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Article

Newcastle Disease Genotype VII Outbreaks in Poultry across Egypt are associated with Enzootic Prevalence of the Virus in Wild Birds

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Abstract: Newcastle disease virus (NDV) genotype VII is a highly pathogenic *Orthoavulavirus* that has caused multiple outbreaks among poultry in Egypt since 2011. This study aimed to investigate the genetic diversity of NDV prevailing in domestic and wild birds in Egyptian governorates. A total of 37 oropharyngeal swabs from wild birds and 101 swabs from domestic bird flocks including chickens, ducks, turkeys, and swans were collected from different geographic regions within 13 governorates during 2019-2020. Virus isolation and propagation via embryonated eggs revealed 91 swab samples produced allantoic fluid containing hemagglutination activity, suggestive of virus presence. The use of RT-PCR targeted to F gene successfully detected NDV in 85 samples. The geographical prevalence of NDV spread to 12 governorates in domestic birds, migratory and non-migratory wild birds. Following whole genome sequencing, we assembled six NDV genome sequences (70 - 99% of genome coverage), including five full F gene sequences. All NDV strains carried high virulence, based on the presence of polybasic amino acids (RRQRF) at the F gene cleavage site. Phylogenetic analysis revealed that the NDV strains belonged to class II within genotype VII.1.1. The presence of genetically similar virulent NDV in wild birds further highlights their role in the dissemination of NDV in poultry populations across Egypt. Continued genomic surveillance in both wild birds and poultry would be necessary for monitoring NDV incursions and genetic diversification.

Keywords: migratory birds; Newcastle disease virus-GVII; poultry; phylogenetics; sequence-independent; single-primer amplification (SISPA); velogenic; whole genome sequencing (WGS)

1. Introduction

Poultry in Egypt has become the substantial source of animal protein. Such a huge industry is challenged by several devastating pathogens. Newcastle Disease (ND) is one of the endemic diseases that still subtract from the outcome of poultry through continuous spread among poultry flocks, even within vaccinated populations. Avian Orthoavulavirus 1 (APMV-1), also known as Newcastle Disease Virus (NDV), is an enveloped negative-sense, single-stranded RNA virus belonging to the family *Paramyxoviridae* – order *Mononegavirales* [1]. The viral genome is around 15,200 base pairs (bp) in length and encodes six different proteins; nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), large RNA polymerase (L), fusion protein (F), and hemagglutinin–neuraminidase (HN) [2,3]. Phylogenetic analysis of F gene sequences of NDVs divided them into two classes (I and II): class I includes avirulent viruses, with a natural reservoir of aquatic wild birds [4], whereas class II contains viruses that have higher genetic and virulence variability with

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at least 20 genotypes (I–XXI, except the recombinant sequence genotype XV) and are known to infect a wide range of domestic and wild birds [2,3,5].

ND was first identified in 1948 [6], with genotype II (NDV-II) being most prevalent. However, since 2011, reported outbreaks revealed a new commonly occurring genotype, NDV-GVII, with a velogenic pathotype and varying incidence among vaccinated flocks of commercial and backyard chickens throughout the Egyptian governorates [7-12].

More than 250 domestic and wild bird species have been shown to be susceptible to NDV infection with varying clinical forms [13-16]. However, virus transmission among wild birds possesses serious risk for both poultry and other wild bird populations [17,18]; virus adaption can encourage new susceptible host species, posing a greater threat to local as well as wider geographical regions [17-19]. Mansour *et al*. [20] reviewed the clinical disease, epidemiology, and evolutionary perspective of NDV among domesticated and wild birds in the view of available research work publications. Virulence of NDV strains varies greatly with the host and to date, ducks are less susceptible to NDV disease [21]; NDV strains of varying virulence and pathogenicity have been isolated from either diseased or apparently healthy ducks, which questions whether ducks are natural reservoirs or less susceptible hosts to NDV [22,23].

The distinctive intermixing of migratory, free-living, and domesticated birds in Egypt might facilitate the direct transmission to commercial flocks and backyard populations besides the evolution power of APMV-1 mutation [20]. Egypt boasts a crucial geographical location for migratory birds, bridging three continents and hosting an essential resting place for those migrating from Europe in the spring and autumn months. It is also considered the second most important migratory pathway for birds in the world, with more than 2 million birds passing through annually [24]. Despite extensive vaccination regimes across commercial and backyard poultry flocks, Egypt have and remain to witness several devastating outbreaks, resulting in huge socio-economic losses [20].

The frequency of virulent strains reported in wild birds on other continents is also increasing [25-27], which questions the possibility that wild birds play a potential role in the spread of virulent NDV in Africa, especially in Egypt. Additionally, low biosecurity measures could endanger the poultry industry, increasing the possibility of disease transmission through wildlife-poultry contact or contaminated fomites [28]. However, there are few reports of ND virus isolation in epizootiological surveys of wild birds, with limited sequences and availability of data [29,30]. Therefore, the role of these birds in the maintenance of the disease is still obscure.

The information about the NDV characterization and the possible transmission from wild birds to the contact poultry is necessary for strategic planning for ND control, particularly in Egypt. In this present study, we investigated the NDV isolates from domestic versus migratory and non-migratory birds and evaluated the potential role of wild birds in NDV transmission to the poultry industry.

2. Materials and Methods

2.1. Study area and sample collection

The aim of this study was to monitor the genotypic diversity the NDV prevailing in the domesticated birds and wild birds (migratory and non-migratory) in Egypt. Active surveillance of wild birds is difficult due to practical, logistical, and financial constraints, thus, we defined clear strategies to obtain samples that would provide us robust sources of targeted NDV prevalence. Therefore, samples were mainly collected from domestic birds (semi-intensive housing system) that could potentially serve as "sentinels" and from wild birds that could serve as a reservoir for virus persistence and transmission to poultry.

The study ran from September 2019 to December 2020 across different governorates (*n=13*) within the country of Egypt, including Dakahlia, Sharkia, Cairo, Suez, Sohag, Asyut, Port-Saied, North Sinai, South Sinai, Alexandria, Ismailia, Damietta, and Gharbia. Oropharyngeal swabs were collected from 101 flocks of domestic birds and 37 from migratory and non-migratory wild birds (total *n=138*).

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2.1.1. Domestic birds

Oropharyngeal swabs collected from the 101 flocks of domestic birds (pool of 3 swabs/ flock) included chickens (*n=60*), ducks (*n=38*), swans (*n=2*), and turkey (*n=1*) of different ages and breeds (**Table 1**). At the time of the farm visit, samples were collected from both apparently healthy and diseased birds. The sick birds that were either dead or diseased were clinically examined in which the signs and post-mortem changes were recorded in addition to the mortality rates.

Table 1. Details of collected samples from domestic birds in Egypt, during 2019-2020.

Species	Breed	No. of flocks	Age/day	Type of poultry flock	Location
Chickens	Cobb	25	23-45	Broiler	Sharkia, Dakahlia, Sohag, Alexandria, Assuit, North and South Sinai
	Sasso	3	40-310	Broiler/Layer	Dakahlia, Sohag, Damietta
	Avian 48	3	30-33	Broiler	Suez, South Sinai
	Hay-line	2	200	Layer	Dakahlia
	Ross	4	28-35	Broiler	Alexandria, North Sinai
	Indian river	3	32-48	Broiler	Suez, North Sinai
	Bovanes	5	210- 252	Layer	Sharkia, Alexandria
	Hubbard	2	27-29	Broiler	Damietta, Port-Said
	Lehman	2	200-330	Layer	South Sinai, Alexandria
	Isapapcoack	2	100	Layer	Sharkia
	Baladi	9	20-40	Native	Sharkia, Sohag, Assuit
Ducks	Muscovy	17	25-360	Broiler/Breeder	Dakahlia, Gharbia
	Mallard	5	187-622	Broiler/Breeder	Dakahlia, Gharbia
	Pekin	16	30-700	Broiler	Sharkia, Dakahlia, Gharbia
Turkeys	Converter	1	47		Sharkia
Swans	Pelican crispus	2	4320-5400		Cairo

2.1.2. Migratory and non-migratory wild birds

A total of 37 apparently healthy wild birds including 14 species were captured randomly via traps or collected from live-bird-markets (LBMs) in different governorates including North Sinai, Dakahlia, Port-Said, Ismailia, and Sharkia, Egypt (**Table 2**). The traps used to capture were baited nets that were erected at the entrances to the lakes at Al Manzala Lake in Port-Said, Bardawil Lake in North Sinai, and Bitter lakes near to Ismailia. The birds caught were classified and scientifically named according to species, order, and breeds by the wildlife scientist at the Faculty of Veterinary Medicine - Suez Canal University. Oropharyngeal swabs were collected largely during the autumn and spring seasons. Sterile cotton swabs (propylene stick) were used to collect samples from the oral cavity of birds in cryovial containing virus transport media (5% glycerol-MEM media pH 7.2 supplemented with 1000IU of penicillin and 1000µg streptomycin per ml) [31]. The samples were kept refrigerated (at 4°C) and transported to the laboratory of the Department of Avian and Rabbit Medicine, Faculty of Veterinary Medicine, Zagazig University, Egypt within 24 hours and stored at -80°C until further processing.

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Table 2. Details of collected samples from migratory and non-migratory wild birds, including order, species, common name, location of capture, collection date, and number of captured birds.

