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Original Research Article

GENETIC CHARACTERIZATION OF EGYPTIAN NEWCASTLE DISEASE VIRUS STRAINS ISOLATED FROM FLOCKS VACCINATED AGAINST NEWCASTLE DISEASE VIRUS, 2014-2015

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Abstract: In the present study, forty-five chicken flocks suspected to be infected with Newcastle disease virus (NDV) in Sharkia Governorate were submitted for clinical and postmortem examination as well as virus isolation and sero-molecular identification. Forty samples were positive for hemagglutinating viruses, 15/40 (37.5%) were confirmed positive isolates against NDV antiserum, 12/40 (30%) were positive isolates against both NDV and AIV (H5) antisera and 9/40 (22.5%) were positive against AIV antiserum using serological identification. Twenty-seven NDV isolates were confirmed positive by molecular identification; twelve of them were confirmed as virulent NDV strains using specific primers. A fragment of 766-bp comprising the 3' end of the M gene and the 5' end of the F gene was sequenced from four amplified products, revealing that these isolates carried the 112RRQKRF117 motif, which is characteristic of virulent strains. The investigated strains were clustered with a class II genotype VIId with large genetic distance with the LaSota strain. This is alarming given the potential evolution of different virulent NDVs and may explain the circulation of these virulent strains among Egyptian poultry farms despite the application of intensive vaccination programs.

Key words: Sharkia; NDV; genotype VIId; poultry; PCR

Introduction

Newcastle disease (ND), one of the most important infectious viral diseases in poultry, still causes major economic losses in the poultry industry. The devastating negative effects of the virus, such as high mortality, reduced egg production, a high condemnation rate and the cost of vaccination (1), are considered major limiting factors on industry growth in many countries due to their substantial socio-economic impact (2). ND is classified by the World Organization for Animal Health (OIE) as a list A disease because it is highly contagious, with high morbidity and mortality in susceptible birds (up to 100%) (3).

Newcastle disease virus (NDV) is an enveloped virus containing linear, non-segmented, negative sense, single-stranded RNA (4). NDV is an avian paramyxovirus-1 (APMV-1), one of the most antigenically distinct avian paramyxoviruses 1-11, genus Avulavirus, family Paramyxoviridae and order Mononegavirales (2,5,6). Based on their pathogenicity in chickens, NDV isolates are categorized into three main pathotypes: lentogenic (low virulence), mesogenic (intermediate virulence) and velogenic (high virulence)

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(7-9). The disease is characterized by respiratory involvement, nervous system impairment, and gastrointestinal and reproductive problems (8-10). Since the first confirmed outbreaks of ND in 1926 in Java, Indonesia, and Newcastle-Upon Tyne, UK (10,11), NDV has continued to re-emerge in both epidemic and endemic forms throughout the world and is a potential cause of sporadic outbreaks in formerly disease-free countries around the world (12).

In Egypt, infection with NDV was recorded for the first time in 1948 (13). Thereafter, many ND outbreaks have been reported, and Egypt has been classified as an endemic country for NDV (14,15). Prevention and control of NDV primarily depend on the strict application of biosecurity measures and intensive vaccination programs that have been successfully used throughout the world for many years (8). Nevertheless, in the last few years, NDV has caused several outbreaks in Egyptian domestic poultry flocks, resulting in massive economic losses (14).

The frequent incidence of NDV infection, even in vaccinated birds, is not only related to improper vaccination or immune suppression but may also be due to viral mutation leading to changes in the genomic sequence of the virus, thus altering its biological properties and virulence (16,17). Therefore, the present study was conducted to genetically characterize field isolates of NDV obtained from Sharkia Govronrate in 2014–2015 and analyze their genetic distance from already-existing vaccine strains.

Material and methods

Flock histories and sample collection

A total of 318 birds representing 45 chicken flocks between 10–240 days of age suspected to be infected with NDV which located in different districts of Sharkia Governorate during 2014–2015 were selected based on the presence of respiratory and/or nervous manifestations as well as greenish diarrhea despite previous vaccination against NDV (Table 1). Birds were subjected to both clinical and postmortem examination before samples collection. Forty-five tissue pools from recently dead birds (3 birds/pool) were collected from the digestive and respiratory organs (trachea, lung, proventriculus and cecal tonsils) under completely aseptic conditions. The samples were ground in sterile iced mortars to create a 10% suspension in PBS. The homogenized suspensions were transferred to centrifuge tubes for centrifugation at 1000 g for 10 min to remove extraneous materials. The supernatants were collected, and antibiotics (streptomycin at 2 mg/ml and penicillin at 2000 IU/ml) were added to inhibit bacterial contamination (18).

