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Isolation and Molecular Characterization of Newcastle Disease Virus Genotype VII Circulating in Egypt (2017-2020)

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ABSTRACT

Despite the frequent use of ND vaccines in Egypt, Newcastle Disease Virus still causes outbreaks in the commercial, backyard, and wild captive poultry. Avian Orthoavula virus 1 (AOaV-1) belongs to the genus Orthoavulavirus and causes ND in a variety of avian species around the world. To explore the evolution of the NDV and evaluate the efficacy of the vaccine regimens currently being used in Egypt's commercial poultry, spleen, brain, ileocecal tonsil, and tracheal tissue specimens were collected from twenty-three vaccinated broiler flocks from four Egyptian governorates (Kafr-Elsheikh, Alexandria, Matrouh, and El-Behera) between 2017 and 2020 for molecular analysis. qRT-PCR was used to characterize the NDV that was propagated in specific pathogen-free embryonated chicken eggs (SPF ECEs). The isolates were screened for other endemic avian respiratory viruses including avian influenza and infectious bronchitis viruses. Five isolates were selected for further genetic characterization, based on the motif of the cleavage site (^{112}RRQKR ↓F^{117}) in the fusion (F) protein. All five isolates were identified as velogenic NDV strains based on partial-length F gene sequences. These isolates are clustered into subgenotype VII.1.1 of the class II of ND viruses, which is the main genotype in the Egyptian poultry industry and in those of many Middle East countries.

Keywords: Fusion protein, Genotype VII, Isolation, NDV and Phylogenetic analysis.

INTRODUCTION

Avian Orthoavula virus 1 virulent strains (AOaV-1, previously termed (NDV) Newcastle disease virus is classified as a List A disease by the World Organization for Animal Health (OIE) (OIE, 2012). Newcastle disease virus is belonging to the Orthoavula virus genus inside the family Paramyxoviridae (Amarasinghe et al., 2017). NDV is a single-stranded negative-sense, nonsegmented enveloped RNA virus, composed of six structural

proteins (phosphoprotein (P), nucleocapsid protein (NP), fusion protein (F), matrix protein (M), haemagglutinin neuraminidase (HN) and the large polymerase (L)) with additional two V and W nonstructural proteins that are transcribed during RNA editing in the P gene (Kolakofsky et al., 2005). There are five different pathotypes for NDV based on the pathogenicity; velogenic (high virulence) which could be subdivided into velogenic viscerotropic and velogenic neurotropic, mesogenic, lentogenic, and finally a symptomatic (OIE, 2012). Based on genetic analysis, NDV strains are divided genetically into 2 separate classes (I and II). Class, I strain have only one genotype and are generally avirulent while class II strains are additional classifications into at least 21 genotypes (Dimitrov et al., 2019).

Although NDV was discovered in Egypt as early as 1948 (Daubney and Mansy, 1948), since 2010, NDV has resulted in catastrophic losses in commercial poultry. Mass vaccination programs in commercial poultry facilities are still with limited success in effectively controlling the disease (Radwan et al., 2013; Hussein et al., 2014; Orabi et al., 2017; El Naggar et al., 2018). Currently, genotypes of NDV circulating in Egypt's poultry industry sectors are mainly three genotypes II, VI, and VII that have been reported in both vaccinated and non-vaccinated birds (Rohaim, 2016; Sabra et al., 2017; Selim et al., 2018; El Naggar et al., 2018). In this study, virulent genotype VII of NDV was isolated and genetically characterized from different broiler flocks within four Egyptian governorates during the period from 2017 to 2020.

MATERIALS AND METHODS

Samples collection and transportation:

Tracheal tissue, brain, iliocecal tonsil and spleen specimens were collected from twenty-three broiler flocks from four Egyptian governorates (Kafr-Elsheikh, Alexandria, Matrouh, and El-Behera) between 2017 and 2020 (Table 1). The individual samples were collected in sterile phosphate-buffered saline (PBS, pH 7.4) mixed with a 50 U/mL penicillin–streptomycin mixture. The suspected broiler flocks exhibited nervous, respiratory signs and watery greenish diarrhea. Samples collection and processing were carried out in strict conformity with animal welfare and health guidelines and legislation. The work was authorized by the Ethics Committee at the Reference Laboratory of Veterinary Quality Control on Poultry Production in Damanhur, Egypt, as part of this process.

NDV molecular screening:

QIAamp Viral RNA Mini Kit was used to extract viral RNA from the samples (QIAGEN, USA in accordance with the manufacturer's guidelines. For NDV screening, q RT-PCR was conducted using primers specific for the M gene (Wise et al., 2004).

Virus Isolation:

Isolation of the virus was carried out for NDV-positive samples in 9-day-old specific-pathogen-free embryonated chicken eggs (SPF-ECE) supplied from SPF farm, Koam Osheim, El-Fayoum, Egypt. The processed samples were inoculated via the allantoic sac according to the standard procedures for three passages as described in (OIE, 2012). Virus propagation was followed up by the hemagglutination assay (HA) on the harvested allantoic fluids (OIE, 2012) and q RT-PCR (Wise et al.,

2004). In addition, the isolates were screened for other endemic avian respiratory viruses including avian influenza (AI) and infectious bronchitis viruses (IBV).