Species	Common name	Number of birds	Collection date (Month/Year)	Location
Corvus cornix	Hooded crow	8	4, 8, 10/2019	Sharkia, Port-Said, Dakahlia
Larus canus	Common gull	1	3/2019	Port-Said
Numenius minutus	Little curlew	1	3/2019	Port-Said
Coturnix ypsilophora	Brown quail	3	4/2019	North Sinai
Streptopelia turtur	European turtle dove	3	4, 10/2019	Port-Said, Sharkia
Nycticorax nycticorax	Black-crowned night heron	2	10/2019	Port-Said
Gallinula chloropus	Common moorhen	2	10/2019	Port-Said
Bubulcus ibis	Cattle egret	8	8, 10/2019	Port-Said, Ismailia, Dakahlia
Anas platyrhynchos	Mallard duck	1	7/2019	North Sinai
Ardenna pacifica	Wedge-tailed shearwater	1	3/2019	Port-Said
Spatula clypeata	Northern Shoveler	2	3/2019	Port-Said
Ardea cinerea	Grey heron	2	3/2019	Port-Said
Porphyrio madagascariensis	Purple gallinule (African swamphen)	2	3, 4/2019	Port-Said
Anthus rubescens	American pipit	1	4/2019	Port-Said

2.2. Sample processing and virus isolation

The swab samples were centrifuged at 3000rpm for 10min, and the supernatants were filtered using 0.22µm sterile filter. After aseptic examination, 200µl of filtrate was inoculated to 10-day-old specific pathogen-free (SPF) embryonated chicken eggs (ECEs) via the allantoic cavity using a standard egg inoculation procedure [31]. 3 fertile eggs per test or negative control sample were used. The inoculated eggs were incubated at 37°C for a maximum of 96 hours with daily monitoring. Embryos that were found dead within the first 24-hour post-inoculation were regarded as non-related unspecific death and were discarded. After 24 hours, any embryo that showed typical signs of virus infection such as haemorrhage, blood vessels coming away from the shell and/or no movement of the embryo during the candling process, were chilled at 4°C immediately. The embryos that remained live until 96-hour post-inoculation (pi) were chilled at 4°C for minimum of 4 hours. Allantoic fluids (AFs) were harvested and subjected to hemagglutination assay (HA) by using 10% (v/v) washed chicken red blood cells (RBCs). Allantoic fluids with positive HA activity were subjected to RT-PCR for NDV detection and sequencing.

2.3. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Viral RNA was extracted either directly from oropharyngeal swabs or HA positive allantoic fluid using the QIAamp MinElute Virus Spin kit (Qiagen GmbH, Hilden, Germany, catalogue no. 57704) in accordance with the manufacturer's guidelines. As positive and negative controls samples, and NDV vaccine strain (La Sota) and allantoic fluid from non-infected embryos were used respectively. The extracted RNAs were reverse transcribed to cDNA using random primers and the assay was processed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, catalogue no. 4268814) following the manufacturer's instructions.

The PCR amplification was performed using primers targeting the fusion (F) gene of NDV (forward: 5'-CACAGCAGGTCGGTGTAGAA-3' and reverse: 5'-TCTCCAAA-TAGGTGGCACGC-3') and conducted using 2X Dream^{Taq} Green PCR Master Mix (Thermoscientific, Catalogue no. K1081). Cycling conditions were performed as a single cycle of initial denaturation at 95°C for 3 min, followed by 30 cycles of 94°C for 30s (denaturation), 60°C for 30s (annealing) and 72°C for 45s (extension), then 72°C for 7 min of final

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extension step. After amplification, the PCR products were resolved on a 1% agarose gel and stained with ethidium bromide; positive amplification products yielded a band size of approximately 306bp. Additionally, the positive HA allantoic fluids were screened for the presence of influenza A virus using the primers targeting the conserved region of influenza M gene [32].

2.4. Sequence-independent single primer amplification (SISPA)

Extracted RNA was used in SISPA reaction as described previously [33]. Briefly, for cDNA: 1µl of 100µM primer K-8N [33] and dNTPs (10µM each) were used in 20µl reaction mixture. Reverse transcription (RT) was performed with SuperScript IV Reverse Transcriptase (ThermoFisher Scientific) at 55°C for 10 min, followed by 80°C for 10 min and then placed on ice. After RT- PCR, 20µl of first-stranded cDNA was heated at 94°C for 3 min, then chilled on ice for 3 min with 10uM of primer K-8N (0.5µL per reaction), and 10µM dNTPs (0.5µl per reaction) in 1× Klenow reaction buffer (NEB) [33]. 1µl of Klenow fragment (NEB) was added and the reaction incubated at 37°C for 60 min. The resulting dsDNA was cleaned using Agencourt AMPure XP beads (Beckman Coulter); purified dsDNA was subsequently used as a template for PCR amplification. 5µl of purified dsDNA was taken forward for sequence-independent PCR amplification, containing 1x Q5 High-Fidelity Master Mix (NEB), 2.5µl of 10µM primer K (5`-GACCATCTAGCGACCTCCAC-3`) and nuclease-free water with a final reaction volume of 50µl. The PCR cycling conditions were as follows: 98°C for 30s, followed by 35 cycles of 98°C for 10s, 55°C for 30s, and 72°C for 1 min, with a final extension at 72°C for 10 min; PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter). Obtained dsDNA was quantified by Qubit dsDNA HS assay (Invitrogen) following manufacturer's instruction and purified dsDNA was subsequently used for genome sequencing.

2.5. Genome sequencing

Nextera XT DNA kit (Illumina) was used following manufacturer's instructions to generate multiplexed paired end sequencing libraries from 1ng of dsDNA, as previously described [33]. Libraries were analysed on a High Sensitivity DNA Chip on the Bioanalyzer (Agilent Technologies), pooled and sequenced on a 2x 300 cycle using the MiSeq Reagent Kit v2 (Illumina, USA).

2.6. Whole genome sequencing data analysis

The raw sequencing reads were analysed as described previously [33,34]. Briefly, the quality of reads were assessed using FastQC (version 0.11.5) and were quality trimmed (employing a quality score of ≥30) with adapter removal using Trim Galore (version 0.5.0) (https://github.com/FelixKrueger/TrimGalore) [35]. *De novo* assembly was performed using SPAdes *de novo* assembler (version 3.10.1) (k-mer 33, 55, and 77) with resulting contigs quality assessed using QUAST (version 5.0.2) [36,37]. Reference-based orientation and scaffolding were performed using Scaffold_builder (version 2.2) [38]. Consensus sequences were recalled based on BWA-MEM mapping of trimmed (but un-normalized) read data to the genome scaffold and parsing of the mpileup alignment; BWA-MEM (version 0.7.17) and Geneious 9.1.2 (https://www.geneious.com) were used to assemble reference genomes [39].

2.7. Phylogenetic analysis

Complete coding sequences of F gene (n=5) were used for comparative genetic analyses. To determine genotypes of NDV viruses, we used previously published NDV reference strains [3] and the maximum likelihood (ML) phylogenetic tree of the F segment was constructed using nucleotide sequences. These sequences were aligned using MUSCLE [40] and ML phylogeny was generated using GTR nucleotide substitution model, with among-site rate variation modelled using a discrete gamma distribution using MEGA 11

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(version 11.0.8) [41]. Bootstrap support values were generated using 500 rapid bootstrap replicates.

3. Results

3.1. Clinical signs and post-mortem lesions

At the time of sampling both migratory and non-migratory wild birds were apparently healthy with no clinical disease signs. However, the clinical disease signs were observed in the domestic birds. In chickens, ruffled feathers, greenish diarrhoea, and respiratory distress (sneezing, swollen eyes, and gasping) were the predominant signs. Cyanosis of the head, comb, and wattles, as well as physical distress was also observed in some chickens. While in ducks, the predominant clinical signs were the nervous distress and greenish diarrhoea. Furthermore, respiratory distress and diarrhoea were apparent in the turkey flock. The post-mortem examination of euthanized birds showed septicaemia and congestion in the internal organs and enteritis with greenish intestinal contents in all examined birds. Haemorrhages were also seen in the caecal tonsils, the mucosa, and the glands of the proventriculus of some chickens.

3.2. Virus identification

Inoculation of SPF-ECEs with swab samples showed that some of embryos died within 96 hours. Among the infected embryos, 80.3% showed diffuse haemorrhages and congestion suggesting possible virus presence, either NDV and/or AIV. The tested harvested allantoic fluids (*n=138*) revealed positive HA activity 65.9% of samples (91/138). The positive HA samples were further confirmed by RT-PCR; NDV was detected in 93.4% (85/91). This corresponded to 61.6% (85/138) of the total samples collected, of which 42% (58/138) were from domestic poultry flocks and 19.6% (27/138) were from wild birds. Finally, the total positive NDV samples of each population were 57.4% (58/101) and 72.9% (27/37) for domestic poultry and wild birds, respectively. From the 85 samples positive for NDV, 83.5% (71/85) were associated with a single infection with NDV, of which 71.8% (51/71) of these samples were from domestic poultry and 28.2% (20/71) wild birds, respectively. These corresponded to 51.4% (71/138) of total combined samples, 50.5% (51/101) of domestic poultry samples, and 54.1% (20/37) of wild bird samples, respectively. Further analysis showed the presence of AIV in 14.5% (20/138) of samples, either as a combined infection of both AIV and NDV 70% (14/20) from both domestic and wild bird samples (7 positive per each) or AIV alone in 30% (6/20) samples collected from domestic birds (**Figure 1 and 2**).