Virus isolation and propagation

A total of 600 (9–11-day-old) embryonated chicken eggs (ECE) obtained from non-vaccinated and non-infected chicken flocks were used for virus isolation and propagation. Processed samples (0.2 ml) were inoculated into the allantoic cavity of the ECE under completely aseptic conditions. All inoculated embryos were incubated at 37ºC for 4–7 days. Daily candling and observation were carried out. Embryos that died within the first 24 hours post-inoculation were discarded. After 24 hours, dead embryos were chilled overnight in a refrigerator for embryonic examination and a direct hemagglutination assay of the harvested allantoic fluid was performed (18). The negative samples were passaged on ECE for another two passages, and samples that showed negative results (no embryonic lesions and negative hemagglutinating activity) after the third passage were recorded as negative samples.

Micro-plate hemagglutination and hemagglutination inhibition assays

Positive hemagglutinating allantoic fluids were subjected to micro-plate hemagglutination and β-hemagglutination-inhibition tests using either NDV (Veterinary Serum and Vaccine Research Institute®) or H5AIV (Harbinweike Biotechnology Development Company®) antisera according to a previously described method (18).

Viral RNA purification and RT-PCR

Viral RNA was extracted from the harvested amino-allantoic fluid which were screened positive HI against NDV antiserum. All the purification steps were performed according to the manufacturer's instructions using a GenJet viral DNA and RNA purification kit®. Three one-step RT-PCR amplification reactions targeting different fragments of the F gene were performed using a one-step Maxime RT-PCR Premix kit®. Different primers were used to amplify three different fragment of NAD genome to diagnose and characterized the isolated strains; A (F:-5'-TTGATGGCAGGCCTCTTGC-3'), B (R:-5'GGAGGATGTTGGCAGCATT-3'); C (R:-5'-AGCGT (C/T) T CTGTCTCCT3'); NDV-M2 (F:-5`TGG AGC CAA ACC CGC ACC TGCGG3`) and NDV-F2(R:-5`GGA GGA TGT TGG CAG CATT3`) (19-20). A and B were used for amplification of 362 bp fragment of F gene for all NDV pathotypes and were tested for all NDV positive HI isolates examined in this study. A and C were used for amplification of 254 bp fragment of F gene of virulent ND strains and were tested for selected 12 NDV isolates according to their geographical distribution and breeding purposes. NDV-M2 and NDV-F2 primers were used for amplification of 766 bp from matrix and F genes and were tested for the same virulent 12 NDV isolates for sequencing purpose. The amplifications were carried out in a BOER thermocycler under the following conditions: 45ºC/30 m (minute) followed by an inactivation step and denaturation at 94ºC/5 m and 40 cycles of 94ºC/30 s (second), 50–54ºC/30 s and 72ºC/1 m with a final extension step at 72ºC/5 m. The amplified fragments were separated on agarose gels (1%) and visualized under ultraviolet light after staining with ethidium bromide.

PCR purification and sequencing

PCR purification was performed using a QIAquick Gel Extraction Kit® Protocol according to the manufacturer's instructions. Sequencing was performed commercially at the Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Animal Health Research Institute, Dokki, Giza, Egypt.

Four choosen 766-bp purified RT-PCR products containing a region comprising both the M and F genes were sequenced in the forward and reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA).

Genomic analysis and phylogenetic tree

Alignment and phylogenic analyses were performed using several software programs such as MEGA (Molecular Evaluation Genetic Analysis, www.megasoftwere. net), BioEdit and DNA Star.

Results

Clinical findings

The examined birds showed one or more of the following signs: respiratory signs in the form of coughing, sneezing, nasal discharge and eye inflammation; nervous signs in the form of paralysis, tremors and torticollis; or digestive disorders such as greenish diarrhea. These signs were accompanied by general signs of illness, such as depression, ruffled feathers and decreased food consumption. Cyanosis of the combs and wattles were also observed. Decreases in egg production among layer flocks sometimes reached 50%. The mortality rate ranged from 8–70%. Necropsies revealed congestion of the brain blood vessels and signs of septicemia in the form of congested subcutaneous blood vessels, congestion of the liver, spleen and lungs, and gallbladder enlargement. Tracheitis and airsacculitis were also observed in the respiratory tract. Hemorrhage on the tips of the proventriculus gland (Figure 1a), greenish mucous content in the gastrointestinal tract, elliptical raised ulcers in the intestine (Figure 1b) and enlarged hemorrhagic cecal tonsils were also observed.

