171, L343, A378 residues) (59)

There are many substitutions were noticed within the neutralizing epitopes at C-terminal globular head of HN protein sequence, all of them are characteristic substitutions for genotype VII.1.1 (40) including the E347K substitution that help the virus to escape from the neutralizing antibodies (60, 61).

Our results might explain the ability of some NDV strains to escape from the immunity that induced by current vaccines (17, 18, 42).

There are three identical substitutions within the six isolates within their fusion peptide sequence (117– 141), which play a vital role in conformation, folding and fusion activities of the F protein (62, 63).

In conclusion, our collective results revealed the continuous evolution of velogenic NDV genotype VII.1.1 in Egypt and highlighting the correlation between the NDV field strains and applied commercial vaccine.

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References

1. USDA. Livestock and poultry: world markets and trade. Washington: United States Department of Agriculture, Foreign Agricultural Service Office of Global Analysis, 2019. https://www. fas.usda.gov/ data/livestock-and-poultry-world-markets-andtrade.

2. Eurostat. Poultry meat production in EU at new high in 2018. Products Eurostat News, 2018. https://ec.europa.eu/eurostat/web/products-eurstat-news/-/DDN-20190325-1

3. U. S. Poultry and egg association. Economic data 2019. Tucker, Georgia, 2019. http://www. poutryegg. org/economic data/

4. Saad AM, Samy A, Soliman MA, Arafa A, Zanaty A, Hassan MK, and Hussein AH (2017). Genotypic and pathogenic characterization of genotype VII Newcastle disease viruses isolated from commercial farms in Egypt and evaluation of heterologous antibody responses. Archives of Virology, 162(7): 1985–1994. DOI: https://doi.org /10.1007/s00705-017-3336-y.

5. Westbury HA (2001). Newcastle disease virus: an evolving pathogen. Avian Pathology, 30:

6. World Health Organization. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Chapter 2.3.14). (2012). Available online at:https://www.oie.int /doc/ged/d12009.pdf (acessed March 8, 2014).

7. Shahid M, Muhammad Q, Al-Ghanim KA, Al-Misned F, and Al-Mulhim N (2020). Isolation, identification and molecular characterization of Newcastle disease virus using SDS-PAGE. Journal of King Saud University -Science, 32(1): 1000-1003. doi: https://doi.org/10.1016/ j.jksus.2019.09.005.

8. Mase M, Imai K, Sanada Y, Sanada N, Yuasa N, Imada T, Tsukamoto K, and Yamaguchi S (2002). Phylogenetic analysis of Newcastle disease virus genotypes isolated in Japan. Journal of Clinical Microbiology, 40: 3826– 3830. doi: https://doi.org/10.1128/jcm.40.10 .3826-3830 .2002.

9. Marks FS, Rodenbusch CR, Okino CH, Hein HE, Costa EF, Machado G, Canal CW, Brentano L, and Corbellini LG (2014). Targeted survey of Newcastle disease virus in backyard poultry flocks located in wintering site for migratory birds from Southern Brazil. Preventive Veterinary Medicine, 116: 197-202. DOI: https://doi.org/10.1016/j.prevetmed.2014.06.001.

10. Kang Y, Li Y, Yuan R, Li X, Sun M, Wang Z, Feng M, Jiao P, and Ren T (2014). Phylogenetic relationships and pathogenicity variation of two Newcastle disease viruses isolated from domestic ducks in Southern China. Virology Journal, 11: 147- 159. DOI: https://doi.org/10.1186/1743-422X-11-147.

11. Diel DG, Susta L, Cardenas Garcia S, Killian ML, Brown CC, Miller PJ, and Afonso CL (2012). Complete genome and clinicopathological characterization of a virulent Newcastle disease virus isolate from South America. Journal of Clinical Microbiology, 50:378–387. doi: https://doi.org/10.1128/JCM.06018-11.

12. Jaganathan S, Ooi PT, Phang LY, Allaudin ZN, Yip LS, Choo PY, Lim BK, Lemiere S, and Audonnet JC (2015). Observation of risk factors, clinical manifestations and genetic characterization of recent Newcastle disease virus outbreak in West Malaysia. BMC Veterinary Research, 11: 219-227. doi: https://doi.org /10.1186/s12917-015-0537-z.