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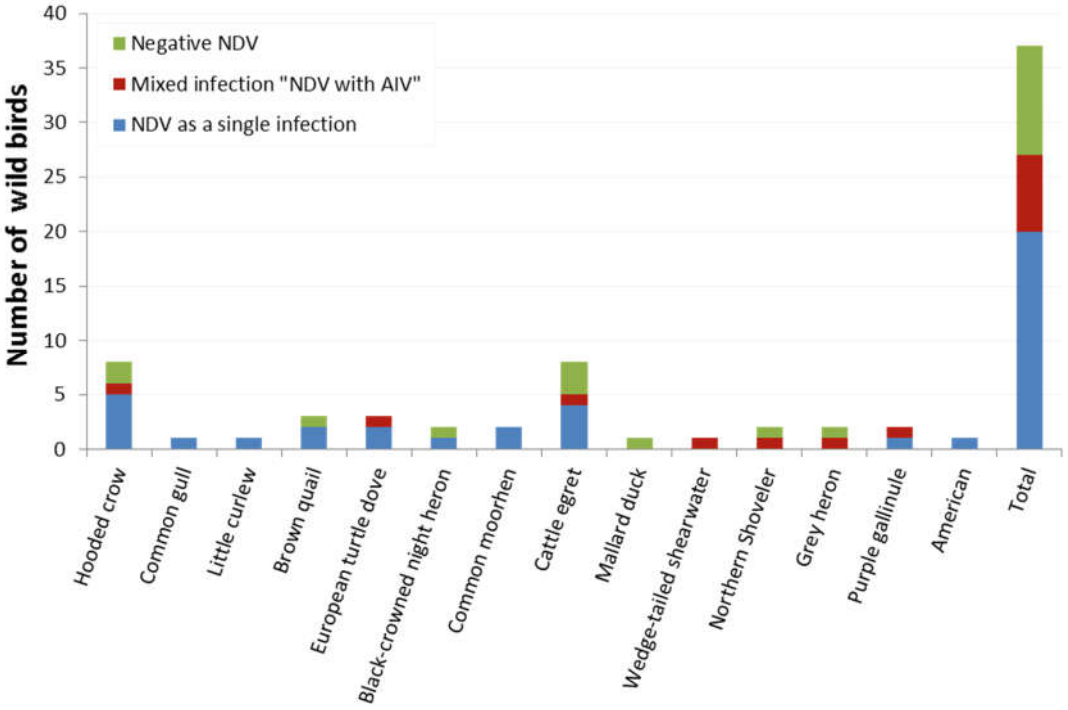

Figure 1. Domestic bird breeds in Egypt positive for NDV as a single natural infection (detected via NDV F gene-specific RT-PCR, blue bars) or mixed natural infection with NDV and AIV (detected via flu M gene-specific RT-PCR, maroon bars). The number of birds negative for NDV is indicated as green bars.

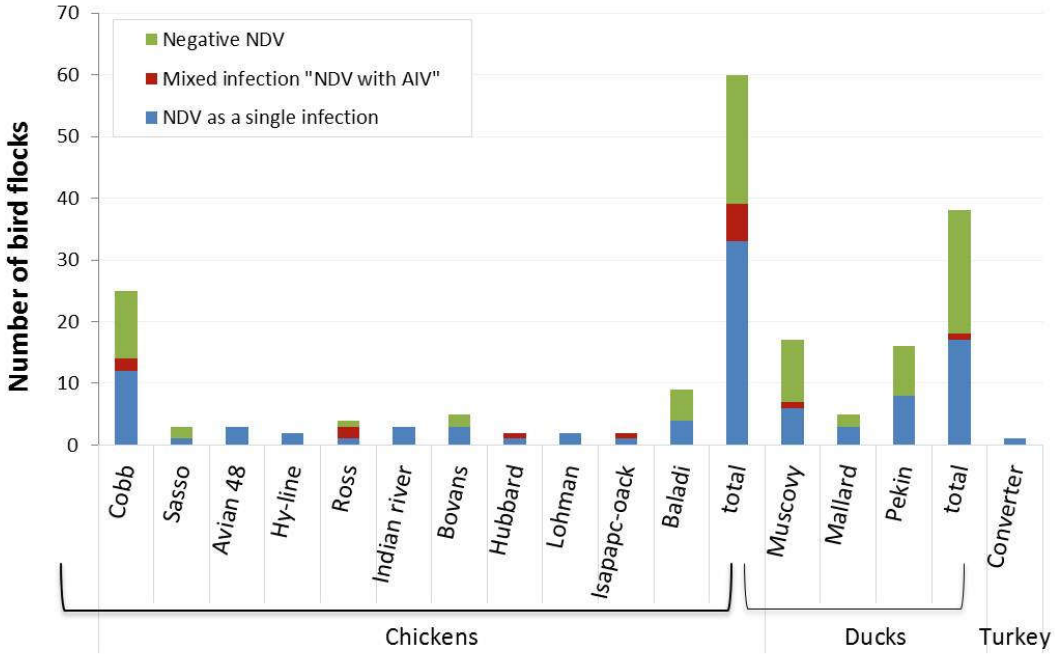

Figure 2. Wild bird breeds in Egypt positive for NDV as single natural infection (detected via NDV F gene-specific RT-PCR, blue bars) or mixed natural infection with NDV and AIV (detected by Flu-M gene-specific RT-PCR, maroon bars). The numbers of birds negative for NDV are indicated as green bars.

3.3. Incidence of NDV based on F gene

The isolation of NDV in the different governorates (*n=13*) of Egypt and the case number analysed (n/n) are shown in **Figure 3**. Among the different species of examined

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domestic birds; 65% (39/60) of chicken flocks (95% CI 51.60% - 76.87%) and 47.4% (18/38) of the duck flocks were positive for NDV infection (95% CI 30.97% - 64.19%). The single turkey flock assessed was also positive for NDV, but swan flocks revealed no virus detection (**Table 3**). Whereas in the wild birds, the NDV infection rate of the species *Larus canus*, *Numenius minutus*, *Streptopelia turtur, Gallinula chloropus*, *Ardenna pacifica*, *Porphyrio madagascariensis*, and *Anthus rubescens* reached 100%, followed by *Corvus cornix* with incidence of 75% (6/8) (95% CI 34.91% - 96.61%) (**Table 4**). Among the 20 NDV isolates used for whole genome sequencing (WGS), 6 viruses were selected at random as representative strains for sequence analysis. The detailed information of the seven isolates is shown in **Table 5**.

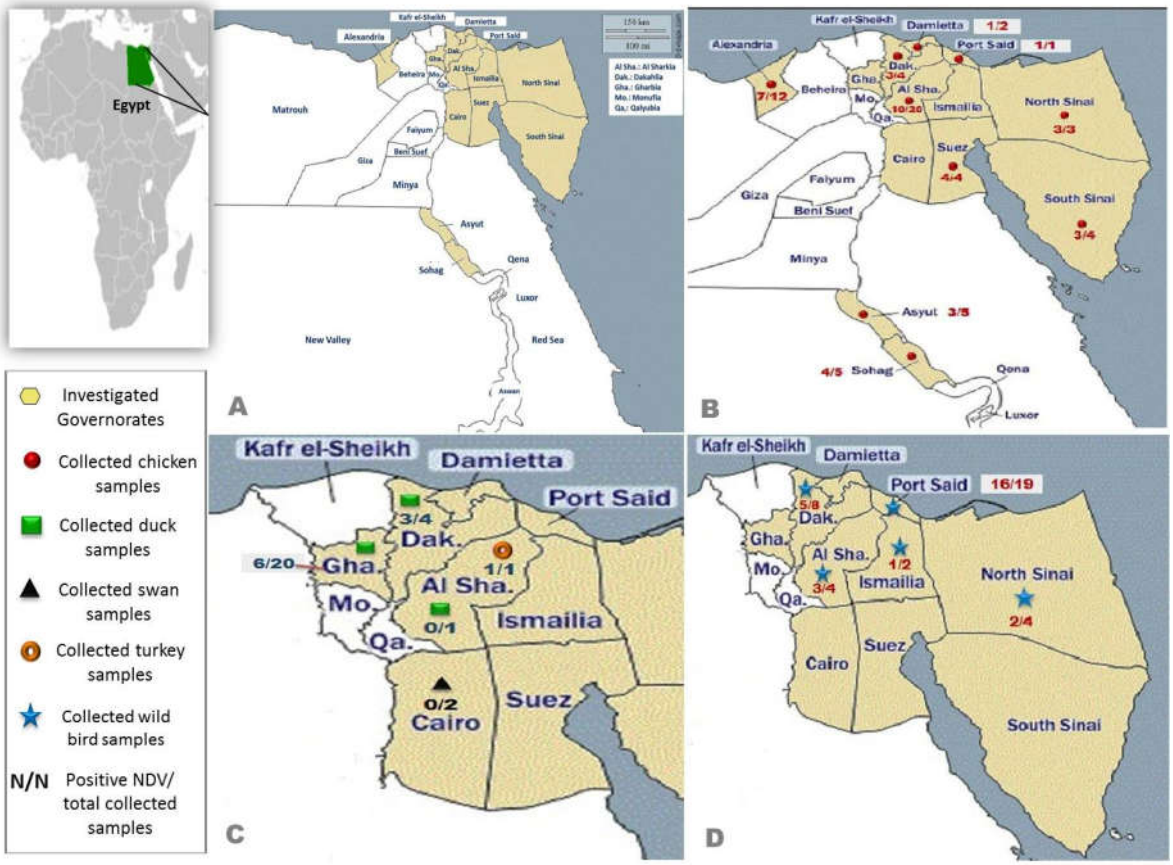

Figure 3. Geographical spread pattern of Egyptian NDV. (**A**) The Egyptian map shows the governorates of collected samples (yellow). (**B**) Distribution of tested chicken (red) samples. (**C**) Distribution of tested duck (green), turkey (orange) and swan (black) samples. (**D**) Distribution of tested wild bird (blue) samples. **N/N** positive NDV to the total collected samples in each governorate.

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Table 3. Incidence of NDV among naturally infected domestic birds, Egypt.

Breeds	No. of flocks	Detected virus(es)			
		No of positive NDV (%)	95% CI	No of positive AIV (%)	95% CI
Chickens					
Cobb	25	14 (56)	34.9-75.6	4 (16)	4.54-36.08
Sasso	3	1 (33.3)	0.84-90.57	0	0
Avian 48	3	3 (100)	29.24-1.00	0	0
Hy-line	2	2 (100)	15.81-1.00	0	0
Ross	4	3 (75)	19.41-99.37	2 (50)	6.76-93.24
Indian river	3	3 (100)	29.24-1.00	0	0
Bovans	5	3 (60)	14.66-94.73	1 (20)	0.51-71.64
Hubbard	2	2 (100)	15.81-1.00	1 (50)	1.26-98.74
Lohman	2	2 (100)	15.81-1.00	0	0
Isapapcoack	2	2 (100)	15.81-1.00	1 (50)	1.26-98.74
Baladi	9	4 (44.4)	13.70-78.80	0	0
Total	**60**	**39 (65)**	**51.60-76.87**	**9 (15)**	7.10-26.57
Ducks					
Muscovy	17	7 (41.2)	18.44-67.08	3 (17.6)	3.80-43.43
Mallard	5	3 (60)	14.66-94.73	0	0
Pekin	16	8 (50)	24.65-75.35	1 (6.3)	0.16-30.23
Total	**38**	**18 (47.4)**	**30.97-64.19**	**4 (10.5)**	**2.94-24.80**
Turkeys					
Converter	1	1 (100)	2.50-1.00	0	0
Swans					
Pelican crispus	2	0	0	0	0

Table 4. Incidence of NDV among naturally infected wild birds, Egypt.