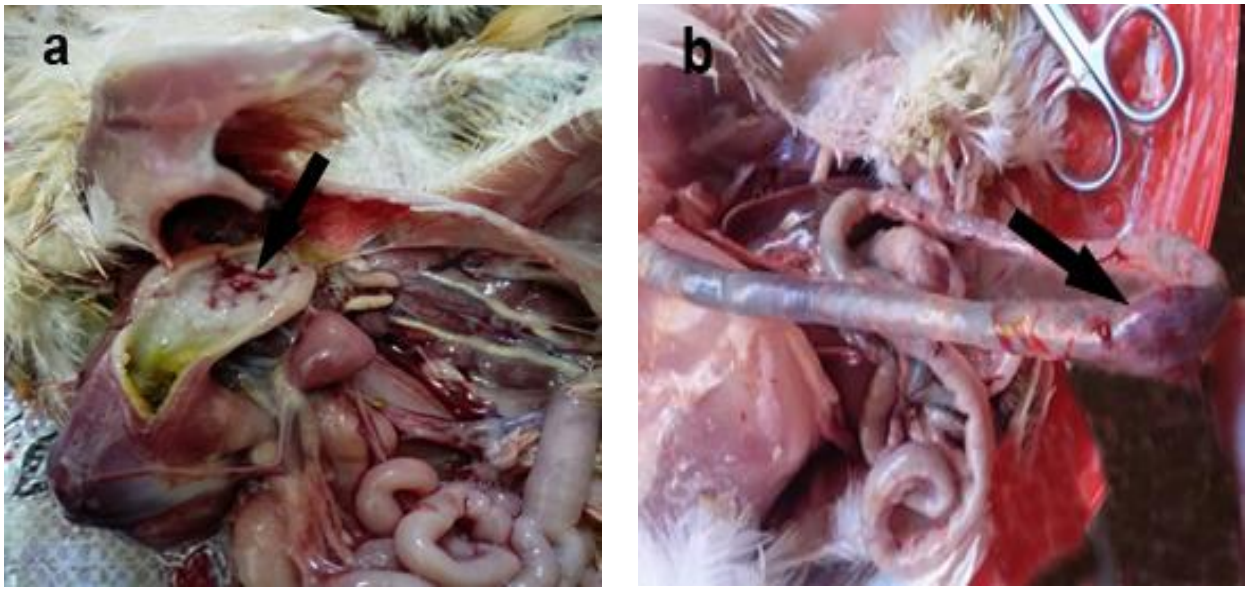

Figure 1: Pathological features of the naturally infected NDV flocks. A: Petechial hemorrhage on the proventriculus glands of 42-day Cobb broiler chick. B: Elliptical ulcer in the intestinal tract in 25 day Hubbard broiler chick

Virus isolation

As shown in Table (1), 40 out of 45 (88.8%) inoculated samples demonstrated positive hemagglutination activity in the harvested allantoic fluid from the first passage. The inoculated embryos showed extensive hemorrhage, congestion and death within 48–96 hours after the first passage. The remains of the negative samples were passaged on ECE for another two passages, and samples that showed negative results (no embryonic lesions and negative hemagglutinating activity) after the third passage were recorded as negative samples.

Micro-hemagglutination inhibition results

Positive HA isolates that were subjected to HI tests against both NDV and AIV (H5) antisera revealed 15/40 (37.5%) positive isolates against NDV antiserum and 12/40 (30%) positive isolates against both NDV and AIV (H5) antisera. Nine isolates (22.5%) were positive against AIV antiserum, and four isolates (10%) did not demonstrate positive activity against either NDV or AIV (H5) antisera (Table 1).

