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Genetic characterization of velogenic and lentogenic Newcastle disease viruses circulating among broiler chickens in Al-wadi Al-gadid Governorate, Egypt (2021)

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ABSTRACT

The present study aimed to investigate circulated Newcastle disease virus (NDV) genotypes, and avian influenza virus (AIV) co-infection among chicken flocks receiving NDV vaccines in Al-Wadi Al-Gadid governorate, Egypt. Ninety-eight clinical samples were collected from diseased chickens in 10 chicken farms during 2021. The virus propagation in embryonated chicken eggs was confirmed serologically by hemagglutination and hemagglutination inhibition tests and molecularly by RT-PCR using specific primers of the Fusion (F) gene and matrix gene of AIV. The results revealed that 15 isolates were NDV-positive, of them, four were AIV co-infected. The F genes amino acid sequence alignments for selected six isolates indicated two cleavage site motifs characteristic to lentogenic (1) and velogenic (5) strains. Also, specific amino acid substitutions in F1 and HRa regions correlated to fusion activity and neutralizing antibody production in the present velogenic isolates were observed. Phylogenetic analysis of the partial F gene sequences revealed the circulation of two genotypes, GII and GVII.1.1 (VIIj), in class II among vaccinated chicken flocks in Al-Wadi Al-Gadid governorate. In addition, velogenic isolates are segregated into a distinct sub-clade from NDVs isolated in southern and northern Egypt. In conclusion, this is the first report on the genetic characterization of NDV among vaccinated chicken flocks in Al-Wadi Al-Gadid governorate. The current data will contribute to the NDV endemicity, genetic evolution, and biosecurity measurements to overcome NDV infection in this promising area for poultry production.

Introduction

Newcastle disease (ND) is a destructive viral disease for the poultry industry which is notifiable to the World Organization of Animal Health (WOAH). ND has been classified as the third most important poultry disease and the eighth most important wildlife disease in 2006 - 2009 (OIE, 2018a). The disease has circulated worldwide and affected several species of domesticated and wild birds (Wang *et al*., 2018; Helen *et al*., 2020; Tran *et al*., 2020; Abozaid and Abdel-Moneim, 2022; Allahyari *et al*., 2022; Sultan *et al*., 2022; Sun *et al*., 2022). It has infected over 240 bird species belonging to 27 orders (Helen *et al*., 2020). Globally, 109 countries from 200 member countries in WOAH have reported outbreaks from ND in 2018 (OIE, 2018b). ND is characterized by respiratory, intestinal, and nervous signs, with a mortality rate up to 100% (Wang *et al*., 2018).

Avian Orthoavulavirus 1 (AOAV-1, previously known as avian paramyxovirus 1 or NDV), which belongs to the genus *Orthoavulavirus*, subfamily *Avulavirinae* in the *Paramyxoviridae* family, is the causative agent of ND (Walker *et al*., 2022). AOAV-1 outbreaks continuously occurred among vaccinated chicken flocks in Egypt although vaccination efforts have been intensively applied (Selim *et al*., 2018; Shakal *et al*., 2020; Tran *et al*., 2020; Dewidar *et al*., 2021).

AOAV-1 is an enveloped virus that has a linear, negative single-stranded RNA. Its genome has 15,186, 15,192, or 15,198 nucleotides in length that encode two non-structural (V and W proteins) and six structural proteins including RNA-dependent RNA polymerase (L) protein, hemagglutinin-neuraminidase protein (HN), fusion protein (F), matrix protein (M), phosphoprotein (P), and nucleoprotein (N). The F and HN proteins play a role in the NDV infection process. The F protein is the key protein that determines NDV strains' virulence and induces neutralizing antibody production (Wang *et al*., 2018). The presence of multiple basic amino acid motifs (112R/K-R-Q-K/R-R116) in the F gene cleavage site followed by phenylalanine (F) at position 117 is a marker for high virulent (velogenic) NDV strains. In contrast, NDV strains with low virulence (lentogenic) usually have 112G/E-K/R-Q-G/E-R116 monobasic amino acid motif followed by leucine (L) at position 117 (WOAH, 2021).

The NDV class I consist of a single genotype with 3 sub-genotypes designated as 1.1.1, 1.1.2, and 1.2 where virus strains are avirulent and mainly isolated from wild birds. On the other hand, class II is composed of 21 distinct genotypes (I - XXI) of avirulent and virulent nature. The unified classification of NDV revealed that genotype VII, one of the most diverse NDV genotypes, is segregated into three sub-genotypes; VII.1.1, VII.1.2, and VII.2 (Dimitrov *et al*., 2019). Sub-genotype VII.1.1 includes VIIb, VIId, VIIe, VIII, and VIIj viruses causing the fourth ND panzootic in Asia, the Middle East, Europe, and Africa after their emergence in the Far East in 1985 (Le *et al*., 2018; Dimitrov *et al*., 2019). The sub-genotype VII.1.1 VIIj (previously named VIId) is the most prevalent sub-genotype in Egypt causing numerous outbreaks in vaccinated poultry farms (Sultan *et al*., 2015; Selim *et al*., 2018; Shakal *et al*., 2020; Tran *et al*., 2020; Dewidar *et al*., 2021; El-morshidy *et al*., 2021).

Co-infection of NDV and avian influenza virus (AIV) has been frequently reported among poultry. This co-infection adversely affects the intensity of clinical signs and shedding virus load (Gowthaman *et al*., 2019; Umar *et al*., 2019; Farzin *et al*., 2023).

To our knowledge, there are no reports concerning the endemicity of NDV infection among vaccinated chicken farms and genetic characteristics in Al-Wadi Al-Gadid governorate, Egypt. Therefore, this study was conducted to isolate and characterize the circulating NDV strains and determine their pathotypes, genotypes, and AIV co-infection among vaccinated chicken farms in Al-Wadi Al-Gadid governorate, Egypt in 2021.

Materials and methods

Study area and sample collection

In this study, a total of 98 samples (brain, trachea, lung, heart, liver, spleen, kidney, intestine, and proventricular) were collected from freshly

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dead broiler chickens shown high mortality (ranging from 70-80%) with respiratory (coughing, sneezing, watery discharge from nostrils labored breathing, gasping, and lethargy) and/or nervous signs from 10 broiler chicken farms (age ranging from 25-35 days) located in Al-Wadi Al-Gadid governorate, Egypt during 2021. The postmortem findings were pinpoint hemorrhages at the tip of proventricular glands, enlarged spleen, hemorrhagic ulcers in the intestinal wall and Payer's batches, petechial hemorrhage in the colon, and greenish intestinal contents. Hemorrhagic lungs, tracheitis, and catarrhal exudates were also observed. The localities in the governorate were selected according to their broiler farms' capacity and history of previously reported mortalities (Fig.1). The collected data included the age of birds, bird strain, flock capacity, the farm rearing system, type of vaccines, and clinical signs was recorded. The collected samples were placed in clean plastic packages and an ice box and stored at -20°C until use.