13. Daubeny R. and Mansy W (1947). The occurrence of Newcastle disease in Egypt. Journal of Comparative Pathology and Therapeutics, 58: 189-200. DOI: https://doi.org/10.1016/S0368-1742(48)80019-6.

14. Hussein HA, Emara MM, Samy AM, and Shalaby MA (2000). Antigenic diversity of Newcastle disease virus isolated from 52 breeder and broiler flocks in Egypt.The Egyptian .J .of Imm .Vet, 2: 3-15. Available at Egyptian Journal of Veterinary Sciences http://www.members.tripod.com/ejimmunology/previous/jun00/jun00-1.html.

15. Mohamed MHA, Kumar S, Paldurai A, and Samal SK (2011). Sequence analysis of fusion protein gene of Newcastle disease virus isolated from outbreaks in Egypt during 2006. Virology Journal, 8: 237-240. doi: https://doi.org/10.1186/1743-422X-8-237.

16. Selim KM, Selim A, Arafa A, Hussein HA, and Elsanousi AA (2018). Molecular characterization of full fusion protein (F) of Newcastle disease virus genotype VIId isolated from Egypt during 2012-2016. Veterinary World, 11(7) 930–938. doi: https://doi.org/10.14202 /vetworld.2018. 930-938.

17. Abd El Aziz M, Abd El-Hamid H, Ellkany H, Nasef S, Nasr S, and El Bestawy A (2016). Biological and molecular characterization of Newcastle disease virus circulating in chicken flocks, Egypt, during 2014-2015. Zagazig Veterinary Journal, 44(1): 9–20. DOI: https://dx.doi.org/10. 21608/zvjz.2016.7827.

18. Ewies SS, Ali A, Tamam SM, and Madbouly HM (2017). Molecular characterization of Newcastle disease virus (genotype VII) from broiler chickens in Egypt. Beni-Suef University Journal of Basic and Applied Sciences, 6

(3): 232-237. DOI: https://doi. org/10.1016/j.bjbas.2017 .04.004.

19. Hassan KE, Shany S, Ali A, Dahshan A, El-Sawah AA, El-kadyM. Prevalence of avian respiratory viruses in broiler flocks in Egypt. Poult Sci. (2016) 95:1271–80. doi: 10.3382/ps/pew068.

20. Mansour SM, Mohamed FF, Eid AA, Mor SK, Goyal SM. Co-circulation of paramyxo-and influenza viruses in pigeons in Egypt. Avian Pathol. (2017) 46:367–75. doi: 10.1080/03079457.2017.1285391.

21. Abd El-Hamid HS, Shafi ME, Albaqami NM, Ellakany HF, Abdelaziz NM, Abdelaziz MN, et al. Sequence analysis and pathogenicity of Avian Orthoavulavirus 1 strains isolated from poultry flocks during 2015–2019. BMC Vet Res. (2020) 16:1–15. doi: 10.1186/s12917-020- 02470-9

22. Shakal M, Maher M, Metwally AS, AbdelSabour MA, Madbbouly YM, Safwat G (2020). Molecular Identification of a velogenic Newcastle Disease Virus Strain Isolated from Egypt. J. World Poult. Res., 10 (2S): 195-202. DOI: https:// dx.doi.org/10.36380 /jwpr.2020.25

23. Alexander DJ. Newcastle disease in the European Union 2000 to 2009. Avian Pathol. (2011) 40:547–58. doi: 10.1080/03079457.2011.618823

24. International Committee on Taxonomy of Viruses (ICTV). Virus Taxonomy. (2018). Available online at: https://talk.ictvonline.org/taxonomy (accessed December 20, 2020).

25. Aldous EW, Mynn JK, Banks J, and Alexander DJ (2003). A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathology, 32: 239–256. doi: https://doi.org/10.1080/0307945031 00009783.