Table 1: Descriptive data and NDV detection steps of the examined chicken flocks

Flock No.	Locality	Breed	Age	Virus Isolation	HI	HI	PCR common type	PCR pathogenic type	PCR primers
					NDV	AIVH5	Common type	Pathotype	M-F primer
1	Zagazig	SASSO	18	+	+	+	+	+	+
2	Menia Kamh	Cobb	41	+	+	-	+	Nd	Nd
3	Zagazig	Native	20	+	+	-	+	Nd	Nd
4	Abo Hamad	SASSO	21	+	+	-	+	Nd	Nd
5	Zagazig	Hubbard	25	+	+	-	+	Nd	Nd
6	Faquos	Hubbard	27	+	+	-	+	Nd	Nd
7	Zagazig	Native	45	-	Nd	Nd	Nd	Nd	Nd
8	Faquos	Cobb	33	+	+	+	+	+	+
9	Elsalhia	Hubbard	25	+	+	-	+	Nd	Nd
10	Elsalhia	Cobb	32	+	-	+	Nd	Nd	Nd
11	Faquos	Lohman (Brown)	180	+	-	+	Nd	Nd	Nd
12	Faquos	Hubbard	24	+	+	+	+	+	+
13	Faquos	Hubbard	31	+	+	+	+	Nd	Nd
14	El-Ibrahemia	Cobb	34	+	+	+	+	+	+
15	Hehia	Cobb	34	+	-	-	Nd	Nd	Nd
16	Faquos	SASSO	22	+	-	-	Nd	Nd	Nd
17	Faquos	Hy-line (Brown)	10	-	Nd	Nd	Nd	Nd	Nd
18	Elsalhia	Lohman (white)	77	+	-	+	Nd	Nd	Nd
19	Elsalhia	Lohman (Brown)	70	+	-	+	Nd	Nd	Nd
20	Faquos	Hy-sex (Brown)	124	+	+	+	+	+	+
21	Abo Kabeir	SASSO	50	+	+	+	+	+	+
22	Abo Kabeir	Cobb	23	+	-	-	Nd	Nd	Nd
23	Abo Kabeir	Cobb	35	+	-	+	Nd	Nd	Nd
24	Abo Kabeir	Saso	30	+	+	+	+	+	+
25	Abo Kabeir	Saso	28	+	+	+	+	Nd	Nd
26	Faquos	Hy-line (Brown)	240	+	-	+	Nd	Nd	Nd
27	Faquos	Hubbard	33	-	Nd	Nd	Nd	Nd	Nd
28	Faquos	Hy-sex (Brown)	86	+	-	+	Nd	Nd	Nd
29	Abo Kabeir	Hy-line (red)	200	+	-	+	Nd	Nd	Nd
30	Hehia	Balady	34	+	+	+	+	Nd	Nd
31	Abo Hamad	Hy-line (Brown)	46	-	Nd	Nd	Nd	Nd	Nd
32	Faquos	Cobb	28	-	Nd	Nd	Nd	Nd	Nd
33	Abo Kabeir	Lohman (Brown)	200	+	-	+	Nd	Nd	Nd
34	Hehia	Cobb	35	+	+	-	+	+	+
35	Faquos	Hubbard	35	+	+	-	+	+	+
36	Abo Kabeir	Baladi	40	-	Nd	Nd	Nd	Nd	Nd
37	Zagazig	Cobb	25	+	+	+	+	+	+
38	Faquos	Hubbard	24	+	+	-	+	Nd	Nd
39	Zagazig	Hubberd	28	+	+	-	+	Nd	Nd
40	Elknayat	Hy-line (Brown)	90	+	+	-	+	Nd	Nd
41	Belbais	Lohaman (white)	28	+	+	+	+	+	+
42	Faquos	Cobb	28	+	+	-	+	Nd	Nd
43	Zagazig	Cobb	35	+	+	-	+	Nd	Nd
44	Menia Kamh	Hubbard	28	+	+	-	+	+	+
45	Zagazig	Cobb	28	+	+	-	+	Nd	Nd

Flock 08: NDV/chicken/Egypt/1/2015, Flock 14: NDV/ chicken/Egypt/2/2015, Flock 37: NDV/chicken/Egypt/3/2015
Flock 44: NDV/ chicken/Egypt/4/2015, Nd: note done

Molecular identification

i. RT-PCR (common type)

RT-PCR using primers A and B resulted in a 362-bp fragment amplified from all 27 NDV tested samples.

ii. RT-PCR (pathotype)

Twelve selected positive RT-PCR isolates were subjected to RT-PCR using primers A and C. A 254-bp fragment was detected in all 12 tested isolates, indicating they were virulent strains.

iii. RT-PCR flanking the M and F genes

The third RT-PCR test was carried on the 12 NDV virulent isolates using the M2 and F2 primer set, which revealed the presence of a 766-bp fragment in the tested samples.

Partial sequencing of the M and F genes

Sequencing of the 766-bp fragment comprising the 3' end of the M gene and the 5' end of the F gene in the four amplified PCR products revealed that they carried the 112 RRQKRF 117 motif, which is characteristic of virulent strains. The four obtained sequences were designated
NDV/chicken/Egypt/1/2015,
NDV/chicken/Egypt/2/2015,
NDV/chicken/Egypt/3/2015 and
NDV/chicken/Egypt/4/2015
and were published in GenBank under the accession numbers KX231852, KX231853, KX231851 and KX231854, respectively.