Fig. 1. Map showing the geographical distribution of chicken farms (red spot) localities in Al-Wadi Al-Gadid governorate from which samples were obtained.

Sample preparation and virus isolation

The samples were transferred to the laboratory of Virology, Faculty of Veterinary Medicine, South Valley University, Qena for virus isolation and identification. 1 g of the organs were homogenized in 9 ml phosphate buffer saline (PBS) containing antibiotics and an antifungal mixture in a 15 ml falcon tube. After overnight incubation at 4ºC samples were clarified by centrifugation at 3000 rpm/5 min at room temperature (RT). A 0.2 ml supernatant was inoculated via an allantoic cavity of 10-day-old embryonated chicken eggs (ECEs) in duplicates and incubated at 37°C in an egg incubator with 55% humidity. ECEs were examined daily for 4 successive days where dead embryos after 24 h or still-alive embryos were chilled at 4°C (WOAH, 2021). Aliquots of the allantoic fluid (AF) were harvested and stored at -20°C for serological and molecular characterization.

Serological detection and identification of NDV

The AFs were examined for hemagglutination (HA) activity by HA test according to the WOAH manual (WOAH, 2021). Briefly, AFs (25 µl) were serially two-fold diluted using PBS (25 µl /each well) in a 96-microtiter plate from wells no. 1 to 10, wells no. 11 and 12 used as negative and positive control, respectively. Then the total volume of each well was adjusted to 50 µl by adding 25 µl of PBS/each well. After that 25 µl of 1% washed chicken red blood cells was added to all wells and incubated at RT for 30-40 min. The samples were defined as negative samples after two blind passages in ECEs without HA activity of their AFs where chicken RBCs were tearing in tilting microtiter plates as the negative control (PBS well no. 11). The positive samples that showed chicken RBCs agglutination with no tearing as positive control (La Sota strain live vaccine well no 12).

The samples showing HA activity were kept for hemagglutination inhibition (HI) test to identify NDV isolates using specific antiserum (obtained from NDV-vaccinated chickens) to NDV (Sultan *et al*., 2016). The HI test was conducted (WOAH, 2021). Two-fold serial dilutions of NDV antiserum (25 µl) were performed in a 96-microtiter plate containing 25 µl PBS/well, 4HA unit (4HAU) of the positive AF (25 µl) was added to wells 1 to 10 and the /mixture was incubated at RT for 30-40 min. After that 25 µl washing chicken red blood cells 1% was added to each well and incubated again at RT for 30-40 min. The isolate showed HA titer and red blood cells agglutination or HI titer less than 24 (<16) was NDV negative. The NDV isolate had HI titer ≥ 16 (WOAH, 2021).

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Viral RNA was extracted from HA positive AFs using an EasyPure viral DNA/RNA extraction kit (TransGen Biotech Ltd, China) following the manufacturer's instructions.

The RT-PCR assay was done using the transScript one-step RT-PCR super mix kit (TransGen Biotech Ltd, China), Primers, forward (APMV-1) 5-TAT ACA CCT CRT CBC ARA CRG G-3 and reverse (APMV-1) 5-ACR AAY TGC TGC ATC TTC CC-3 (Tran *et al*., 2019) used to amplify 403 bp of the NDV F gene, including the F gene cleavage site. While IAVM1 5- AGC GTA GAC GCT TTG TC-3 and IAVM2 5-GAC GAT CAA GAA TCC AC-3 forward and reverse primers, respectively, (Straick *et al*., 2000) targeting M gene were used to investigate the presence of AIV co-infection in NDV positive samples. The RT-PCR reaction volume of 20 µl contains 10 µl of TransGen one-step reaction mixture, 0.4 µl of each primer (20 pmol), 0.4 µl of enzyme mix, 5 µl of extract RNA and 3.8 µl of RNase-free water.

The thermal profile was one cycle of initial RT at 45°C/45 min, one cycle of initial denaturation at 95°C/2 min, followed by 30 cycles of denaturation at 95°C/30 sec, annealing at 54°C/30 sec and extension at 72°C/1 min, and one cycle of final 72°C/5min to detect NDV F gene. The thermal profile used to detect the M gene of AIV was RT at 45°C/45 min (one cycle), 90°C for 5 min initial denaturation; 40 cycles of 92°C for 1 min; 50°C for 1 min, and 72°C for 1 min, and one cycle of final extension 72°C for 7 min using Thermal-cycler (A200 gradient, Japan).

The PCR product was evaluated by electrophoresis in a 1.5% agarose gel and 1× tris-acetate EDTA (TAE) buffer stained with ethidium bromide (0.5 µg/ml), and visualized by ultraviolet transilluminator, and photographed using gel documentation system (MicroDOC, Cleaver scientific, Ltd, UK) with Canon digital camera.

PCR product sequencing and data analysis

The amplified 403 bp product of selected NDV partial F genes was cut off from the gel, and purified using QIAquick PCR purification kits (Qiagen GmbH, Hilden Germany) according to the manufacturer's instructions. Then the purified products were sequenced by an applied biosystems prism3130 automated DNA sequencer (Hitachi, Japan) using a BigDye terminator v.3.1 cycle sequencing kit (ABI, Foster City, CA, USA). The nucleotide sequence alignment and deduced amino acid sequence prediction were conducted by Clustal W, Ver., 1.4 using Bioedite package, Ver., 7.2. software. The molecular phylogenetic tree was constructed using the Maximum composite likelihood method based on the General Time Reversible model. The bootstrap consensus tree was inferred from 1000 replicates using Mega 6.0 software (Tamura *et al*., 2013).