26. Ashraf A, Shah MSU, Habib M, Hussain M, Mahboob S, and Al-Ghanim K (2016). Isolation, identification and molecular characterization of highly pathogenic Newcastle disease virus from field outbreaks. Brazilian Archives of Biology and Technology, 59: e16160301. DOI: https:// doi.org/10.1590/1678-4324-2016160301.

27. de Leeuw OS, Koch G, Hartog L, Ravenshorst N, Peeters BPH. Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin–neuraminidase protein. J Gen Virol. (2005) 86:1759–69.doi: 10.1099/vir.0.80822-0

28. Czeglédi A, Ujvári D, Somogyi E, Wehmann E, Werner O, Lomniczi B.Third genome size category of avian paramyxovirus serotype 1 (Newcastledisease virus) and evolutionary implications. Virus Res. (2006) 120:36–48.doi: 10.1016/j.virusres.2005.11.009

29. Peeters BP, de Leeuw OS, Koch G, and Gielkens AL (1999). Rescue of Newcastle disease virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. Journal of Virology, 73:5001–5009. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC11 2544/.

30. Russell, P.H., Alexander, D.J., 1983. Antigenic variation of Newcastle disease virus strains detected by monoclonal antibodies. Arch. Virol. 75, 243–253. https://doi.org/10.1007/BF01314 890.

31. Ballagi-Pordany, A., Wehmann, E., Herczeg, J., Belak, S., Lomniczi, B., 1996. Identification and grouping of Newcastle disease virus strains by restriction site analysis of a region from the F gene. Arch. Virol. 141, 243–261. https:// doi.org/10.1007/BF01718397.

32. Dimitrov KM, Abolnik C, Afonso CL, Albina E, Bahl J, Berg M, Briand FX, Brown IH, Choi KS, Chvala I, Diel DG, Durr PA, Ferreira HL, Fusaro A, Gil P, Goujgoulova GV, Grund C, Hicks JT, Joannis TM, Torchetti MK, Kolosov S, Lambrecht B, Lewis NS, Liu H, Liu H, McCullough S, Miller PJ, Monne I, Muller CP, Munir M, Reischak D, Sabra M, Samal SK, Servan de Almeida R, Shittu I, Snoeck CJ, Suarez DL, Van Borm S, Wang Z, Wong FYK. Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. Infect Genet Evol. 2019 Oct; 74:103917. doi: 10.1016/j.meegid.2019 .103917. Epub 2019 Jun 11. PMID: 31200111; PMCID: PMC6876278.

33. Toyoda, T., Sakaguchi, T., Hirota, H., Gotoh, B., Kuma, K., Miyata, T., Nagai, Y., 1989. Newcastle disease virus evolution II. Lack of gene recombination in generating virulent and avirulent viruses. Virology 169, 273–283. https://doi.org/ 10.1016/0042-6822(89)90152-9.

34. Herczeg, J., Wehmann, E., Bragg, R.R., Dias, P.M.T., Hadjiev, G., Werner, O., Lomniczi,B., 1999. Two novel genetic groups (VIIb and VIII) responsible for recent Newcastle disease outbreaks in southern Africa, one (VIIb) of which reached southern Europe. Arch. Virol. 144, 2087–2099. https://doi.org/10.1007 /s007050050624.

35. Miller, P.J., Decanini, E.L., Afonso, C.L., 2010. Newcastle disease: evolution of genotypes and the related diagnostic challenges. Infect. Genet. Evol. 10, 26–35. https://doi.org/10. 1016/j.meegid.2009.09.012.

36. Cattoli, G., Fusaro, A., Monne, I., Molia, S., Le Menach, A., Maregeya, B., Nchare, A., Bangana, I., Maina, A.G., Koffi, J.N., Thiam, H., Bezeid, O.E., Salviato, A., Nisi, R., Terregino, C., Capua, I., 2010. Emergence of a new genetic lineage of Newcastle disease virus in west and Central Africa–implications for diagnosis and control. Vet. Microbiol. 142, 168–176. https://doi.org/10.1016 /j.vetmic.2009.09.063.

37. Diel, D.G., da Silva, L.H., Liu, H., Wang, Z., Miller, P.J., 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. https://doi.org/10.1016/j. meegid.2012.07.012.