Alignment and sequence analysis

Alignment and sequence analysis of the four sequenced viral fragments was carried out on a selected region including the cleavage site. The truncated 413-bp fragment, spanning 5' (1–413) 3' within the F gene, was analyzed using MEGA 6.06 software. When the four sequences were aligned together, they showed a high similarity with nucleotide identity ranging from 98.3% to 99.5%. When the four sequenced strains were compared with 37 other NDV reference strains (Table 2), they were shown to be closely related to EG/ CK/ NDV/ 19/Luxor-Esna/2011, NDV/ chicken/ VRLCU138/ Egypt/2012 and NDV/ Chicken/ China/ SDYT03/2011 with average identities of 99.2%, 98.3% and 98.3%, respectively. Nucleotide identity with the LaSota reference strain ranged from 79.4–80.5%, identity with B1 ranged from 79.2–80.4% and identity with Komarov ranged from 79.2–80.4%. As shown in Figure 2, the above-mentioned variations in nucleotide identity led to several amino acid substitutions, either when the four sequences were compared together or were compared with other reference strains. NDV/ chicken/ Egypt/ 1/2015 and NDV/ chicken/ Egypt/ 3/2015 shared two amino acid substitutions at positions (4k→4R) and (13L→13P) with NDV/ Chicken/ China/ SDYT03/2011. Other substitutions were present at position 28 (L→S) in NDV/ chicken/ Egypt/1/2015 and at position 43 (V → I) in NDV/ chicken/ Egypt /3/2015. The other two studied strains were 100% identical with each other and with NDV/ Chicken/China/ SDYT03/2011. When the four studied strains were compared with the LaSota strain, 27 amino acid substitutions at different positions were found. Regarding the cleavage site motif, the four studied strains contained the same cleavage site specific to NDV virulent strains such as NDV/ Chicken/ China/ SDYT03/ 2011.

Table 2: Newcastle disease reference strains used in the phylogenetic analysis

Strain	Accession number	Genotype
NDV/99-0655	AY935494	I
NDV/99-1997PR-32	AY935497	I
NDV/Queensland-V4	AF217084	I
NDV/lreland/Ulster/67	AY562991	I
NDV/LaSota	AY845400	II
NDV/clone30	Y18898	II
NDV/Ostrich/lsmailia/2010	JN 193503	II
N DV/B1	NC-002617	II
NDV/ turkey/USA/VGGA/89	AY289002	II
NDV/chicken/Cameroon/CAE08-061/2009	HF969123	II
NDV/chicken/Egypt/4/2006	FJ969395	II
APMV-1/chicken/U.S.(TX)/GB/1948	GU978777	II
NDV/chicken/Egypt/2/2006	FJ969393	II
NDV/ Beadutte C	X04719	II
NDV/Chicken/Egypt/1/2005	FJ9393I3	II
NDV/Komarov	KT44590l	I
NDV/JS/9/05/Go	FJ430160	III
APMVI-australia-victoria	M21881	III
NDV/Herts/33	AY741404	IV
NDV/ltalien	EU293914	IV
NDV/rAnhinga	EF065682	V
NDV/ mixed species/U.S./Largo/71	AY562990	V
NDV/ASTR/74	Y190I2	VI
NDV/chicken/U.S.(CA)/1083(Fontana)/72	AY562988	VI
NDV/chicken/Egypt/2/2015	KX231853	VII
NDV/chickenEgypt/4/2015	KX23I854	VII
NDV/chicken/Egypt/1/2015	KX231852	VII
NDV/chicken/Egypt/3/2015	KX231851	VII
NDV-F388-RLQP-CH-EG-I4	KP316016	VII
EG/CK/NDV/19/Luxor-Esna/2011	KP 119144	VII
NDV-B161-RLQP-CH-EG-I2	KM288620	VII
NDV/B131/RLQP/CH-EG- 12	KM288610	VII
NDV/Chicken/China/SDYT03/2011	JQ015297	VII
NDV/turkey/lsrael/111/2011	JN979564	VII
NDV/chicken/VRLCU138/Egypt/2012	JX885868	VII
NDV/QH1	FJ751918	VIII
NDV/Mali-ML029_07/2007	JF966386	XIV
NDV/chicken/Togo AK018'2009	JX390609	XVII
NDV/Chicken/lvoryCoast/CIV08-069/2007	HF969127	XVIII
NDV/Chicken/CentralAfrican Republic/CAF09-014/2008	HF969125	XVII
NDV/chicken/Nigeria/N IE10-124/2011	HF969168	XVII