Species	Common Name	No. of birds	Detected virus(es)			
			No. of positive NDV (%)	95% CI	No. of positive AIV (%)	95% CI
Corvus cornix	Hooded crow	8	6 (75)	34.91-96.61	1 (12.5)	0.32-52.65
Larus canus	Common gull	1	1 (100)	2.50-1.00	0	0
Numenius minutus	Little curlew	1	1(100)	2.50-1.00	0	0
Coturnix ypsilophora	Brown quail	3	2 (66.7)	9.43-99.16	0	0
Streptopelia turtur	European turtle dove	3	3 (100)	29.24-1.00	1 (33.33)	0.84-90.57
Nycticorax nycticorax	Black-crowned night heron	2	1 (50)	1.26-98.74	0	0
Gallinula chloropus	Common moorhen	2	2 (100)	15.81-1.00	0	0
Bubulcus ibis	Cattle egret	8	5 (62.5)	24.49-91.48	1 (12.5)	0.32-52.65
Anas platyrhynchos	Mallard duck	1	0	0	0	0
Ardenna pacifica	Wedge-tailed shearwater	1	1 (100)	2.50-1.00	1 (100)	2.50-1.00
Spatula clypeata	Northern shoveler	2	1 (50)	1.26-98.74	1 (50) *	1.26-98.74
Ardea cinerea	Grey heron	2	1 (50)	1.26-98.74	1 (50) *	1.26-98.74
Porphyrio madagascariensis	Purple gallinule (African swamphen)	2	2 (100)	15.81-1.00	1 (50)	1.26-98.74
Anthus rubescens	American pipit	1	1 (100)	2.50-1.00	0	0
Total		**37**	**27 (72.97)**	**55.88-86.21**	**7 (18.9)**	**7.96-35.16**

(*) the same birds were infected with NDV and AIV.

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Table 5. The details of whole genome sequencing of NDV Egyptian strains of this study.

Name of isolates	Breed	Locality	Descriptive Data					Sequencing	Accession number
			Date	Age/ days	Flock density	Mortality rate/3 days	Clinical finding		
NDV/Chicken/Egypt/NOR/ZU-NM76/2019	Ross	North Sinai	03/2019	33	14000	600	Respiratory signs, greenish diarrhoea, septicaemia	Approx. 99% genome coverage (103 nucleotides gap), Full F gene	OP219678
NDV/Chicken/Egypt/ALEX/ZU-NM97/2019	Ross	Alexandria	05/2019	30	5000	62	Respiratory signs, greenish diarrhoea, haemorrhages in caecal tonsils and proventriculus	Approx. 90% genome coverage, Full F gene	OP219679
NDV/Chicken/Egypt/ALEX/ZU-NM99/2019	Ross	Alexandria	05/2019	25	6000	85	Respiratory signs, greenish diarrhoea, haemorrhages in caecal tonsils and proventriculus	Approx. 99% genome coverage (150 nucleotides gap), Full F gene	OP219680
NDV/Duck/Egypt/DAK/ZU-NM09/2019	Mallard (Domestic)	Dakahlia	03/2019	300	3300	185	Nervous signs, greenish diarrhoea, congestion in parenchymatous organs	Approx. 99% genome coverage (153 nucleotides gap), Full F gene	OP219681
NDV/Black-crowned night heron/Egypt/POR/ZU-NM85/2019	Migratory	Port-Said	09/2019	N/A	N/A	N/A	Apparently healthy without PM lesions	Approx. 90% genome coverage, Full F gene	OP219682
NDV/Duck/Egypt/DAK/ZU-NM54/2019	Muscovy	Dakahlia	03/2019	60	4000	113	Greenish diarrhoea, septicaemia	Approx. 70% genome coverage (3918 gap), Partial F gene	OP219683

3.4. Phylogenetics

Only full F gene sequences were included in phylogenetic analysis (**Table 5**). Among the five NDV isolates analysed, three isolates derived from chickens, one domestic duck, and a black-crowned night heron in Egypt in 2019 were included in phylogenetic analysis. The homology analysis of the five viral genome sequences demonstrated that the F genes shared very high nucleotide sequence identity of 98.04 – 100% (**Table S1**). Two chicken isolates NDV/Chicken/Egypt/NOR/ZU-NM76/2019 and NDV/Chicken/Egypt/ALEX/ZU-NM99/2019 clustered together with NDV/Black-crowned_night_heron/Egypt/POR/ZU-NM85/2019, a wild bird isolate, whereas the remaining chicken isolate, NDV/Chicken/Egypt/ALEX/ZU-NM97/2019, clustered together with mallard duck isolate NDV/Duck/Egypt/DAK/ZU-NM09/2019 isolate (**Figure S1**). In addition, homology analysis was also performed using the whole NDV genome sequences obtained in this study. Two chicken and one duck isolates (99% genome coverage) along with Black-crowned night heron isolate (90%) were included in this analysis (**Table 5**). A high 98.63% identity was found between NDV/Chicken/Egypt/NOR/ZU-NM76/2019 and NDV/Chicken/Egypt/ALEX/ZU-NM99/2019, followed by 96.87 – 97.18% of nucleotide sequence identity between the chicken isolates and duck isolate,

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NDV/Duck/Egypt/DAK/ZU-NM09/2019 (**Table S2**). Although we did not assemble a complete genome sequence of wild bird isolate (NDV/Black-crowned night heron/Egypt/POR/ZU-NM85/2019), however the homology analysis showed 95.63 – 96.19% identity with two chicken isolates and 94.48% nucleotide identity with the duck isolate (**Table S2).**

All isolates belonged to class II and clustered with NDV isolates within genotype VII.1.1.j (**Table S1**). Phylogenetic analysis of other representative isolates of genotype VII.1.1j strains shared a high F-gene nucleotide similarity (97.83% - 99.49%) with those isolated in Egypt between 2012-2016 (**Figure S3** and **Table S3**). The isolates also share high similarity with 2017 genotype VII Israeli strains and 2011 Chinese isolates (97.95% - 98.01% similarity). The F protein cleavage site sequence of all five isolates contained polybasic residues, (RRQRF) confirming their high virulence pathotype. The comparative alignment of the complete F protein amino acid sequence of the isolated strains against each other revealed four frequent residue substitutions (N/S, T/A, N/S and R/K) at positions, 30, 90, 258 and 480, respectively, resulting in independent sequences (**Figure S2**).

4. Discussion

Global distribution of NDV and epidemiological analysis portrays genotype VII as the predominant genotype and is responsible for the fourth major NDV panzootic worldwide with often fatal consequences following infection of susceptible birds [42,43]. The poultry industry in Egypt is equally suffering massive economic losses due to NDV-GVII infections, despite delivering mass vaccination schedules to all commercial flocks. Continued prevalence of NDV-GVII in wild birds plays a critical role in the dissemination and evolution of NDV [44]. Surveillance and reporting of NDV in wild birds has been limited yet is important for the assessment of potential disease risks to the poultry flocks. As such, this study developed the epidemiological picture and dissemination of NDV in domestic and wild bird populations throughout 13 governorates of Egypt.–

Previous studies suggest that many wild birds may act not just as a reservoir of low virulence strains [17,45,46], but may also play a critical role in the epidemiology of different variants NDV strains persisting in Africa, including VNDV strains responsible for poultry outbreaks [47]. Therefore, we anticipated that samples from wild birds may mostly contain low virulence strains; however, the current results, contradicted this hypothesis and all wild birds sampled were positive for VNDV-GVII, confirmed by the presence of polybasic residues at the cleavage site in the F gene. Additionally, all NDV positive samples collected from poultry flocks were also all VNDV-GVII.

The incidences of NDV among domestic birds were 65% and 47.4% in chicken and duck flocks, respectively; similar levels of NDV prevalence in domestic poultry (52.4%) was found in a cohort epidemiological study by Abozaid *et al*., [48] The observed higher prevalence of NDV in wild birds (72.9%) compared to domestic birds (57.4%) was surprising. A similarly high prevalence of NDV infection in wild birds was recorded by Ameji *et al*. [49] with 60%. Conversely, low detection rate of NDV was recorded in wild birds with percentage of 5.18% [50]; 3.06% [47], and 3.77% [29]. In view of a few studies carried out in Egypt on wild bird species, velogenic NDV sub-genotype VII.1.1 was isolated from cattle egret and house sparrows that were collected from the vicinity of poultry farms with a history of NDV infection [30]. Among the 85 samples positive for NDV, the majority 71 (61.6%) contained NDV alone but 14 samples (38.4%) contained both NDV and AIV. Such incidences of NDV and AIV co-infections are not rare and have been widely reported in many regions of Egypt, with higher rates of occurrence where both pathogens are enzootic poultry and wild birds [8].

In the current study, all our isolates that included apparently healthy migratory birds (Black-crowned night heron) and diseased domestic ducks, had a velogenic NDV configuration of the cleavage site (RRQRF), conflicting findings of previous studies, performed in various countries, which mainly isolated lentogenic NDVs from wild birds [51-55] and domestic ducks [4,54,56]; Mohammed *et al*., [29] detected a lentogenic motif at the cleavage

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site in all NDV strains isolated from wild pintail, Northern shoveler and Laughing doves. However, recent studies isolated several virulent strains of NDV from wild birds [57-59]. Similarly, Meng *et al*., [60], Rehman *et al*., [61], Waziri *et al*., [62], with Zahid *et al*., [63] recorded that the velogenic strains of NDV showed closer identity with genotype VII from apparently healthy ducks.