38. Courtney, S.C., Susta, L., Gomez, D., Hines, N.L., Pedersen, J.C., Brown, C.C., Miller, P.J., 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. https://doi. org/10.1128/JCM.02393-12.

39. Wang JY, Liu WH, Ren JJ, Tang P, Wu N, Wu HY, and Liu H J (2015). Characterization of emerging Newcastle disease virus isolates in China. Virology Journal, 12(1): 119. DOI: https://doi.org/10.1186 /s12985-015-0351-z.

40. Mansour SMG, ElBakrey RM, Mohamed FF, Hamouda EE, Abdallah MS, Elbestawy AR, Ismail MM, Abdien HMF and Eid AAM (2021) Avian Paramyxovirus Type 1 in Egypt: Epidemiology, Evolutionary Perspective, and Vaccine Approach. Front. Vet. Sci. 8:647462. doi: 10.3389/fvets.2021.647462.

41. Dimitrov KM, Ramey AM, Qiu X, Bahl J, and Afonso CL (2016). Temporal, geographic, and host distribution of avian paramyxovirus 1 (Newcastle disease virus). Infection, Genetics and Evolution, 39: 22–34. DOI: https://doi.org/10.1016 /j.meegid.2016.01.008.

42. Kattenbelt JA, Meers J, and Gould AR (2006). Genome sequence of the thermostable Newcastle disease virus (strain I-2) reveals a possible phenotypic locus. Veterinary Microbiology, 114: 134-141. DOI: https://doi.org/10.1016/j. vetmic.2005.10.041.

43. OIE Manual (2018). Newcastle Disease, Chapter 2.3.14. OIE, Paris. Available at: https:// www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.14_NEWCASTLE_DIS.pdf.

44. Wise GM, David LS, Bruce SS, Janice CP, Dennis AS, Daniel JK, Darrell R K, and Erica S (2004). Development of a Real-Time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. Journal of Clinical Microbiology, 42 (1) 329-338. doi: https://doi.org/10.1128/JCM.42.1.329-338.2004.

45. Munir, M., Abbas, M., Khan, M.T. et al. Genomic and biological characterization of a velogenic Newcastle disease virus isolated from a healthy backyard poultry flock in 2010. Virol J 9, 46 (2012). https://doi.org/10.1186/1743-422 X-9-46.

46. Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41:95-98. Available at: https:// www.scienceopen.com/document?vid=690e7265 -f2ce-4d9c-82a2-399cca46fbef.

47. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30(12):2725-2729. doi:10.1093/molbev/mst 197.

48. Munir M, Cortey M, Abbas M, Qureshi ZU, Afzal F, Shabbir MZ, Khan MT, Ahmed S, Ahmad S, Baule C, Ståhl K, Zohari S, Berg M (2012) Biological characterization and phylogenetic analysis of a novel genetic group of Newcastle disease virus isolated from outbreaks in commercial poultry and from backyard poultry flocks in Pakistan. Infect Genet Evol 12:1010–1019. https ://doi.org/10.1016/j.meegi d.2012.02.015.

49. Abd Elfatah, K.S.; Elabasy, M.A.; El-khyate, F.; Elmahallawy, E.K.; Mosad, S.M.; El-Gohary, F.A.; Abdo, W.; Al-Brakati, A.; Seadawy, M.G.; Tahoon, A.E.; et al. Molecular Characterization of Velogenic Newcastle Disease Virus (Sub-Genotype VII.1.1) from Wild Birds, with Assessment of Its Pathogenicity in Susceptible Chickens. Animals 2021, 11, 505. https://doi.org/10.33 90/ani11020505

50. Damena D, Fusaro A, Sombo M, Belaineh R, Heidari A, Kebede A, Kidane, M, and Chaka H (2016). Characterization of Newcastle disease virus isolates obtained from outbreak cases in commercial chickens and wild pigeons in Ethiopia. Springer Plus, 5: 476-453. DOI: https://doi.org/ 10.1186/s40064-016-2114-8.