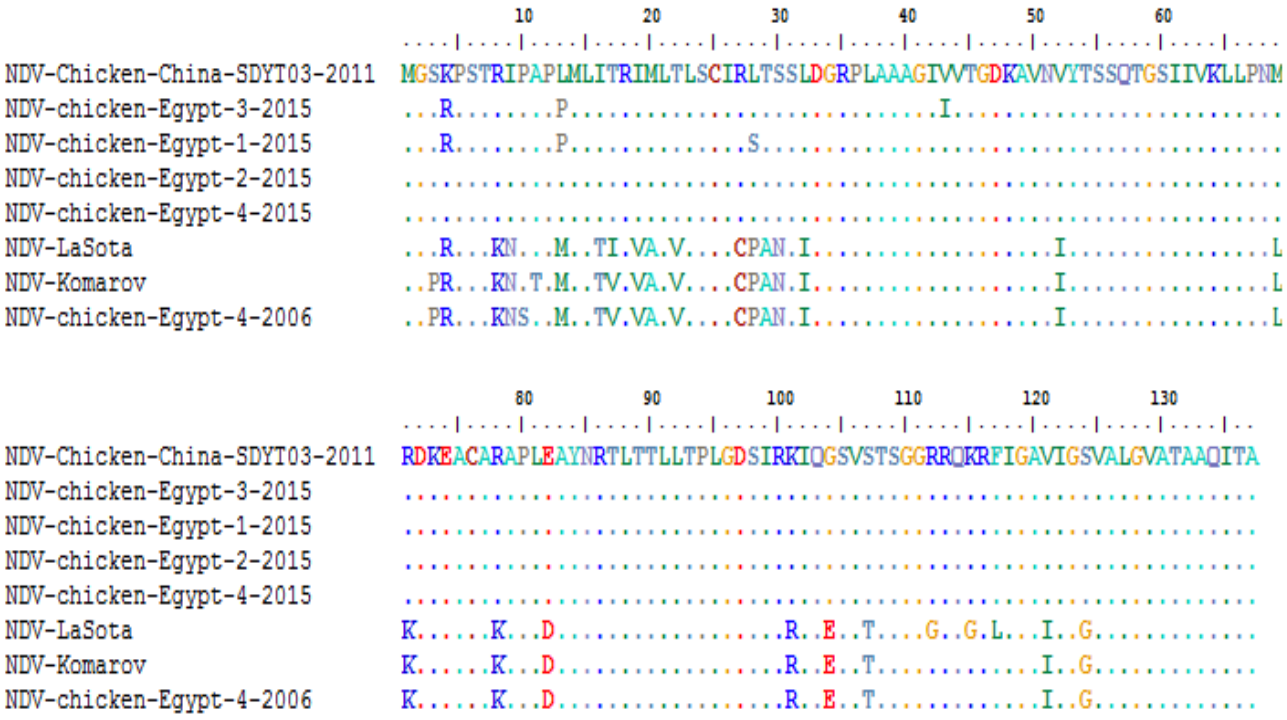

Figure 2: F protein amino acids alignment between the 4 studied strains and other vaccine and reference strains. The alignment was performed using bioedit software

According to the phylogenetic analysis of the studied strains, the nucleotide sequences of 41 strains isolated from different areas of the world, including vaccine strains, were aligned. As shown in Figure 3, the four studied strains clustered with genotype VII (d) of previously published velogenic NDV strains. The nearest neighbors for the four studied strains were EG/CK/NDV/19/Luxor-Esna/2011, NDV/F388/RLQP/CH-EG/14, NDV/chicken/VRLCU138/Egypt/2012, NDV/turkey/Israel/111/2011 and NDV/Chicken/China/SDYT03/2011, with average distances of 0.9%, 1.4%, 1.6%, 1.6% and 1.9%, respectively. The average distance between the four studied strains and the lentogenic LaSota vaccine was 24%, while the average distance was 22% in the case of the mesogenic Komarov vaccine.