As NDV is an acute contagious disease affecting birds of all ages [64], the clinical observations of this study revealed that domestic birds including chickens, ducks and turkey infected with virulent strains of NDV (VNDV) experience cyanosis of comb and wattles, greenish diarrhoea, respiratory and nervous distress general septicaemia and haemorrhages in the internal organs, with variable degrees of severity. These clinical and postmortem observations correlate to disease manifestation findings of birds infected with VNDV in other studies [10,65-67]. However, all examined migratory and non-migratory wild birds that were positive for NDV had no apparent clinical disease signs or post-mortem lesions, which are coherent with the findings recorded in previous studies related to ND infections in wild birds [30,58].

Ducks are another important species to be given more interest, as they present varied clinical disease pattern under NDV infection. In the current study, NDV as a single infection was isolated from 17 clinically diseased ducks and only one flock was co-infected with NDV and AIV. In recent years, many NDV strains were pathogenic in waterfowls, and natural ND cases in ducks have been gradually increasing [23,68-71]. Experimental infection of Muscovy ducks with NDV genotype VII led to only 5% mortality despite significant tracheal and cloacal shedding, however close contact chickens experienced severe symptoms with higher mortality rates, emphasizing the role of ducks as effective carriers of NDV [72]. Additionally, virulent NDVs of genotype VII with AIV were identified in two out of six investigated duck farms suffering from respiratory manifestation and high mortality in Egypt [73]. The current study illustrated the dramatic change in the expansion of the VNDV infection in ducks which not only emphasizes the fact that ducks are effective carriers of NDV but also serve as potential reservoir hosts, susceptible to NDV and clinically display outcomes of the disease. Additionally, the evidence suggests that the clinical disease signs or pathological outcome varies greatly with host species, age and NDV strain.

Phylogenetic analysis revealed that all sequences of the 7 examined strains of domestic poultry: NDV/Chicken/Egypt/NOR/ZU-NM76/2019, NDV/Chicken/Egypt/ALEX/ZU-NM93/2019, NDV/Chicken/Egypt/ALEX/ZU-NM97/2019 and NDV/Chicken Egypt/ALEX/ZU-NM99/2019, those from ducks: NDV/Duck/Egypt/DAK/ZU-NM09/2019 and NDV/Duck/Egypt/DAK/ZU-NM54/2019, and one from migratory wild birds: "NDV/Black-crowned_night_heron/Egypt/POR/ZU-NM85/2019" were velogenic and clustered together in class II within sub-genotype VII.1.1, with 98.04 – 100% nucleotide identity. Additionally, high F-gene nucleotide similarity was detected with isolates from China in 2009 (97.89% - 98.08%). NDV genotype VII is the predominant genotype in poultry in the Middle East with most NDV isolates from wild birds additionally assigned to this genotype [52]. Other previous studies concluded that the predominant NDV is NDV sub-genotype VII.1.1 and causing several outbreaks in Egypt [10,67,74-76]. The close phylogenetic relationship between circulating NDV strains in migratory wild birds and those detected in domestic poultry suggest an enhanced role of wild birds and their ability to transmit the virus cross-species among different hosts and across continents.

Also, the studied strains were highly similar with the sequences of NDV-GVII.1.1 isolates from neighbouring countries such as those in Israel, 2017 and China, 2011 (98.11% - 98.01%); genotype VII was the predominant genotype in China of NDV during 2000–2015 [77-79]. Field strains isolated in Israel since 2000 mainly belong to sub-genotypes VIIb, VIId, and VIIi of genotype VII (class II) [42,80,81]. These findings suggest that this genotype remains endemic and circulating in many countries. Lately, genotype VII.1.1 of virulent strains NDV has been causing severe diseases outbreaks in poultry in several countries [82-85]. Dimitrov *et al*. [3] reported that a single genotype (VII.1.1) was responsible for the fourth NDV panzootic.

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Few amino acid (aa) substitutions (N30S, T90A, N258S, and R480K) were found in the entire length of F protein in APMV-1 isolates from chicken, duck, and wild birds as compared with each other. The substitution particularly in the surface-exposed aa residues in the signal peptide cleavage site of NDV F protein (position: 30) is considered to be highly variable as this region must undergo constant positive selection [86]. Orabi et al., [87] detected that the N30 residue appears to be conserved in all vaccine and Egyptian field strains clustered within genotype II; two isolates from this study "NDV/Duck/Egypt/DAK/ZU-NM09/2019" and "NDV/Chicken/Egypt/ALEX/ZU-NM97/2019" shared such residue. This suggested that some ND viruses of genotype VII in Egypt may induce a new selection profile based on mutation categories of field virus isolates in the presence of vaccines of different genotypes [87,88].

Being F protein is considered two subunits (F1 and F2), the F1 subunit is included in the position (117 → 533), and the F2 subunit is in the position (32 → 116), in which the cleavage site present at position 112-116 [86,87]. In the current study, there are two aa substitutions in the F2 subunit (position: 90) and F1 subunit (position: 258) which might initiate a change in the fusion protein. The aa substitutions at residue 90 (T→N) in the F2 subunit and its location near the cleavage site motif which might initiate change in the antigenicity. Preceding studies suggested that aa substitutions in the F protein at residues located in the helix sheet near the cleavage site motif (78 and 79) may alter the antigenicity [71,89,90].

The hypervariable regions of the fusion peptide unsurprisingly had three-heptad repeat regions HRa, HRb, and HRc spanning at positions 143–185, 268–299, and 471–500, respectively [86,91]. Three isolates (NDV/Chicken/Egypt/NOR/ZU-NM76/2019, NDV/Black-crowned night heron/Egypt/POR/ZU-NM85/2019, and NDV/Chicken/Egypt/ALEX/ZU-NM99/2019) showed the substitution R480K within the HRc, a domain essential for virus fusion together with HRa and HRb. Although the strains of NDV genotype VII isolated during 2016 in Egypt had a conserved amino acid residue (R480), but recorded substitution at closely aa position N469S and discussed that they induced high mortality among infected chickens [87]. Earlier reports indicated that the fusion activity of NDV may be affected by the aa substitutions in the fusion peptide and HR regions of the F protein [10,92]. Further study is required to investigate the viral fitness following such substitutions detected within the F protein of NDV of the isolates of this study.

Based on NDV genotypic characterization, NDV-II and VII lineages were mainly found in domestic poultry (chickens) and NDV-VI lineage in pigeons and doves [93-95]. Results of this study revealed that current NDV-VII.1.1 with the velogenic character is circulating amongst wild and domestic birds in Egypt, raising concern to the disease picture outcomes recorded in the domestic ducks. Additionally, the high antigenic and genetic identities of the NDV strains isolated from geographically separated hosts within study may reflect the possible role of wild and migratory birds in the maintenance and transmission cycle of NDV between and within domesticated poultry in Egypt, further proved by previous authors for different wild birds in other regions around the world [96-98]. As such, ND could be included in the important disease list affecting ducks in Egypt. The continuous surveillance of NDV traits among migratory and free-living birds simultaneously with domestic avian species remains critical for the implementation of appropriate protective vaccines as well as risk assessments of virus dissemination from wild birds to poultry. Necessitating more improved biosecurity systems reduce the virus transmission cycle between wild birds and domestic poultry.

Supplementary Materials: Table S1: The genetic relatedness among the NDV strains. Phylogenetic analysis of a total of 130 NDV strains, including our five isolates (in bold), was performed based on the full F gene coding region. The analysis included 125 representative sequences that belong to different genotyping groups; **Table S2**: The genetic relatedness among the NDV isolates sequenced in this study. Phylogenetic analysis was done based on full genome sequence. Sequence similarities are represented as pairwise percentages coloured from white to green for genetic dissimilarity to

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similarity; **Table S3:** Genetic similarity of closely branched strains from phylogenetic analysis of the fusion (F) protein gene from **Figure S3**. Pairwise comparisons shown as percentage similarity of the open-reading frame; **Figure S1**: A maximum-likelihood phylogenetic tree of the full fusion, F gene sequences of members of the Class II Newcastle disease virus strains. The analysis involved 130 nucleotide sequences created by an international consortium of NDV experts for the needs of objective classification of NDV isolates [3]. Five samples sequenced in this study (red) clustered with China, Liaoning 2009, a group VII strain. GTR nucleotide substitution model, with among-site rate variation modelled using a discrete gamma distribution was performed to generate evolutionary history. Evolutionary analyses were conducted in MEGA11; **Figure S2**: Multiple sequence alignment of fusion protein (F) of Newcastle disease virus (NDV) strains sequenced in this study. Differences in amino acid compositions between the NDV strains are highlighted with position number listed above. Amino acids are coloured by default using RasMol 'amino' colour scheme according to traditional amino acid properties. Identical amino acids are indicated by a (.); **Figure S3**: Phylogenetic tree of the nucleotide sequences of the fusion (F) protein gene of Newcastle disease virus strains representing the genotype VII subgroup viruses (*n=559*). The evolutionary history was generated using GTR nucleotide substitution model, with among-site rate variation modelled using a discrete gamma distribution and 500 bootstrap replicate tests. The analysis involved 559 nucleotide sequences, including five F gene sequences in this study (red). Closely related strains outside of the Egyptian strains are an Israeli strain (blue) and a Chinese strain (purple). Visualised and edited in FigTree.

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Institutional Review Board Statement: All procedures involving animals were performed adopting applicable international, national, and/or institutional guidelines for the care and use of animals for research. The Ministry of Environment for wild bird capture uses the procedures outlined in the Guide for the Care and Use of birds in scientific research. Those who capture wild birds have a hunting identity card and apply the technical and environmental conditions issued by the Ministry of Environment by Law No. 4 of 1994 and based on Ministerial Resolutions No. 1270 of 8/9/2018 and No. 209 of 6/8/2019.

Informed Consent Statement: Not applicable.