51. Ganar, K., Das, M., Sinha, S., Kumar, S (2014). Newcastle disease virus: current status and our understanding. Virus Research, 184: 71–81. doi: https://doi.org /10.1016/j.virusres.2014.02. 016.

52. Collins MS, Bashiruddin JB, and Alexander DJ (1993). Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. Archives of Virology, 128: 363-370. DOI: https://doi.org /10.1007/BF01309446.

53. Kolakofsky D, Roux L, Garcin D, Ruigrok RW (2005) Paramyxovirus mRNA editing, the 'rule of six' and error catastrophe: a hypothesis. J Gen Virol 86(7): 1869–1877. https://doi.org/10.1099/vir.0.80986-0

54. El Naggar RF, Rohaim MA, Bazid AH, Ahmed KA, Hussein HA, Munir M. Biological characterization of wild-bird-origin avian avulavirus 1 and efficacy of currently applied vaccines against potential infection in commercial poultry. Arch Virol. 2018 Oct;163(10): 2743-2755. doi: 10.1007/ s00705-018-391 6-5. Epub 2018 Jun 19. PMID: 29922856.

55. Alexander DJ, Senne DA (2008) Newcastle disease, other avian paramyxoviruses, and pneumovirus infections. In: Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE (eds) Diseases of poultry, 12th edn. Iowa State University Press, Ames, pp 75–116

56. Yusoff K, Nesbit M, McCartney H, Meulemans G, Alexander DJ, Collins MS, et al. Location of neutralizing epitopes on the fusion protein of Newcastle disease virus strain Beaudette C. J Gen Virol. (1989) 70:3105–9. doi: 10.1099/ 0022-1317-70-11-3105.

57. Liu XF, Wan HQ, Ni XX, Wu YT, Liu WB. 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. (2003) 148:1387–403. doi: 10.1007/s00705-003-0014-z.

58. Ismail NM, El-Deeb AH, Emara MM, Tawfik HI, Wanis NA, Hussein HA. Prime-boost vaccination strategy against avian influenza and Newcastle disease viruses reduces shedding of the challenge viruses. Virus Dis. (2018) 29:324–32. doi: 10.1007/s13337-018-0463-3.

59. Römer-Oberdörfer A, Veits J, Werner O, Mettenleiter TC (2006) Enhancement of pathogenicity of Newcastle disease virus by alteration of specific amino acid residues in the surface glycoproteins F and HN. Avian Dis 50(2):259–263. https://doi.org/10.1637/7471-11150 5r.1

60. Cho SH, Kim SJ, Kwon HJ. Genomic sequence of an antigenic variant Newcastle disease virus isolated in Korea. Virus Genes. (2007) 35:293–302. doi: 10 .1007/s11262-007-0078-z.

61. Hu S,Wang T, Liu Y,Meng C,Wang X,Wu Y, et al. Identification of a variable epitope on the Newcastle disease virus hemagglutinin–neuraminidase protein. Vet Microbiol. (2010) 140:92–7. doi:10.1016/j.vetmic .2009.07.029.

62. Sergel TA, McGinnes LW, Morrison TG. A single amino acid change in the Newcastle disease virus fusion protein alters the requirement for HN protein in fusion. J Virol. (2000) 74:5101–7. doi:10.1128/JVI .74.11.5101-5107.2000.

63. Sergel TA, McGinnes LW, Morrison TG. Mutations in the fusion peptide and adjacent heptad repeat inhibit folding or activity of the Newcastle disease virus fusion protein. J Virol. (2001) 75:7934–43. doi:10.1128/JVI .75.17.7934-7943.2001.