Accession numbers

The obtained nucleotide sequences were deposited in the GenBank

under following accession numbers; EG/NDV/Ch/Alwadi-algadid/3/2021 (OR267171), EG/NDV/Ch/Alwadi-algadid/54/2021 (OR267172), EG/NDV/Ch/Alwadi-algadid/103/2021 (OR267173), EG/NDV/Ch/Alwadi-algadid/125/2021 (OR267174), EG/NDV/Ch/Alwadi-algadid/140/2021 (OR267175), and EG/NDV/Ch/Alwadi-algadid/118/2021 (OR267176)

Results

NDV isolation and identification

Out of 98 ND-suspected field samples propagated in 10 days old ECE via allantoic cavity, 15 AFs were HA positive with virus titer ranging from 23 (1/8) to 210 (1/1024). All HA positive embryos died within 48 - 96 hrs post-inoculation. The HA positive AF was inhibited by anti-NDV hyper-immune serum in the HI test. The HI titers of the positive viruses were from 24 (1/16) to 210 (1/1024) as shown in Table 1.

Molecular detection of NDV F gene and M gene of AIV co-infection

By using specific primers targeting the amplification of 403bp F including the cleavage site all HA positive isolates (15 isolates) and control positive sample (La Sota strain vaccine) were positive showing amplification products of 403bp (Table 1 and Fig. 2). Only four samples of the DNV positive samples were co-infected with AIV showing PCR products of 601 bp (Table 1 and Fig. 3). From them 6 representative NDV isolates were selected for genetic analyses based on localities, farm capacity, bird strain, vaccination program variations, clinical signs, HA and HI titers, and AIV co-infection as shown in Tables 2 and 3.

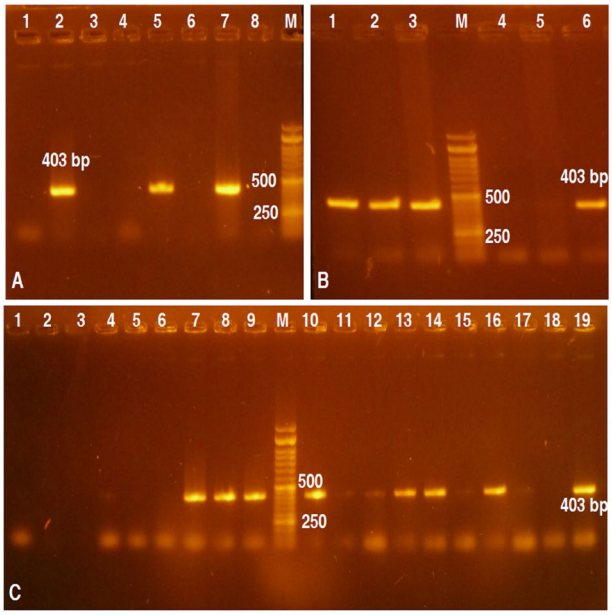

Fig. 2. The RT-PCR products (403bp) of NDV F gene in 1.5% agarose gel stained with ethidium bromide (0.5µg/ml) after electrophoresis and photographed. A) Lane M: 100bp DNA ladder. Lanes 2,5, and 7: NDV positive isolates. Lanes 1,3-4, 6, and 8: negative samples. B) Lane M: 100bp DNA ladder. Lanes 1-3, and 6: positive NDV isolates. Lanes 4-5: negative samples. C) Lane M: 100 bp DNA ladder. Lanes 7-10, 12-14, 16, and 19: positive NDV isolates. Lanes 1-6, 11, 15, and 17-18: negative samples.

Nucleotides and deduced amino acids analysis for NDV partial F genes

Amplified NDV F gene fragments (403bp) of selected 6 isolates were sequenced and resulting nucleotide sequences were aligned with NDV

Table 1. Results of serological (HA and HI tests) and molecular (RT-PCR) identification of obtained NDV isolates and AIV co-infection from different localities in Al-Wadi Al-Gadid governorate.

No	Sample ID	Location	Serological results		Molecular results	
			HA titer	HI titer	NDV	AIV
1	3	Ein Yaseen	1024	1024	403 bp	-ve
2	54	Koriem	64	512	403 bp	601 bp
3	101	Zirzara	1024	<16 [a]	403 bp	601 bp
4	103	Zirzara	1024	512	403 bp	-ve
5	107	Zirzara	1024	32	403 bp	-ve
6	108	Zirzara	512	32	403 bp	-ve
7	114	Zirzara	1024	64	403 bp	-ve
8	118	Elsabat elsharky	64	1024	403 bp	601 bp
9	119	Elsabat elsharky	512	256	403 bp	-ve
10	120	Elsabat elsharky	1024	128	403 bp	-ve
11	124	Elsabat elsharky	128	32	403 bp	601 bp
12	125	Elsabat elsharky	8	<16	403 bp	-ve
13	135	Shark elbalad	1024	256	403 bp	-ve
14	140	El kharga 2	128	1024	403 bp	-ve
15	148	El kharga 2	256	1024	403 bp	-ve

a Negative HI results.

Table 2. The data of selected NDV isolates obtained from vaccinated chickens in the Al-Wadi Al-Gadid governorate was used for sequencing.

Sample ID	Farm Capacity	Bird Type	Age (days)	Vaccination (Age/days)	Location	Clinical Signs
3	6000	Arbo	27	Hitchner (7) Clone 30 (17)	Ein Yaseen	Sneezing, coughing, depression, and death
54	6000	Arbo	25	Hitchner (7) Clone 30 (17)	Koriem	Sneezing, nasal discharge, coughing, greenish watery diarrhea and depression
103	6000	Cup	30	Hitchner (7) Clone 30 (17)	Zirzara	Gasping, green diarrhea, low weight and lethargy
118	2700	Even	30	Hitchner (8) Clone 30 (18)	Elsabat elsharky	Green diarrhea, low in weight, and lethargy
125	2700	Even	30	Hitchner (8) Clone 30 (18)	Elsabat elsharky	Green diarrhea, low in weight, and lethargy
140	4500	Arbo	35	Clone 30 (17)	El kharga 2	Gasping and whitish diarrhea

F gene sequences that have been retrieved from GenBank using blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The results revealed nucleotides and amino acids identities of 81.8 – 100% among representative isolates. Interestingly, isolate EG/NDV/Alwadi algadid/3/2021 showed high similarity in nucleotides and amino acids of 99.6% and 100% to La Sota and Clon_30 vaccine strains, respectively, (Table 4). On the other hand the remaining isolates EG/NDV/Alwadi algadid/54/2021, EG/NDV/Alwadi algadid/103/2021, EG/NDV/Alwadi algadid/118/2021, EG/NDV/Alwadi algadid/125/2021, and EG/NDV/Alwadi algadid/140/2021 had high identities of 96.8 – 97.7% in both nucleotides and amino acids with Chicken/Egypt/Luxor/2011/1 and NDV/Luxor/168/2012 velogenic strains which were isolated from vaccinated chickens in Luxor governorates in 2011 and 2012, respectively, (Table 4).