Data Availability Statement: participant data will be made available upon reasonable requests directed to the corresponding author. Proposals will be reviewed and approved by the investigator and collaborators on the basis of scientific merit. After approval of a proposal, data can be shared through a secure online platform after signing a data access agreement.

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Supplementary material

Suppl. Table 1. The genetic relatedness among the NDV strains. Phylogenetic analysis of a total of 130 NDV strains, including our five isolates (in bold), was performed based on the full F gene coding region. The analysis included 125 representative sequences that belong to different genotyping groups. Representative datasets were created by an international consortium of NDV experts for the needs of objective classification of NDV isolates [3].

	NDV/Chicken/Egypt/ALEX/ZU-NM99/2019	NDV/Chicken/Egypt/ALEX/ZU-NM97/2019	NDV/Chicken/Egypt/NOR/ZU-NM76/2019	NDV/Black-crowned night heron/Egypt/POR/ZU-NM85/2019	NDV/Duck/Egypt/DAK/ZU-NM09/2019
NDV/Chicken/Egypt/ALEX/ZU-NM99/2019		98.014	100	100	98.014
NDV/Chicken/Egypt/ALEX/ZU-NM97/2019	98.014		98.014	98.014	100
NDV/Chicken/Egypt/NOR/ZU-NM76/2019	100	98.014		100	98.014
NDV/Black-crowned night heron/Egypt/POR/ZU-NM85/2019	100	98.014	100		98.014
NDV/Duck/Egypt/DAK/ZU-NM09/2019	98.014	100	98.014	98.014	
XIV.2_XIV_b_HF969187_chicken_Nigeria_NIE08_453_2008	86.883	87.425	86.883	86.883	87.425
XIV.2_XIV_b_HF969210_chicken_Nigeria_NIE10_139_2011	86.763	87.365	86.763	86.763	87.365
XIV.2_XIV_b_KY171990_chicken_Nigeria_KD_TW_03T_N45_720_2009	86.883	87.485	86.883	86.883	87.485
XIV.1_XIV_a_JN872165_chicken_Niger_VIR_1377_7_2006	87.304	87.545	87.304	87.304	87.545
XIV.1_XIV_a_HF969205_turkey_Nigeria_NIE09_2071_2009	87.064	87.485	87.064	87.064	87.485
XIV.1_XIV_a_JQ039386_chicken_Nigeria_VRD08_36_2008	87.545	87.906	87.545	87.545	87.906
XVII_XVII_b_HF969194_chicken_Nigeria_NIE08_2199_2009	87.665	87.906	87.665	87.665	87.906
XVII_XVII_a_HF969176_chicken_Nigeria_NIE10_310_2011	87.786	87.605	87.786	87.786	87.605
XVII_XVII_a_HF969191_chicken_Nigeria_NIE08_2042_2009	87.786	87.365	87.786	87.786	87.365
XII.2_XII_b_MF278927_goose_China_FS_SS_292_2013	88.207	88.267	88.207	88.207	88.267
XII.2_XII_b_JN627504_goose_China_GD_12_2011	88.267	88.327	88.267	88.267	88.327
XII.2_XII_b_JN627507_goose_China_GD_1003_2010	88.568	88.508	88.568	88.568	88.508
XII.1_XII_a_KU594618_chicken_Peru_Arequipa_VFAR_81_2015	87.064	87.004	87.064	87.064	87.004
XII.1_XII_a_KU594615_chicken_Peru_Apurimac_50009_2005	88.448	88.147	88.448	88.448	88.147
XII.1_XII_a_KU594616_gamecock_Peru_Lurin_40871_2004	88.448	88.147	88.448	88.448	88.147
XVIII.1_XVIII_a_JX518885_chicken_Mali_ML57051T_2010	88.387	88.568	88.387	88.387	88.568
XVIII.1_XVIII_a_FJ772455_Mauritania_1532_14_2006	88.628	88.809	88.628	88.628	88.809
XVIII.1_XVIII_a_JF966389_guinea_fowl_Mali_ML038_2007	88.207	88.387	88.207	88.207	88.387
XVIII.2_XVIII_b_JX518886_chicken_Mali_ML57072T_2010	89.29	88.748	89.29	89.29	88.748
XVIII.2_XVIII_b_HF969218_chicken_Ivory_Coast_CIV08_42_2007	88.869	88.688	88.869	88.869	88.688
XVIII.2_XVIII_b_HG326600_village_weaver_Ivory_Coast_CIV08_32_2006	89.11	88.929	89.11	89.11	88.929
XIII.2.2_XIII_b_KM056349_chicken_India_ndv42_gopalpura_4_2013	87.304	87.124	87.304	87.304	87.124
XIII.1.1_XIII_a_JN942043_roller_Tanzania_47385_11_2010	87.485	87.605	87.485	87.485	87.605

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XIII.1.1_XIII_a_MF409241_chicken_Zambia_Chiwoko_2015	88.508	88.688	88.508	88.508	88.688
XIII.1.1_XIII_a_JN942034_ostrich_South_Africa_45445_3_1995	89.651	89.591	89.651	89.651	89.591
XIII.1.2_XIII_a_JQ267584_chicken_Iran_EMM_2_2008	89.35	89.29	89.35	89.35	89.29
XIII.1.2_XIII_a_JQ267579_chicken_Iran_EMM_7_2011	89.23	89.29	89.23	89.23	89.29
XIII.1.2_XIII_a_JQ267585_chicken_Iran_EMM_1_2008	89.11	89.17	89.11	89.11	89.17
XIII.2.2_XIII_b_KT734767_chicken_India_Polashbari_2014	88.147	88.267	88.147	88.147	88.267
XIII.2.2_XIII_b_KX372707_chicken_India_Nagpur_3_2011	87.244	87.244	87.244	87.244	87.244
XIII.2.1_XIII_b_GU182323_chicken_Pakistan_SPVC_Karachi_43_2008	87.545	87.726	87.545	87.545	87.726
XIII.2.1_XIII_b_GU182331_chicken_Pakistan_SPVC_Karachi_33_2007	88.327	88.387	88.327	88.327	88.387
XIII.2.1_XIII_b_KF113338_chicken_Pakistan_University_Diagnostic_Lab_12_2010	88.207	88.387	88.207	88.207	88.387
VII.2_VII_h_MF622047_chicken_South_Africa_RBWW_3_2013	89.17	88.989	89.17	89.17	88.989
VII.2_VII_i_KU862293_Parakeet_Pakistan_Karachi_AW_1_2014	90.554	90.193	90.554	90.554	90.193
VII.2_VII_i_HQ697254_chicken_Indonesia_Banjarmasin_10_2010	90.854	90.493	90.854	90.854	90.493
VII.1.2_VII_f_DQ227246_goose_China_Jiangsu_JS02_1999	92.96	92.78	92.96	92.96	92.78
VII.1.2_VII_f_AY028995_fowl_China_A7_1996	93.081	92.9	93.081	93.081	92.9
VII.1.2_VII_f_GQ338309_pigeon_China_18_2003	93.562	93.381	93.562	93.562	93.381
VII.1.1_VII_l_KX268351_chicken_Iran_Behshahr_2015	93.682	93.201	93.682	93.682	93.201
VII.1.1_VII_j_KC542905_chicken_China_Liaoning_1_2009_2009	98.075	97.894	98.075	98.075	97.894
VII.1.1_VII_e_AB853927_chicken_Japan_Ibaraki_SG106_1999	94.344	93.923	94.344	94.344	93.923
VII.1.1_VII_b_EF589133_pheasant_China_98_Guizhou_1998	95.307	95.247	95.307	95.307	95.247
VII.1.1_VII_d_EF579733_chicken_China_Shandong_Pyan_2004	94.344	94.043	94.344	94.344	94.043
VII.2_VII_k_KY747479_chicken_Namibia_5620_2016	89.29	89.11	89.29	89.29	89.11
VII.2_VII_a_JN986837_chicken_Netherlands_152608_ancestral_1993	91.937	91.637	91.937	91.937	91.637
XI_XI_HQ266602_chicken_Madagascar_MG_725_2008	82.07	81.889	82.07	82.07	81.889
XI_XI_JX518882_chicken_Madagascar_MGMNJ_2009	81.408	81.227	81.408	81.408	81.227
XI_XI_JX518884_chicken_Madagascar_MGS1595T_2011	81.889	81.829	81.889	81.889	81.829
II_II_GU978777_chicken_USA_TX_GB_1948	82.912	83.032	82.912	82.912	83.032
II_II_AF077761_chicken_USA_Lasota_1946	82.912	83.032	82.912	82.912	83.032
II_II_JN872151_chicken_USA_Hitchner_B1_1947	83.032	83.153	83.032	83.032	83.153
XXI_VI_l_KC205479_chicken_Ethiopia_ETHMG1C_2011	85.68	85.319	85.68	85.68	85.319
X.1_X_a_FJ705468_mottled_duck_USA_TX_130_2011	84.271	83.848	84.271	84.271	83.848
X.1_X_a_KX857716_Redhead_USA_ndv42_AI09_4117_2009	84.055	83.634	84.055	84.055	83.634
X.2_X_b_FJ705466_mallard_99_376_1999	84.15	84.15	84.15	84.15	84.15
X.2_X_b_KX857721_Mallard_USA_MN_AI10_3434_2010	83.755	83.755	83.755	83.755	83.755
V.1_V_b_JN872194_chicken_Honduras_498109_15_2007	84.717	84.717	84.717	84.717	84.717
V.1_V_b_JN942027_fighting_cock_Nicaragua_95066_9_2001	85.499	85.86	85.499	85.499	85.86
V.1_V_b_JN872189_parrot_USA_Coast_8278_1982	86.763	86.763	86.763	86.763	86.763
V.2_V_c_JQ697744_chicken_Mexico_NC04_635_2010	87.417	87.477	87.417	87.417	87.477
V.2_V_c_EU518682_Dove_Mexico_Distrito_Federal_462_2004	87.665	87.605	87.665	87.665	87.605
V.2_V_c_EU518684_chicken_Mexico_Estado_de_Mexico_466_2006	87.365	87.425	87.365	87.365	87.425
XIX_V_a_FJ705456_cormorant_USA_MN_92_40140_1992	85.723	85.723	85.723	85.723	85.723
XIX_V_a_JN942024_cormorant_USA_WI_272409_2003	86.161	85.68	86.161	86.161	85.68