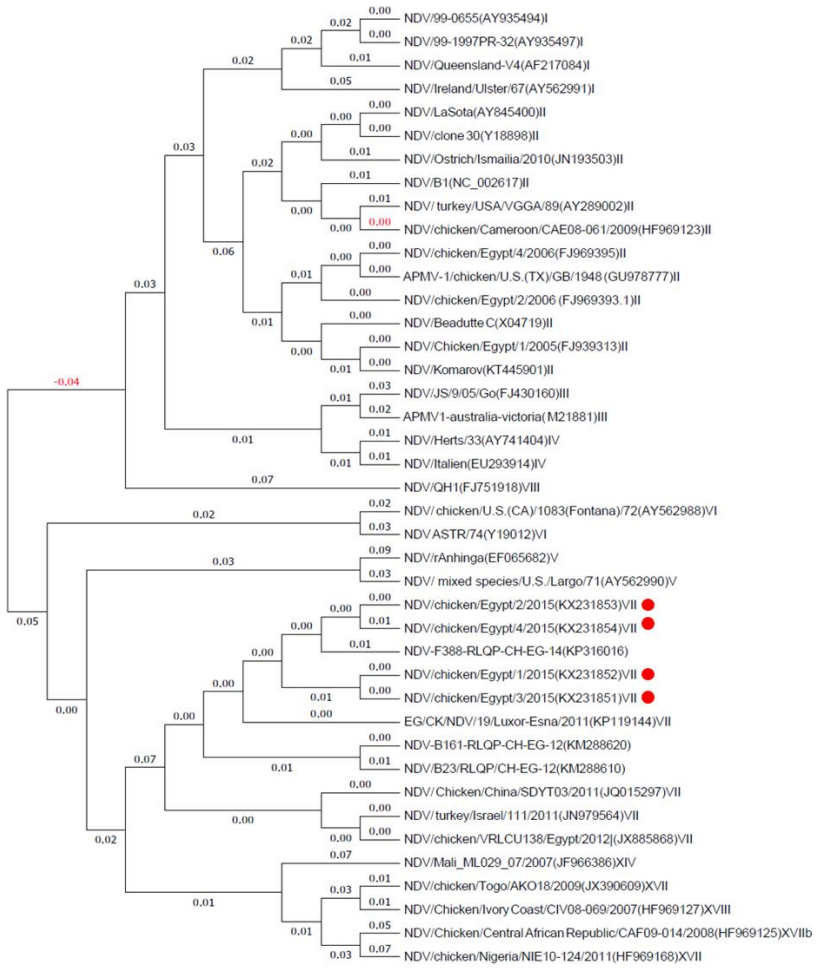

Figure 3: Phylogenetic analysis of the nucleotide sequence of NDV strain based on partial F-gene. The four sequences in this study are labelled. Phylogenic relationship through a bootstraps trail of 1000 was determined with the MEGA Version 6 using Cluster W alignment algorism and neighbour-joining method for tree construction.

Discussion

Undoubtedly, ND has been considered as one of the highly destructive disease (12). Not only the highly contagious nature of disease but also the high morbidity and mortality rate play a vital role to classify ND as list A disease by OIE (21). Since 1948, ND has been recognized as an endemic disease after it was discovered for the first time in Egypt (13). The virus still causes severe outbreaks on poultry farms a cross Egypt, although intensive vaccination programs against NDV has been continuously applied either on large scale or small scale poultry farming.

The genetic composition of the NDV genome isolated from chicken flocks in Sharkia Governorate was studied previously in 2005-2006 (15). However, it is of interest to identify the changes characterizing NDV in the past 10 years. Clinically, the examined birds presented respiratory signs including sneezing, coughing, and nasal discharge; nervous signs manifesting as torticollis, opisthotonus, and leg paralysis; and/or digestive troubles such as greenish diarrhea surrounding the cloaca. Cyanosis of the comb and wattles was also observed. Postmortem examination revealed congestion (brain, trachea, lungs and liver) and hemorrhages (proventriculus, intestine and cecal tonsils). Thickening of the air sacs and greenish mucous content in the intestinal tract were observed. The above-mentioned clinical findings were previously shown to be associated with

NDV and AIV virus infection by many authors (7,22,23). The presence of elliptical ulcers in the intestinal tract was reported in 20 poultry flocks as a characteristic pathogenomic lesion of vvNDV (23).

Viral inoculation in ECE led to congestion and hemorrhages in chicken embryos with deaths between 48–96 hours post-inoculation as well as positive HA activity in the allantoic fluids of forty investigated samples. Similarly, Alexander (8) stated that NDV induces hemorrhage and mortality in chicken embryos as well as hemagglutinating activity in chicken RBCS. However, these embryonic lesions and viral HA activity are potentially attributable to any of the avian paramyxoviruses or avian influenza viruses (21). Therefore, it is necessary to confirm the presence of NDV using an HI test due to its low cost, availability and simplicity (24,25). In the present study, the HI results revealed 15 flocks were infected by NDV alone, 12 flocks were infected by both NDV and AIV (H5), and only 9 flocks were infected by AIV (H5) alone. In Nigeria, a serological survey using an HI test proposed that a concurrent infection between pathogenic avian influenza and NDV was possible (26).

In the current study, the RT-PCR technique was utilized to schematically identify, characterize and pathotype the isolated NDVs. First, the A+B primer set was used for the preliminary identification of NDVs via amplification of the 362-bp fragment of the F gene, which is conserved in all NDV pathotypes (20). Twenty-seven isolates were confirmed as positive for NDV via PCR amplification. NDV isolates are divided into three groups based on their virulence in poultry: velogenic (high virulence), mesogenic (moderate virulence) and lentogenic (low virulence) (3,18). Second, another set of primer pairs (A+C), which included conserved forward primer and reverse primers representing the cleavage site of the F gene for virulent strains, was used to amplify a 256-bp fragment only in cases of virulent NDV (20). Our selected 12 samples were based on their geographical distribution and breeding purpose were tested and confirmed as positive virulent strains. These data confirmed that RT-PCR was a successful and rapid tool for NDV strain characterization and pathotyping during outbreaks (20,27).

Because all conserved NDV-positive samples originated from flocks that were subjected to intensive NDV vaccination programs, including live and inactivated vaccine, pathotyping and genotyping of the circulating virus among these flocks was a critical issue. The OIE defines an outbreak of ND as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that has multiple basic amino acids at the C-terminus of the F2 protein and a phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term multiple basic amino acids refers to three or more basic amino acids, specifically lysine (K) or arginine (R), present in residues 113–116 and phenylalanine (F) at position 117 (21,28). Failure to demonstrate the characteristic amino acid residue pattern described above requires characterization of the isolated virus with an ICPI test (21).