The alignment of deduced amino acid sequences studied isolates with closely related vaccine strains and velogenic strains indicated two F gene cleavage site motifs; one is 109S-G-G-G–R–Q–G-R-L117 characteristic to EG/NDV/Alwadi algadid/3/2021 isolate. The other amino acid motif was 109S-G-G-R–R–Q–K-R-F117 for the remaining NDV 5 isolates (Table 3 and Fig. 4), which have several specific mutated amino acids in positions 52 L instead of I (L52I), V79A, and A90T (numbering based on full F gene of La Sota strain). Also, unique amino acid substitutions were observed for these 5 isolates in positions P161T, R171G, and R183Q for EG/NDV/Alwadi algadid/54/2021 isolate and A121I, C124G, and L129V for EG/NDV/Alwadi algadid/140/2021 isolate (Fig. 4).

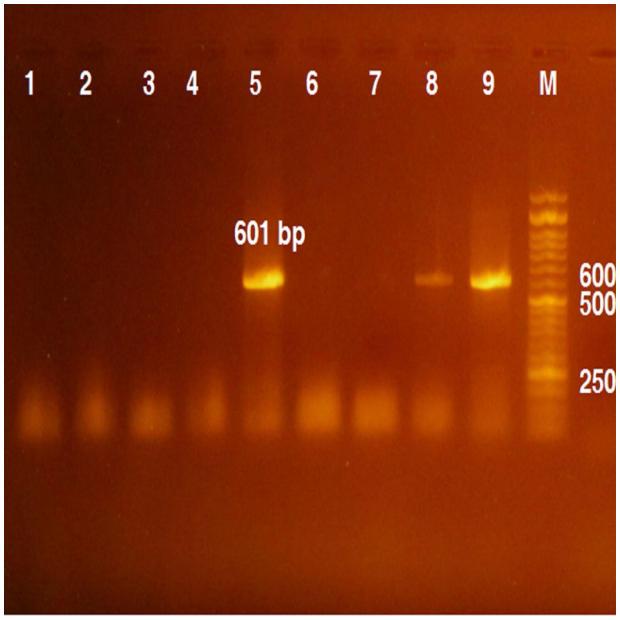

Fig. 3. The RT-PCR products (601bp) of AIV M gene in 1.5% agarose gel stained with ethidium bromide (0.5µg/ml) after electrophoresis and photographed. Lane M: 100bp DNA ladder. Lanes 5 and 8-9: positive AIV isolates. Lanes 1-4, and 6-7: negative samples.

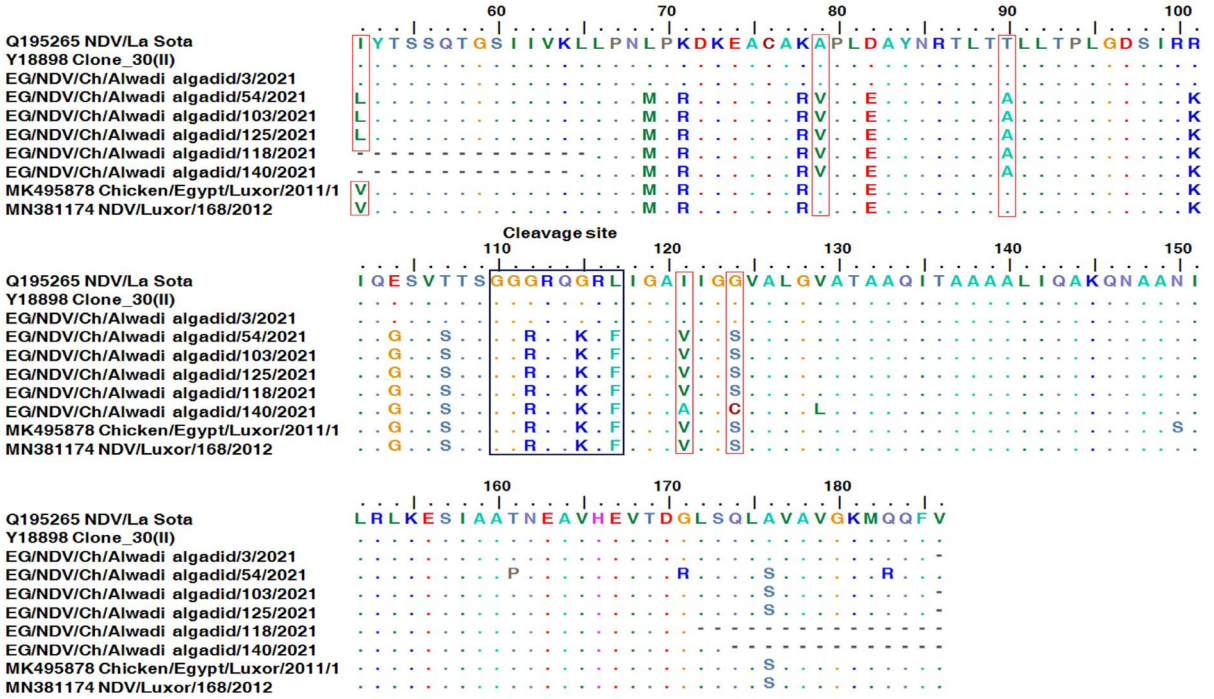

Fig. 4. Aligning deduced amino acid sequences of the partial F genes of NDV isolates from chicken farms in Al-Wadi Al-Gadid governorate, La Sota, Clone_30, and velogenic strains isolated from Luxor governorate. The amino acid positions are shown above based on La Sota F gene (accession number Q195265) amino acid numbering. The F protein cleavage site amino acid motifs of the present isolates (closed box) are indicated. Unique amino acids of the current isolates in the closed red box. Dots indicated identical amino acids while letters indicated different amino acids from La Sota strain.

Table 3. Serological, AIV co-infection, pathogenicity features, and cleavage site motifs of the selected NDV isolates obtained from chickens in the Al-Wadi Al-Gadid governorate in 2021.