Supplementary Table 1: Evolutionary distances analysis between study isolates, other Egyptian isolates, and most commonly used ND vaccines based on complete F protein gene sequence. The number of base substitutions per site from averaging all sequence pairs is shown. Analysis was conducted using the Maximum composite Likelihood model. 28 nucleotide sequences represent the full-length F protein gene of study isolates (n=6), other Egyptian isolates (n=17), and most commonly used ND vaccines (n=5). Evolutionary analyses were conducted in MEGA6

F protein gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
	No. of base substitutions per site																											
1 JF950510.1_LaSota																												
2 KM056356.1_Avinew	0.11																											
3 Y18898.1_Clone_30	0.00	0.11																										
4 AF309418.1_HB1	0.01	0.11	0.01																									
5 AY935499.2_I-2	0.13	0.04	0.13	0.13																								
6 KY075882.1_NDV/chicken/Egypt/Damietta9/2016	0.20	0.16	0.20	0.19	0.18																							
7 MK673139.1_chicken/Egypt/Sohag/66/1030/2011	0.19	0.16	0.19	0.19	0.17	0.01																						
8 MG717684.1_NDV/teal/Egypt/SDU-3/2016	0.19	0.16	0.19	0.19	0.18	0.01	0.00																					
9 MG717686.1NDV/teal/Egypt/SDU-4/2016	0.19	0.16	0.19	0.19	0.17	0.01	0.00	0.00																				
10 MW285792.1_NDV/Chicken/Egypt/AR2178-14/2013	0.19	0.16	0.19	0.19	0.17	0.01	0.00	0.01	0.00																			
11 MG717683.1_NDV/quail/Egypt/SDU-2/2016	0.19	0.16	0.19	0.19	0.18	0.01	0.00	0.00	0.00	0.00																		
12 MK495900.1_Chicken/Egypt/Luxor/2012/23	0.19	0.16	0.19	0.19	0.17	0.01	0.00	0.00	0.00	0.00	0.00																	
13 MK495901.1_Chicken/Egypt/Luxor/2012/24	0.19	0.16	0.19	0.19	0.17	0.01	0.00	0.00	0.00	0.00	0.00	0.00																
14 MK495893.1_Chicken/Egypt/Luxor/2012/15	0.19	0.16	0.19	0.19	0.17	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00															
15 MK495886.1_Chicken/Egypt/Luxor/2012/5	0.19	0.16	0.19	0.19	0.17	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00														
16 MK495896.1_Chicken/Egypt/Luxor/2012/18	0.20	0.16	0.19	0.19	0.17	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00													
17 MK005975.1_chicken/Egypt/Sohag/1/1012/2011	0.19	0.16	0.19	0.19	0.17	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.01												
18 KY075881.1_NDV/chicken/Egypt/Ismailia8/2016	0.20	0.16	0.20	0.19	0.18	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01											
19 MK495907.1_Chicken/Egypt/Qena/2012/3	0.20	0.16	0.19	0.19	0.18	0.01	0.01	0.01	0.00	0.01	0.01	0.00	0.00	0.00	0.01	0.01	0.01	0.01										
20 MG717686.1_NDV/teal/Egypt/SDU-4/2016	0.19	0.16	0.19	0.19	0.17	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00									
21 MK495903.1_Chicken/Egypt/Luxor/2012/26	0.19	0.16	0.19	0.19	0.17	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00								
22 MK495904.1_Chicken/Egypt/Luxor/2012/27	0.19	0.16	0.19	0.19	0.17	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00							
23 MZ826277 CK/Egypt/FAY-ZU/Dec-2019	0.20	0.17	0.20	0.20	0.18	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01						
24 MZ826278 CK/Egypt/GIZ-ZU/Feb-2020	0.20	0.16	0.19	0.19	0.18	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01					
25 MZ826279 CK/Egypt/FAY-ZU/Apr-2020	0.20	0.16	0.20	0.19	0.18	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.00				
26 MZ826280 CK/Egypt/BENI-ZU/Oct-2020	0.20	0.16	0.19	0.19	0.18	0.01	0.01	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.00			
27 MZ826281 CK/Egypt/MON-ZU/Nov-2020	0.19	0.15	0.19	0.19	0.16	0.04	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.04	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03		
28 MZ8262782 CK/Egypt/FAY-ZU/Nov-2020	0.20	0.16	0.19	0.19	0.17	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.03	