On the other hand, avirulent viruses contain the 112 G/E-K/R-Q-G/E-R-L 117 motif at the F protein cleavage site, which fails to meet the above-mentioned criteria for virulent strains (29). Therefore, sequencing of the 766-bp fragment from four PCR products obtained from virulent NDV isolates (spanning position 980 of the M gene to position 485 of the F gene) revealed that they contained the F-gene cleavage site characteristic of virulent NDV strains (112RRQKRF117). Based on genome length and the F gene sequence, NDV strains have been classified into two classes within a single serotype: class I (nine genotypes, 1–9) and II (eighteen genotypes, I–XVIII) (30,31). Currently, the circulating strains associated with disease outbreaks worldwide predominantly derive from genotypes V, VI and VII of class II (6).

Interestingly, phylogenetic analysis of the nucleotide sequence of the F gene clustered the four sequenced strains with class II genotype VII strains (EG/CK/NDV/19/Luxor-Esna/2011, NDV/F388/RLQP/CH/EG/14 and NDV/chicken/VRLCU138/Egypt/2012), which are responsible for recent NDV outbreaks at other areas in Egypt (32,33). The four sequenced strains demonstrate low distance and high

identity with EG/ CK/ NDV/ 19/Luxor-Esna/2011 and Chicken /China / SDYT03/2011 (98.8–99.5% and 97.5–98.5%, respectively). Analysis of the obtained results clearly indicates a considerable rate of mutation among circulating NDVs between 2005 and now. Saad and colleagues recorded the presence of class II genotype II among chicken flocks in Sharkia Governorate (34). Later, a similar study performed on samples obtained from 2005–2006 in the same Governorate concluded that the NDV circulating in the Sharkia Governorate clustered with class II genotype II and demonstrated high identity with the LaSota strain (93.4–98.2%) (15).

In the current study, the four studied strains clustered with class II genotype VII and demonstrated a lower range of identity (79.2–80.6%) and an overall amino acid distance of 24.4% with the vaccine strains, including the LaSota strain. This finding is very similar to a previous study in which a distance matrix analysis of the F gene from NDV genotype VII strains isolated from Malaysia indicated an overall amino acid distance of 21% with the genotype II LaSota vaccine and 12.8% with genotype VIII isolates (35). The large genetic distance with the LaSota strain may be alarming regarding the potential evolution of different virulent NDV and may explain the circulation of these virulent strains among Egyptian poultry farms despite the application of intensive vaccination programs. On the other hand, other studies have suggested that the circulation of virulent NDV among vaccinated flocks may be attributed to poor flock immunity due to inadequate vaccination practices and concurrent infection with immuno-suppressive agents rather than antigenic variation (36-38).

Moreover, the studied strains had several amino acid substitutions with the previously isolated strain from the same locality (NDV/Chicken/Egypt/4/2006). A previous study described NDV genotype VII strains isolated from China as immune response-escaping antigenic variants (39). Furthermore, previous studies concluded that superior protection against genotype VII is achieved using the closest vaccine to the circulating field isolates considering the induction of a higher and earlier immune response, protection against morbidity and mortality, and reduction of challenge virus shedding (33,40). The presence of specific amino acid residues may help in subtyping NDV genotypes. Hence, genotype VII is sub-classified into 5 subtypes (genotypes VIIa, b, c, d and e), each with a specific molecular characterization. One example of a genotype VIId isolate involves a unique pattern consisting of V52-for-I, K101-for-R, S176-for-A and Y314-for-F (39). In our study, the four sequences were genotype VIId. Interestingly, this subtyping method provides researchers with highly valuable information about the origin and epidemiology of the studied strains. For example, genotypes VIIa and b represent viruses that emerged in 1970 in the Far East and spread to Europe and Asia or South Africa, respectively. Another example is genotypes VIIc and d, which originated from China, Kazakhstan and South Africa (40,41). Therefore, there is a strong probability that the studied strains are from China or Middle Eastern countries such as Israel and enter Egypt through infected poultry products or wild birds.

Conclusion

In conclusion, genotype VIId class II strains are the currently circulating virus strains responsible for NDV outbreaks among poultry farms in Sharkia Governorate. Despite current vaccination regimens against NDV, this virus continues to impact the Egyptian poultry industry. Further investigation to evaluate the efficacy of the available vaccines against challenge with recently isolated viruses is recommended, as is the improvement of vaccine application to ensure proper vaccination.

Conflict of interest

The authors have no conflict to declare.

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