Sample ID	Serology [a]		Co-infection		Cleavage site	Pathogenicity
	HA	HI				
EG/NDV/ Al-Wadi Al-Gadid/3/2021	2^9	2^9	NDV	-ve	GRQGRL	Lentogenic
EG/NDV/Al-Wadi Al-Gadid/54/2021	2^6	2^8	NDV	AIV	RRQRRF	Velogenic
EG/NDV/Al-Wadi Al-Gadid/103/2021	2^9	2^8	NDV	-ve	RRQRRF	Velogenic
EG/NDV/Al-Wadi Al-Gadid/118/2021	2^6	2^9	NDV	AIV	RRQRRF	Velogenic
EG/NDV/Al-Wadi Al-Gadid/125/2021	2^3	($<2^4$)	NDV	-ve	RRQRRF	Velogenic
EG/NDV/Al-Wadi Al-Gadid/140/2021	2^7	2^9	NDV	-ve	RRQRRF	Velogenic

a The HA and HI titers are expressed as the reciprocal of the highest dilution given a positive result.

Molecular phylogenetic analysis of NDV isolates partial F genes

The evolutionary history for the current isolates in comparison with those retrieved from GenBank categorized the isolates into two classes; class I includes EG/NDV/Alwadi algadid/3/2021 together with La Sota and Clon_30 vaccine strains and class II encompassed other isolates. The EG/NDV/Alwadi algadid/54/2021, EG/NDV/Alwadi algadid/103/2021, EG/NDV/Alwadi algadid/118/2021, EG/NDV/Alwadi algadid/125/2021, and EG/NDV/Alwadi algadid/140/2021 were grouped with NDV isolates of genotype VII.1.1 and sub-genotype VIIj forming separated clade between northern and southern Egyptian NDV isolates (Fig. 5) with further three sub-clades for the 5 isolates.

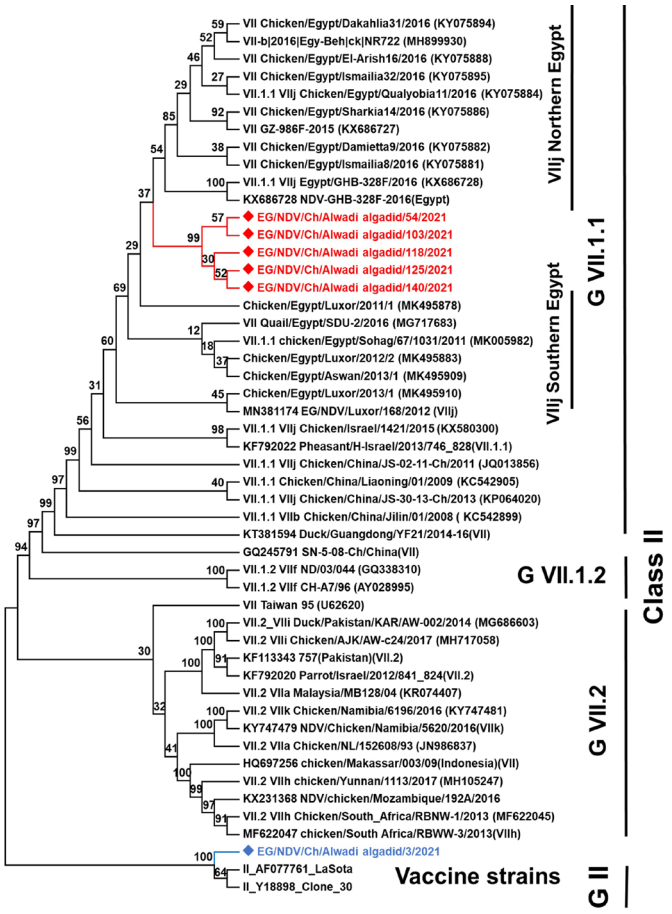

Fig. 5. The evolutionary phylogenetic tree based on partial F gene nucleotide sequences (372 bases) for NDV isolates (red diamonds for velogenic isolates and a blue diamond for lentogenic isolate) isolated in Al-Wadi Al-Gadid in the present study and other strains retrieved from GenBank. All gaps were removed, and the tree was constructed by Maximum composite likelihood method based on the General Time Reversible model with bootstrap 1000 replicates using Mega 6.0 software. NDV class and genotypes are indicated in the phylogenetic tree.

Discussion

ND is a detrimental economic problem for poultry producers worldwide making a great obstacle to efforts carried out for poultry industry improvements. In Egypt, numerous outbreaks of NDV are continuously taking place among chicken flock farms conducting intensive vaccination programs all over the country (Tran *et al*., 2020; Dewidar *et al*., 2021; El-morshidy *et al*., 2021). Also, several experiments have been performed to evaluate efficient vaccination regimes and develop vaccines from circulated NDV strains to control ND outbreaks (Chong *et al*., 2010; Hu *et al*., 2011; Bello *et al*., 2020; Hassan *et al*., 2020; Zhang *et al*., 2021; Lebdah *et al*., 2024). In addition, documented studies stated that the prevalence of velogenic NDV strains among wild birds added extra risk factors for NDV circulation in Egypt (Elbestawy *et al*., 2019; Sultan *et al*., 2022). Counting these factors together reinforces the necessity of continuous investigations of NDV among poultry and follows their genetic evolution. To our knowledge, no documented reports on NDV strains circulated among vaccinated chicken farms in the Al-Wadi Al-Gadid governorate although poultry investments were rapidly grown in this area because of governmental trends to establish the strategy of sustainable agriculture development aimed to reach self-sufficiency of poultry production. The main objective of the present research work is the isolation, identification, pathotyping, and genotyping of NDV isolates from infected vaccinated chicken flocks in the Al-Wadi Al-Gadid governorate in 2021.

So, clinical samples were obtained from 10 chicken farms distributed at different locations in Al-Wadi Al-Gadid governorate where NDV isolation and characterization were conducted. Interestingly, two isolates HI negative ($<2^4$) were RT-PCR NDV positive, and the F gene sequence analysis to one of them indicated velogenic strain. This result agrees with Hu *et al.* (2011) and Hassan *et al.* (2020) who reported that circulating velogenic strains showed a lower affinity to neutralized antibodies produced by heterogeneous vaccine strains than antibodies produced by homogenous velogenic strains. Also, four NDV isolates were AIV co-infected this result is consistent with Gowthaman *et al.* (2019); Umar *et al.* (2019); Farzin *et al.* (2023) who reported NDV and AIV co-infection in chickens. This may be attributed to poor biosecurity measurements applied in chicken farms.