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XIX_V_a_KC433530_cormorant_USA_FL_41105_2012	85.379	85.259	85.379	85.379	85.259
XXI.2_VI_i_KU377535_Turtle_dove_Italy_12VIR1876_1_2012	85.86	85.56	85.86	85.86	85.56
XXI.2_VI_i_JN638234_dove_Italy_11RS98_102VIR_2011	85.8	85.379	85.8	85.8	85.379
XXI.2_VI_i_KU377533_Turtle_dove_Italy_10VIR7155_2010	85.74	85.319	85.74	85.74	85.319
XVI_XVI_JX915242_chicken_Dominican_Republic_28138_4_1986	85.018	85.018	85.018	85.018	85.018
XVI_XVI_JX186997_chicken_Dominican_Republic_867_2008	83.694	84.055	83.694	83.694	84.055
I.2_I_b_KC503453_American_green_winged_teal_USA_AK_44493_716_2009	84.176	84.116	84.176	84.176	84.116
I.2_I_b_AY965079_duck_Russia_FarEast_2713_2001	84.717	84.777	84.717	84.717	84.777
I.2_I_b_HG326605_spur_winged_goose_Nigeria_NIE08_121_2008	84.416	84.416	84.416	84.416	84.416
I.1.1_I_a_M24693_chicken_Australia_Queensland_1966	85.078	84.958	85.078	85.078	84.958
I.1.1_I_a_AY935490_chicken_Australia_2_1334_2002	85.078	84.898	85.078	85.078	84.898
I.1.1_I_a_AY935495_chicken_Australia_99_868_hi_1999	85.439	85.199	85.439	85.439	85.199
I.1.2.2_I_d_KC503476_northern_pintail_USA_AK_44500_136_2009	84.116	83.875	84.116	84.116	83.875
I.1.2.2_I_d_AB465607_chicken_Japan_Ishi_1962	85.018	84.958	85.018	85.018	84.958
I.1.2.2_I_d_KC503479_redpoll_Russia_Nikita_530_FFNK2_2008	83.935	83.815	83.935	83.935	83.815
I.1.2.1_I_c_KX352834_gull_Russia_Tyva_14_2014	82.671	82.732	82.671	82.671	82.732
I.1.2.1_I_c_EF564816_redknot_USA_NJ_A_101_1383_2001	83.273	83.454	83.273	83.273	83.454
I.1.2.1_I_c_GQ918280_black_headed_gull_Sweden_1994	84.176	84.296	84.176	84.176	84.296
XXI.1.2_VI_m_KY042141_Pigeon_Pakistan_Jallo_Lahore_221B_2016	86.161	86.522	86.161	86.161	86.522
XXI.1.2_VI_m_KU862298_pigeon_Pakistan_Lahore_AW_2_2015	86.221	86.522	86.221	86.221	86.522
XXI.1.2_VI_m_KY042135_Pigeon_Pakistan_22A_2015	86.643	87.064	86.643	86.643	87.064
III_III_GU182327_chicken_Pakistan_SPVC_Karachi_1_1974	84.838	84.958	84.838	84.838	84.958
III_III_EF201805_avian_Mukteswar_1940	85.078	85.199	85.078	85.078	85.199
III_III_MH996904_pigeon_Bulgaria_Novo_Selo_1161_1995	85.018	85.138	85.018	85.018	85.138
XXI.1.1_VI_g_KY042136_Pigeon_Pakistan_Lahore_125_2015	88.267	88.267	88.267	88.267	88.267
XXI.1.1_VI_g_JF824032_pigeon_Russia_Vladimir_687_2005	88.207	87.906	88.207	88.207	87.906
XXI.1.1_VI_g_KY042132_Pigeon_Egypt_73_OP_G29_2015	88.026	87.846	88.026	88.026	87.846
XX_VI_c_KY042142_quail_Korea_88_M_1988	88.267	88.207	88.267	88.267	88.207
XX_VI_c_AB853928_chicken_Japan_Ibaraki_SM87_1987	88.387	88.327	88.387	88.387	88.327
XX_VI_c_AF458016_chicken_China_ZhJ_2_1986	88.573	88.513	88.573	88.573	88.513
VI.2.1.2_VI_h_HG424627_pigeon_Nigeria_NIE13_92_2013	87.545	87.605	87.545	87.545	87.605
VI.2.2.2_VI_e_FJ480825_pigeon_China_PG_JS_1_2005	87.545	87.665	87.545	87.545	87.665
VI.2.2.2_VI_e_JX244794_pigeon_China_100_2008	87.966	88.087	87.966	87.966	88.087
VI.2.2.2_VI_e_KJ607163_pigeon_China_LJS_1_2004	87.846	87.846	87.846	87.846	87.846
VI.1_VI_b_AF109885_domestic_fowl_Great_Britain_GB1168_1984	88.508	88.568	88.508	88.508	88.568
VI.1_VI_b_FJ410145_pigeon_USA_NY_1984	88.448	88.387	88.448	88.448	88.387
VI.1_VI_b_FJ865434_pigeon_China_S_1_2002	88.267	88.327	88.267	88.267	88.327
VI.2.2.1_VI_f_JN872180_waterfowl_USA_TX_209682_2002	87.665	87.846	87.665	87.665	87.846
VI.2.2.1_VI_f_JN872182_pigeon_USA_12339_1998	87.605	87.545	87.605	87.605	87.545
VI.2.2.1_VI_f_JX901312_pigeon_USA_101_2001	87.485	87.184	87.485	87.485	87.184
VI.2.1.2_VI_h_HG326604_pigeon_Nigeria_NIE09_1898_2009	87.665	87.485	87.665	87.665	87.485
VI.2.1.2_VI_h_JX518532_laughing_dove_Kenya_B2_Isiolo_2012	87.786	87.244	87.786	87.786	87.244

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VI.2.1.1.1_VI_n_MG018211_ECDO_USA_TX_1185_kidney_26981_3_A_2015	86.522	86.402	86.522	86.522	86.402
VI.2.1.1.1_VI_a_JX901367_pigeon_USA_PA_810_2008	87.545	87.726	87.545	87.545	87.726
VI.2.1.1.1_VI_a_JX901351_pigeon_USA_NJ_721_2007	87.726	87.786	87.726	87.726	87.786
VI.2.1.1.2.2_VI_k_MG840654.1_pigeon_China_Ningxia_2068_2016	86.101	86.041	86.101	86.101	86.041
VI.2.1.1.2.2_VI_k_KT163262_pigeon_China_SH_167_2013	86.643	86.582	86.643	86.643	86.582
VI.2.1.1.2.2_VI_k_JX901124_pigeon_Belgium_11_09620_2011	86.883	86.823	86.883	86.883	86.823
VI.2.1.1.2.1_VI_j_JX094510_pigeon_China_sms12_2012	87.064	87.064	87.064	87.064	87.064
VI.2.1.1.2.1_VI_j_JX901110_pigeon_Belgium_248_1998	88.327	88.387	88.327	88.327	88.387
VI.2.1.1.2.1_VI_j_JX486553_pigeon_China_LHLJ_110813_2011	87.906	87.726	87.906	87.906	87.726
IX_IX_AF458009_chicken_China_FJ_1_1985	85.732	85.973	85.732	85.732	85.973
IX_IX_FJ436303_chicken_China_ZJ_1_1986	85.62	85.74	85.62	85.62	85.74
IX_IX_FJ436302_chicken_China_F48E8_1948	85.68	85.8	85.68	85.68	85.8
IV_IV_AY741404_Fowl_UK_Herts_1933	87.425	87.425	87.425	87.425	87.425
IV_IV_MH996900_pullet_Bulgaria_Plovdiv_1153_1959	86.703	86.342	86.703	86.703	86.342
VIII_VIII_FJ751918_chicken_China_QH1_1979	87.485	87.665	87.485	87.485	87.665
XVI_XVI_JX915243_chicken_Mexico_Queretaro_452_1947_1947	88.628	88.929	88.628	88.628	88.929
VIII_VIII_AY734534_chicken_Argentina_Trenque_Lauquen_1970	88.267	88.508	88.267	88.267	88.508
VIII_VIII_JX012096_Malaysia_AF2240_1960	88.748	88.929	88.748	88.748	88.929

Suppl. Table 2. The genetic relatedness among the NDV isolates sequenced in this study. Phylogenetic analysis was done based on full genome sequence. Sequence similarities are represented as pairwise percentages, coloured from white to green for genetic dissimilarity to similarity.

	NDV/Chicken/Egypt/ALEX/ZU-NM97/2019	NDV/Black-crowned night heron/Egypt/POR/ZU-	NDV/Duck/Egypt/DAK/ZU-NM09/2019	NDV/Chicken/Egypt/NOR/ZU-NM76/2019	NDV/Chicken/Egypt/ALEX/ZU-NM99/2019
NDV/Chicken/Egypt/ALEX/ZU-NM97/2019		94.147	97.035	96.85	97.096
NDV/Black-crowned night heron/Egypt/POR/ZU-NM85/2019	94.147		94.481	95.628	96.194
NDV/Duck/Egypt/DAK/ZU-NM09/2019	97.035	94.481		96.873	97.182
NDV/Chicken/Egypt/NOR/ZU-NM76/2019	96.85	95.628	96.873		98.627
NDV/Chicken/Egypt/ALEX/ZU-NM99/2019	97.096	96.194	97.182	98.627	

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Suppl. Table 3. Genetic similarity of closely branched strains from phylogenetic analysis of the fusion (F) protein gene from **Suppl. Figure 3**. Pairwise comparisons shown as percentage similarity of the open-reading frame.