Supplementary Table 2: Evolutionary distances analysis between study isolates, other Egyptian isolates, and most commonly used ND vaccines based on complete HN protein gene sequence. The number of base substitutions per site from averaging all sequence pairs is shown. Analysis was conducted using the Maximum composite Likelihood model. 28 nucleotide sequences represent the full-length HN protein gene of study isolates (n=6), other Egyptian isolates (n=17), and most commonly used ND vaccines (n=5). Evolutionary analyses were conducted in MEGA6

HN protein gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
	No. of base substitutions per site																											
1 JF950510.1_LaSota																												
2 KM056356.1_Avinew	0.11																											
3 Y18898.1_Clone_30	0.00	0.11																										
4 AF309418.1_HB1	0.01	0.11	0.01																									
5 AY935499.2_I-2	0.13	0.05	0.13	0.12																								
6 MG717686.1_NDV/teal/Egypt/SDU-4/2016	0.23	0.18	0.23	0.23	0.19																							
7 MG717683.1_NDV/quail/Egypt/SDU-2/2016	0.23	0.18	0.23	0.23	0.18	0.01																						
8 MK495908.1_Chicken/Egypt/Qena/2012/4	0.24	0.19	0.24	0.24	0.19	0.01	0.01																					
9 MK673139.1_chicken/Egypt/Sohag/66/1030/2011	0.23	0.18	0.23	0.23	0.19	0.00	0.01	0.01																				
10 MK495885.1_Chicken/Egypt/Luxor/2012/4	0.24	0.19	0.23	0.23	0.19	0.00	0.01	0.01	0.01																			
11 MK495906.1_Chicken/Egypt/Qena/2012/2	0.24	0.19	0.23	0.23	0.19	0.01	0.01	0.00	0.01	0.01																		
12 MK495883.1_Chicken/Egypt/Luxor/2012/2	0.24	0.19	0.23	0.23	0.19	0.00	0.01	0.01	0.01	0.01	0.01																	
13 MK495888.1_Chicken/Egypt/Luxor/2012/8	0.23	0.19	0.23	0.23	0.19	0.00	0.01	0.01	0.01	0.01	0.01	0.00																
14 MK495897.1_Chicken/Egypt/Luxor/2012/19	0.23	0.19	0.23	0.23	0.19	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00															
15 MW285792.1_NDV/Chicken/Egypt/AR2178-14/2013	0.23	0.19	0.23	0.23	0.19	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00														
16 MK495903.1_Chicken/Egypt/Luxor/2012/26	0.23	0.18	0.23	0.23	0.19	0.00	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00													
17 MK495904.1_Chicken/Egypt/Luxor/2012/27	0.23	0.19	0.23	0.23	0.19	0.00	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00												
18 MK495909.1_Chicken/Egypt/Aswan/2013/1	0.23	0.18	0.23	0.23	0.19	0.00	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00											
19 MK495901.1_Chicken/Egypt/Luxor/2012/24	0.23	0.19	0.23	0.23	0.19	0.00	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00										
20 MK495902.1_Chicken/Egypt/Luxor/2012/25	0.23	0.19	0.23	0.23	0.19	0.00	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00									
21 MK495907.1_Chicken/Egypt/Qena/2012/3	0.23	0.19	0.23	0.23	0.19	0.00	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00								
22 MG717684.1_NDV/teal/Egypt/SDU-3/2016	0.23	0.18	0.23	0.23	0.19	0.00	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00							
23 MZ826283_CK/Egypt/FAY-ZU/Dec-2019	0.23	0.19	0.23	0.23	0.19	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01						
24 MZ826284_CK/Egypt/GIZ-ZU/Feb-2020	0.23	0.19	0.23	0.23	0.19	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00					
25 MZ826285_CK/Egypt/FAY-ZU/Apr-2020	0.23	0.19	0.23	0.23	0.19	0.01	0.02	0.02	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01				
26 MZ826286_CK/Egypt/BENI-ZU/Oct-2020	0.23	0.19	0.23	0.23	0.19	0.01	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01			
27 MZ826287_CK/Egypt/MON-ZU/Nov-2020	0.23	0.19	0.23	0.23	0.19	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01		
28 MZ826288_CK/Egypt/FAY-ZU/Nov-2020	0.23	0.19	0.23	0.23	0.19	0.01	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.00	0.01	