The amino acid sequence motif at the cleavage site of the precursor F0 glycoprotein is the critical site for major changes in virus virulence. F0 precursor glycoprotein of NDV cleaved into F1 and F2 during virus replication, this cleavage can be affected by proteases that are present in a wide range of host tissue and organs recognizing a single arginine, trypsin-like enzymes (Afonso, 2021). Also, the amino acid sequence of the F protein cleavage site determines the NDV virulence where virulent viruses have multiple basic amino acid residues of at least three arginine or lysine between residues 113 and 116 (112K/R-K/R-Q-K/R-R116) at the C-terminus of the F2 protein and phenylalanine (F) at residue 117,

Table 4. Nucleotide identity (Upper half) and amino acid identity (lower half) among NDV strains isolated in this study from Al-Wadi Al-Gadid and other strains.

Starin ID	1	2	3	4	5	6	7	8	9	10
1-Q195265 Lasota	ID	100	83	83	99.6	82	82.8	82.8	82	82.8
2-Y18898 Clone_30	99.6	ID	82.8	83	100	82	82.5	82.5	81.8	82.5
3-MK495878 Chicken/Egypt/Luxor/2011/1	83	82.8	ID	99	82.8	97	97.8	97.8	96.8	97.8
4-MN381174 NDV/Luxor/168/2012	83	82.8	99	ID	82.8	97	97.8	97.8	96.8	97.8
5-EG/NDV/Ch/Alwadi algadid/3/2021	99.6	100	82.8	82.8	ID	82	82.5	82.5	81.8	82.5
6-EG/NDV/Ch/Alwadi algadid/54/2021	82	81.8	97	97	81.8	ID	99	99	98	99
7-EG/NDV/Ch/Alwadi algadid/103/2021	82.8	82.5	97.8	97.8	82.5	99	ID	100	99	100
8-EG/NDV/Ch/Alwadi algadid/125/2021	82.8	82.5	97.8	97.8	82.5	99	100	ID	99	100
9-EG/NDV/Ch/Alwadi algadid/140/2021	82	81.8	96.8	96.8	81.8	98	99	99	ID	99
10-EG/NDV/Ch/Alwadi algadid/118/2021	82.8	82.5	97.8	97.8	82.5	99	100	100	99	ID

which is the N-terminus of the F1 protein. On the other hand, lentogenic strains are considered to have a monobasic F cleavage site (112G/E-K/R-Q-G/E-R116) and a Leucine (L) residue at position 117 (WOAH, 2021). In the current study, 5 isolates (EG/NDV/Alwadi algadid/54/2021, EG/NDV/Alwadi algadid/103/2021, EG/NDV/Alwadi algadid/118/2021¸ EG/NDV/Alwadi algadid/125/2021, and EG/NDV/Alwadi algadid/140/2021) were velogenic strains with polybasic amino acids at their F gene cleavage site and phenylalanine amino acid at position 117. The other isolate (EG/NDV/Alwadi algadid/3/2021) was a lentogenic strain with a single basic amino acid at its F gene cleavage site and a leucine amino acid at position 117.

Mutations at amino acid residues 72, 78, and 79 are located next to a major hydrophilic region and close to the only cysteine residue at position 76 in the F2 region, which is necessarily included in the disulfide linkage to FI (Yusoff *et al*., 1989). Also, amino acid substitutions at positions 160 and 171 were observed in the F1 slightly hydrophilic region (Yusoff *et al*., 1989) as well as mutated amino acid in 161 which contributed to the fusion activity of the F gene (Toyoda *et al*., 1988). All velogenic isolates had amino acid R78K substitution in the F gene epitope as velogenic strains isolated from Luxor governorate. Interestingly the EG/NDV/Alwadi algadid/54/2021 isolate showed characteristic mutated amino acids P161T, R171G, and R183Q in the heptad repeat a (HRa) epitopes. In addition to unique amino acid substitutions L52I, V79A, and A90T were detected in current velogenic NDV isolates F2 region but not in other velogenic strains in southern Egypt. Also, the EG/NDV/Alwadi algadid/140/2021 isolate had three specific amino acid substitutions A121I, C124G, and L129V. These mutations may account for virus virulence and neutralizing antibody avoidance.

To our knowledge, few studies have reported the occurrence of lentogenic NDV strains among commercial chicken farms in Egypt (Mohamed *et al*., 2011; El-Habbaa *et al*., 2017). Although lentogenic strains infection outcomes showed mild or no clinical signs among infected chickens their possible reversion to virulent strains by passage contributes to virus evolution (Shengqing *et al*., 2002; Sahoo *et al*., 2022). It is noteworthy that, the lentogenic strain (EG/NDV/Alwadi algadid/3/2021) showed 100% identity in amino acid nucleotide sequences to Clone_30 and phylogenetically clustered with Clone_30 and La Sota strains in genotype II.

The current study revealed for the first time the circulation of velogenic genotype VII.1.1, VIIj, and lentogenic genotype II NDV strains among chicken flocks in Al-Wadi Al-Gadid governorate exhibiting respiratory and gastrointestinal signs despite receiving vaccination regimes. Also, amino acid substitutions correlated to neutralizing antibody epitopes, virulence, and virus evolution. It is known that vaccination of birds against NDV protects from high mortality and severe clinical signs but does not prevent virus shedding. So, the virus spreads among birds, and co-infection with other pathogens exaggerates pathogenicity.

Conclusion

Two genotypes of NDV strains, genotype II (lentogenic) and VII.1.1 (velogenic VIIj), are circulating among vaccinated chicken farms in Al-Wadi Al-Gadid governorate. The velogenic strains possess unique amino acid substitutions in F1 and HRa regions distinguishing them from velogenic strains in southern Egypt and contributing to neutralization antibody production and fusion activity. These results will be helpful for molecular epidemiology and biosecurity implementation to control NDV infection in the Al-Wadi Al-Gadid governorate which is a promising area for poultry investments.

Conflict of interest

The authors have no conflict of interest to declare.

References

Abozaid, K.G.A., Abdel-Moneim, A.S., 2022. Epidemiological surveillance of Newcastle disease virus in Egypt-a 6-year cohort study. Trop. Anim. Health Prod. 54, 243. https://doi.org/10.1007/s11250-022-03234-3.

Afonso, C.L., 2021. Virulence during Newcastle disease viruses cross species adaptation. Viruses 13, 110. doi: 10.3390/v13010110.

Allahyari, E., Allymehr, M., Molouki, A., Mehrabadi, M.F., Talebi, A., 2022. Molecular characterisation and phylogenetic study of the fusion gene of Newcastle disease viruses isolated from broiler farms of Iran in 2018-2019. Bulgarian Journal of Veterinary Medicine 25, 21-32. Doi:10.15547/bjvm.2020-0041.