	Egypt_76_chicken_2019	Egypt_85_night_heron_2019	Egypt_9_domestic_duck_2019	Egypt_97_chicken_2019	Egypt_99_chicken_2019
KY510687.1_NDV/chicken/IS/2/2017	98.014	98.014	97.954	97.954	98.014
JQ015297.1_NDV/chicken/China/SDYT03/2011	98.014	98.014	97.954	97.954	98.014
KU365650.1_NDV-FU4-EGYPT-NLQP-2014	98.255	98.255	98.195	98.195	98.255
KU365653.1_NDV-FU5-EGYPT-NLQP-2014	97.834	97.834	97.894	97.894	97.834
KY075892.1_NDV/chicken/Egypt/Ismailia29/2016	98.315	98.315	99.458	99.458	98.315
KY075888.1_NDV/chicken/Egypt/El-Arish16/2016	98.616	98.616	98.436	98.436	98.616

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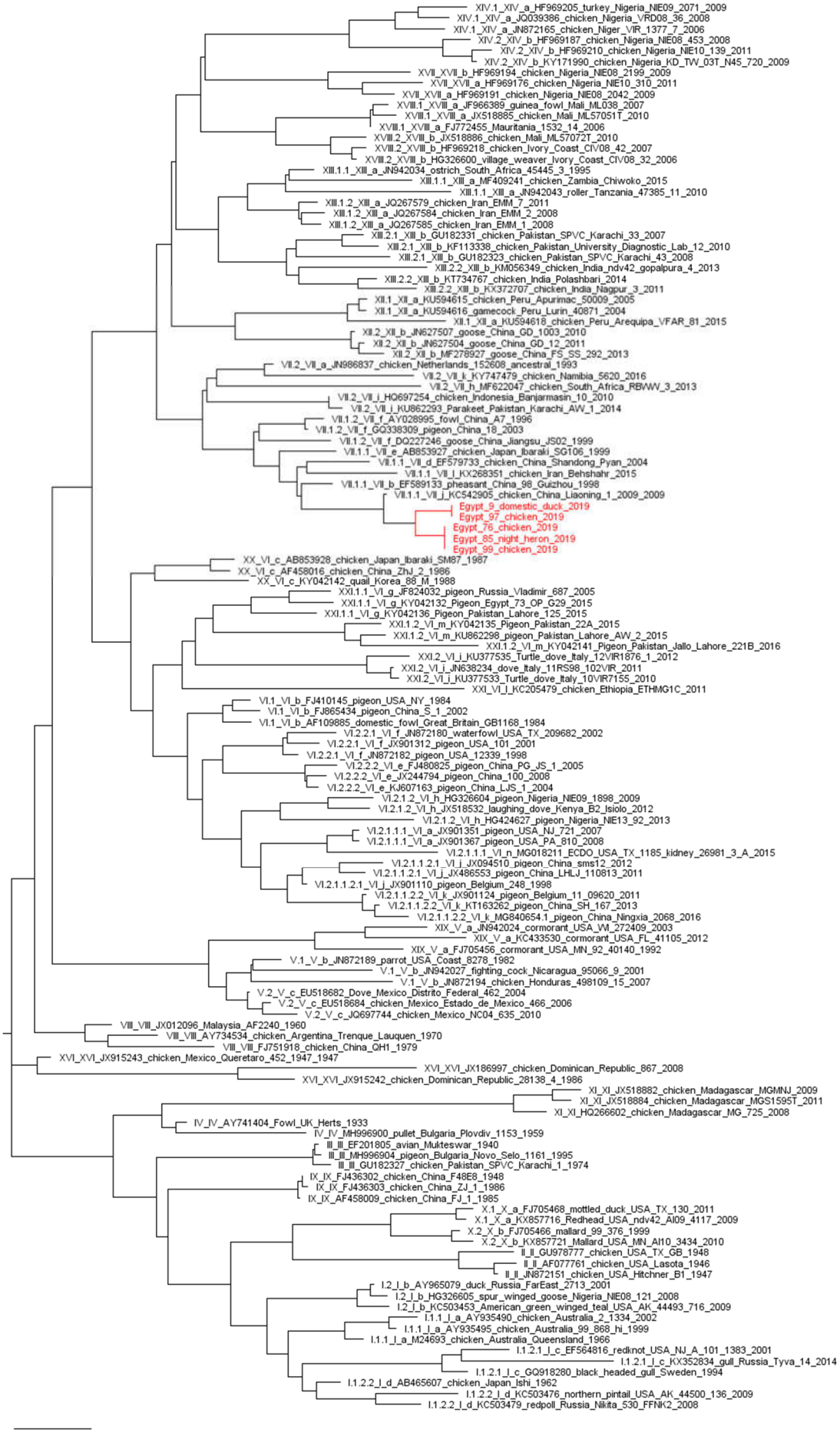

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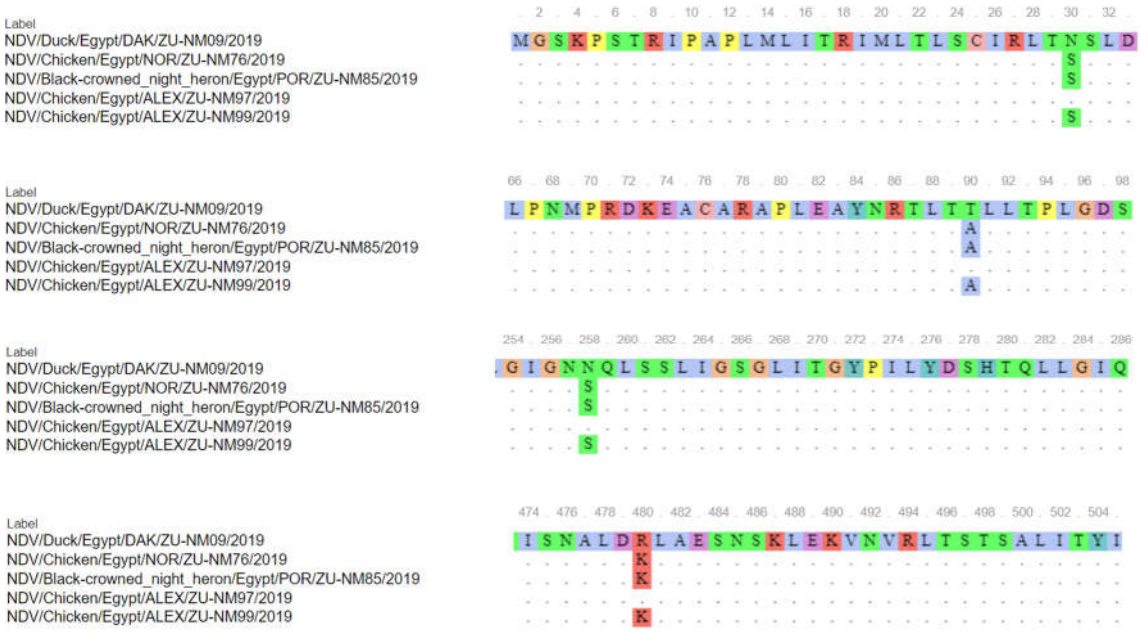
Suppl. Figure 1. A maximum-likelihood phylogenetic tree of the full fusion, F gene sequences of members of the Class II Newcastle disease virus strains. The analysis involved 130 nucleotide sequences created by an international consortium of NDV experts for the needs of objective classification of NDV isolates (Dymitrov et al, 2019). Five samples sequenced in this study (red) clustered with China, Liaoning 2009, a group VII strain. GTR nucleotide substitution model, with among-site rate variation modelled using a discrete gamma distribution was performed to generate evolutionary history. Evolutionary analyses were conducted in MEGA11.



Suppl. Figure 2. Multiple sequence alignment of fusion protein (F) of Newcastle disease virus (NDV) strains sequenced in this study. Differences in amino acid compositions between the NDV strains are highlighted with position number listed above. Amino acids are coloured by default using RasMol 'amino' colour scheme according to traditional amino acid properties. Identical amino acids are indicated by a (.).

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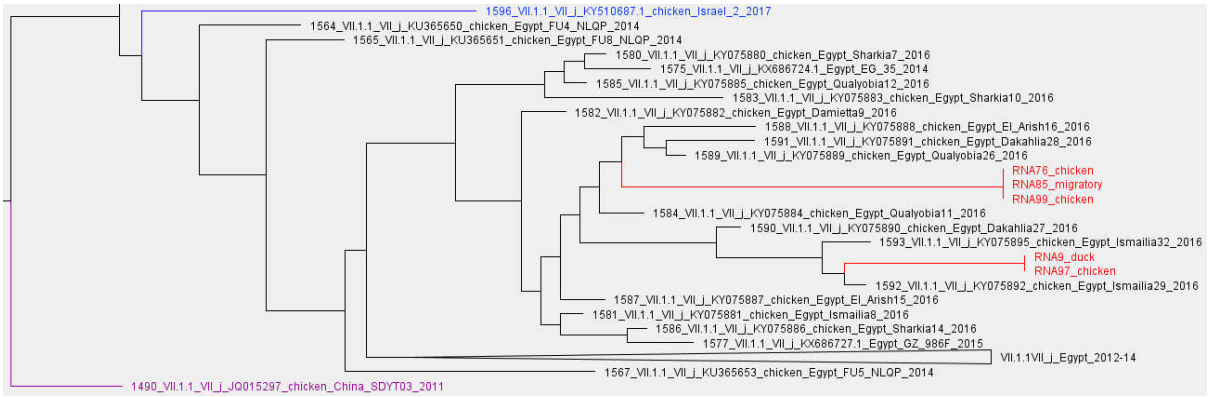

Suppl. Figure 3. Phylogenetic tree of the nucleotide sequences of the fusion (F) protein gene of Newcastle disease virus strains representing the genotype VII subgroup viruses (*n=559*). The evolutionary history was generated using GTR nucleotide substitution model, with among-site rate variation modelled using a discrete gamma distribution and 500 bootstrap replicate tests. The analysis involved 559 nucleotide sequences, including five F gene sequences in this study (red). Closely related strains outside of the Egyptian strains are an Israeli strain (blue) and a Chinese strain (purple). Visualised and edited in FigTree.