Bello, M.B., Mahamud, S.N.A., Yusoff, K., Ideris, A., Hair-Bejo, M., Peeters, B.P.H., Omar, A.R., 2020. Development of an Effective and Stable Genotype-Matched Live Attenuated Newcastle Disease Virus Vaccine Based on a Novel Naturally Recombinant Malaysian Isolate Using Reverse Genetics. Vaccines (Basel) 8, 270. doi:10.3390/vaccines8020270.

Chong, Y.L., Padhi, A., Hudson, P.J., Poss, M., 2010. The effect of vaccination on the evolution and population dynamics of avian paramyxovirus-1. PLoS Pathogens 6, e1000872.

Dimitrov, K.M., Abolnik, C., Afonso, C.L., Albina, E., Bahl, J., Berg, M., Briand, F.X., Brown, I.H., Choi, K.S., Chvala, I., 2019. Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. Infect. Genet. Evol. 74,103917. doi: 10.1016/j.meegid.2019.103917.

Dewidar, A.A., El-sawah, A., Shany, S.A., Dahshan, A.-H. M., Ali, A., 2021. Genetic characterization of genotype VII.1.1 Newcastle Disease viruses from commercial and backyard broiler chickens in Egypt. Ger. J. Vet. Res. 1, 11-17. Doi:https://doi.org/10.51585/gjvr.2021.4.0025.

Elbestawy, A.R., Ellakany, H.F., El-Hamid, H.S.A., Zedan, R.E., Gado, A.R., Sedeik, M.E., Abd El-Hack, M.E., Saadeldin, I.M., Alowaimer, A.N., Ba-Awadh, H.A., Swelum, A.A., 2019. Muscovy ducks infected with velogenic Newcastle disease virus (genotype VIId) act as carriers to infect in-contact chickens. Poult Sci. 98, 4441-4448. doi: 10.3382/ps/pez276.

El-Habbaa, A.S., El-Bagoury, G.F., El-Adaway, S.F., El-Mahdy, S.S., 2017. Isolation, pathotyping and genotyping of Newcastle disease virus from broiler chickens in Egypt. J. of Virol. Sci. 1, 114-122.

El-Morshidy, Y., Abdo, W., Elmahallawy, E.K., Abd El-Dayem, G.A., El-Sawak, A., El-Habashi, N., Mosad, S.M., Lokman, M.S., Albrakati, A., Abou, A.S., 2021. Pathogenesis of Velogenic Genotype VII.1.1 Newcastle Disease Virus Isolated from Chicken in Egypt via Different Inoculation Routes: Molecular, Histopathological, and Immunohistochemical Study. Animals (Basel) 11, 3567. doi: 10.3390/ani11123567.

Farzin, H.R., Ajam, G., Asgharzadeh, M., Zanguei Motlagh, Z., Ghasemitabas, S., Tabatabaeizadeh, S.E., Zibaee, S., Jamshidian-Mojaver, M., 2023. The role of the major viral pathogens in a respiratory disease outbreak of broiler flocks in Eastern Iran. Iran J Vet Res. 24, 205-214. doi: 10.22099/IJVR.2023.45051.6632.

Hassan, M.I., Abd El-Azeem, M.W., Selim, A., Sultan, S., 2020. Molecular and biological characterization of the immunological potency of Newcastle disease virus oil emulsion-inactivated vaccines prepared from field isolate obtained from vaccinated chickens outbreak. Braz J. Microbiol. 51, 815-826. Doi: 10.1007/s42770-019-00203-1.

Helen, O.A., Ismaila, S., Paul, A.A., Chinwe, J.A., 2020. Molecular characterization and phylogenetic analysis of Newcastle disease virus isolated from poultry in North Central States of Nigeria. Journal of Veterinary Medicine and Animal Health 12, 20 -26. https://doi.org/10.5897/JVMAH2019.0825.

Hu, Z., Hu, S., Meng, C., Wang, X., Zhu, J., Liu, X., 2011.Generation of a genotype VII Newcastle disease virus vaccine candidate with high yield in embryonated chicken eggs. Avian Dis. 55, 391-397. doi: 10.1637/9633-122410-Reg.1.

Gowthaman, V., Singh, S.D., Dhama, K., Ramakrishnan, M.A., Malik, Y.P.S., Gopala Krishna Murthy, T.R., Chitra, R., Munir, M., 2019. Co-infection of Newcastle disease virus genotype XIII with low pathogenic avian influenza exacerbates clinical outcome of Newcastle disease in vaccinated layer poultry flocks. Virus Disease 30, 441-452. Doi:10.1007/s13337-019-00533-6.

Le, X.T.K., Doan, H.T.T., Le, T.H., 2018. Molecular analysis of Newcastle disease virus isolates reveals a novel XIId subgenotype in Vietnam. Arch. Virol. 163, 3125-3130. Doi:10.1007/s00705-018-3961-0.

Lebdah, M.A., Abdallah, A., Hamouda, E.E., Elseddawy, N.M., ElBakrey, R.M., 2024 Protective effectiveness of two vaccination schemes against the prevalent Egyptian strain of Newcastle disease virus genotype VII. Open Vet. J. 14, 32-45. Doi:10.5455/OVJ.2024.v14.i1.5.

Mohamed, M.H., Kumar, S., Paldurai, A., Samal, S.K., 2011. Sequence analysis of fusion protein gene of Newcastle disease virus isolated from outbreaks in Egypt during 2006. Virol. J. 8, 237. Doi:10.1186/1743-422X-8-237.

OIE, 2018a. OIE-Listed diseases, infections and infestations in force in 2018. World Organ. Anim. Heal.

OIE, 2018b. World Animal Health Information System WAHIS Portal: Animal Health Data. https://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/world-animal-health-information-system/

Selim, K.M., Selim, A., Arafa, A., Hussein, H.A,, Elsanousi, A.A., 2018. Molecular characterization of full fusion protein (F) of Newcastle disease virus genotype VIId isolated from Egypt during 2012-2016. Vet World 11, 930-938. Doi: 10.14202/vetworld.2018.930-938.

Sahoo, N., Bhuyan, K., Panda, B., Behura, N.C., Biswal, S., Samal, L., Chaudhary, D., Bansal, N., Singh, R., Joshi, V.G., Jindal, N., Mahajan, N.K., Maan, S., Ravishankar, C., Rajasekhar, R., Radzio-Basu, J., Herzog, C.M., Kapur, V., Mor, S.K., Goyal, S.M., 2022. Prevalence of Newcastle disease and associated risk factors in domestic chickens in the Indian state of Odisha. PLoS One 17, e0264028. Doi:10.1371/journal.pone.0264028.

Shakal, M., Maher, M., Metwally, A.S., Abdelsabour, M.A., Madbbouly, Y.M., Safwat, G., 2020. Molecular Identification of a Velogenic Newcastle Disease Virus Strain Isolated from Egypt. J. World Poult. Res. 10, 195-202. https://dx.doi.org/10.36380/jwpr.2020.25.

Shengqing, Y., Kishida, N., Ito, H., Kida, H., Otsuki, K., Kawaoka, Y., 2002. Generation of velogenic Newcastle disease viruses from a nonpathogenic waterfowl isolate by passaging in chickens. Virology 301, 206–11. Doi:10.1006/viro.2002.1539.

Sultan, S., Osman, N., Ahmed, I.A., S Ibrahim, R., Sabra, M., 2015. Phylogenetic Characterization of Velogenic Newcastle Disease Viruses Isolated from Field Outbreaks among Vaccinated broiler chickens in Upper Egypt. Assiut Veterinary Medical Journal 61, 126-135. Doi:10.21608/avmj.2015.170195.

Sultan, S., Osman, N., Mohamed, M.A., Ahmed I.A., Abdallah, R.A.M., Faraouk, M., Taha, M., 2016. Infectious bursal disease vaccine ameliorates velogenic Newcastle disease virus infection in immunopotentiated chickens. Comp. Clin. Pathol. 25, 91–100. https://doi.org/10.1007/s00580-015-2145-5.

Sultan, S., Eldamarany, N.M.I., Abdelazeem, M.W., Fahmy, H.A., 2022. Active Surveillance and Genetic Characterization of Prevalent Velogenic Newcastle Disease and Highly Pathogenic Avian Influenza H5N8 Viruses Among Migratory Wild Birds in Southern Egypt During 2015-2018. Food Environ Virol. 4, 280-294. Doi:10.1007/s12560-022-09532-1.

Sun, J., Ai, H., Chen, L., Li, L., Shi, Q., Liu, T., Zhao, R., Zhang, C., Han, Z., Liu, S., 2022. Surveillance of Class I Newcastle Disease Virus at Live Bird Markets in China and Identification of Variants with Increased Virulence and Replication Capacity. J Virol. 96, e00241-22. https://doi.org/10.1128/jvi.00241-22.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725-2729. doi: 10.1093/molbev/mst197.

Toyoda, T., Gotoh, B., Sakaguchi, T., Kida, H., Nagai, Y., 1988. Identification of amino acids relevant to three antigenic determinants on the fusion protein of Newcastle disease virus that are involved in fusion inhibition and neutralization. J. Virol. 62, 4427-30. doi: 10.1128/JVI.62.11.4427-4430.1988.

Tran, T.H.G., Van, H.D., Duy, T.D., Iskender, S., Mairambek, I., Omatsu, T., Katayama, Y., Mizutani, T., Ozeki, Y., Takeda, Y., Ogawa, H., Imai, K., 2019. Molecular characterization of a virulent strain of Newcastle disease virus isolated from a diseased chicken in Kyrgyzstan in 2016. Japanese Journal of Veterinary Research 67, 263–273. https://doi.org/10.14943/jjvr.67.4.263.

Tran, G.T.H., Sultan, S., Osman, N., Hassan, M.I., VAN Dong, H., Dao, T.D., Omatsu, T., Katayama, Y., Mizutani, T., Takeda, Y., Ogawa, H., Imai, K., 2020. Molecular characterization of full genome sequences of Newcastle disease viruses circulating among vaccinated chickens in Egypt during 2011-2013. J. Vet. Med. Sci. 82, 809-816. Doi:10.1292/jvms.19-0623.

Umar, S., Teillaud, A., Aslam, H.B., Guerin, J.L., Ducatez, M.F., 2019. Molecular epidemiology of respiratory viruses in commercial chicken flocks in Pakistan from 2014 through to 2016. BMC Vet. Res. 15, 351. Doi:10.1186/s12917-019-2103-6.

Walker, P.J., Siddell, S.G., Lefkowitz, E.J., Mushegian, A.R., Adriaenssens, E.M., Alfenas-Zerbini, P., Dempsey, D.M., Dutilh, B.E., García, M.L., Curtis Hendrickson, R., Junglen, S., Krupovic, M., Kuhn, J.H., Lambert, A.J., Łobocka, M., Oksanen, H.M., Orton, R.J., Robertson, D.L., Rubino, L., Sabanadzovic, S., Simmonds, P., Smith, D.B., Suzuki, N., Van Doorslaer, K., Vandamme, A.M., Varsani, A., Zerbini, F.M., 2022. Recent changes to virus taxonomy ratified by the International Committee on Taxonomy of Viruses. Arch. Virol. 167, 2429-2440. Doi:10.1007/s00705-022-05516-5.

Wang, Y., Zhang, H., Gu, C., Cheng, G., Zhang, W., Mehmood, K., Hu, X., 2018. Phylogenetic and pathotypic characterization of a Newcastle disease virus strain isolated from ducks and pigeons in hubei, China. Revista Brasileira de Ciência Avícola 20, 145-152. https://doi.org/10.1590/1806-9061-2016-0459.

WOAH., 2021.Terrestrial Manual, NEWCASTLE DISEASE (INFECTION WITH NEWCASTLE DISEASE VIRUS). https://www.woah.org/fileadmin/Home/fr/Health_standards/tahm/3.03.14_NEWCASTLE_DIS.pdf.

Yusoff, K., Nesbit, M., McCartney, H., Meulemans, G., Alexander, D.J., Collins, M.S., Emmerson, P.T., Samson, A.C., 1989. Location of neutralizing epitopes on the fusion protein of Newcastle disease virus strain Beaudette C. J. Gen. Virol. 70, 3105-9. Doi:10.1099/0022-1317-70-11-3105.

Zhang, X., Bo, Z., Meng, C., Chen, Y., Zhang, C., Cao, Y., Wu, Y., 2021. Generation and Evaluation of Recombinant Thermostable Newcastle Disease Virus Expressing the HA of H9N2 Avian Influenza Virus. Viruses 13, 1606. Doi:10.3390/v13081606.