Genetic characterization and Sequencing:

Five positive NDV isolates were chosen. Partial F-gene gene amplification by using standard RT-PCR (Aldous et al., 2003). The purified PCR products were sequenced directly using ABI PRISM Big Dye Terminator version 3.1 and Qiagen PCR Clean-Up kit (Qiagen, USA) according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

Sequence analysis and phylogeny:

The obtained sequences were further aligned and analyzed together with other sequences collected from the NCBI GenBank that representing all the genotype VII subgroups and as best fitted Model, the phylogenetic analysis was carried out using Clustal W multiple alignment MEGA 6 program (Tamura et al., 2013) and maximum likelihood analysis (1,000 replicates for bootstrap).

RESULTS

NDV molecular screening of the tested samples:

Seventeen samples tested positive for NDV by using the qRT-PCR assay while the remaining six samples were negative (Table 2).

Virus isolation, propagation, and identification:

Five NDV-positive samples were successfully isolated through inoculation into the allantoic cavities of 9-day-old SPF-ECE (five eggs/ sample) for three serial passages. Embryo mortalities between 36 and 48 hours were noticed throughout the different passages. HA activity mean 2^6 HA units was detected in the allantoic fluids of the five samples. The five NDV isolates were negative for both AI and IBV by qRT-PCR testing.

Phylogeny and genetic analysis of the NDV isolates:

Partial F-gene amplification was conducted for the five isolates using degenerate primers and submitted to GenBank under the accession numbers OK000492, OK000493, OK000494, OK000495 and OK000496 respectively. Our results confirmed that all the five isolates (NDV.VII.1.1/Egy-Elbeh/ELH.1/2020, NDV.VII.1.1/Egy-Elbeh/ELH.2/2020, NDV.VII.1.1/Egy-Alex/ELH.3/2019, NDV.VII.1.1/Egy-Matr/ELH.4/2018 and NDV.VII.1.1/Egy-Matr/ELH.5/2018 shared the cleavage site motif 112RRQKR ↓F117, which confirmed the virulent nature of these isolates. Meanwhile, our isolated strains are belonging to class II/ genotype VII; sub genotype VII.1.1 formerly classified as VII.b (Figure 1. A & B).

Nucleotide alignment of 350 bp of the five isolates showed a high relation between the five isolates with identity ranging between 99-100% (Figure 1 C). Amino acids alignment showed the high relationship between the five viruses with identity ranged between 98-100 (Figure 1 D). Moreover, there are three viruses NDV.VII.1.1/Egy-Alex/ELH.3/2019, NDV.VII.1.1/Egy-Matr/ELH.4/2018 and NDV.VII.1.1/Egy-Matr/ELH.5/2018 had similarity of 100% at the level of both nucleotide and amino acids. On the other hand, compared to the vaccines strains there was distance ranged between 14% to 26 % (Table 3)

Table (1): History of samples collected from different broiler Egyptian flocks during 2017- 2020 for NDV detection:

Sample ID	Governate/Year	Age of birds/ days	Clinical signs &pm lesions	Number of birds	Mortality%
1	Elbehira2020	15	Coughing& torticollis	50	100
2	Elbehira2020	20	Ruffled feathers, depression&sneezing	200	50
3	Elbehira2020	15	Edema of the head and wattles, nervous signs such as paralysis and torticollis and respiratory signs	200	75
4	Alexandria 2019	17	Greenish diarrhea &petechial Hemorrhages of proventriculus	300	50
5	Matrouh 2018	23	Respiratory signs with depression, watery greenish diarrhea	8000	100
6	Kafr-ElSheikh2018	20	Respiratory signs	200	10
7	Elbehira2018	37	Depression, nervous signs	1000	30
8	Elbehira2017	16	Nervous signs and respiratory	500	20
9	Matrouh2017	35	Respiratory signs	2000	5
10	Elbehira2017	13	Nervous signs and respiratory	5500	25
11	Matrouh2017	16	Respiratory signs with depression, watery greenish diarrhea	6000	30
12	Kafr-ElSheikh2017	22	Respiratory signs	70	5
13	Kafr-ElSheikh2017	40	Edema of the head and wattles, nervous signs	100	10
14	Alexandria 2017	39	respiratory signs and watery greenish diarrhea	50	5
15	Alexandria 2017	18	Nervous signs and respiratory signs	1000	15
16	Elbehira2017	22	Nervous signs and respiratory	170	10
17	Elbehira2017	15	respiratory signs and watery greenish diarrhea	70	100
18	Matrouh2017	14	Nervous signs and respiratory	1500	20
19	Matrouh2017	30	respiratory signs	4000	10
20	Elbehira2017	29	Respiratory signs with depression, watery	800	30

			greenish diarrhea		
21	Elbehira2017	40	Nervous signs and respiratory	70	5
22	Gharbia 2017	27	Respiratory signs, edema of the head and wattles,	100	10
23	Gharbia 2017	14	respiratory signs and watery greenish diarrhea	100	10

Table (2): NDV results of examined farms by RT-PCR (M-gene):

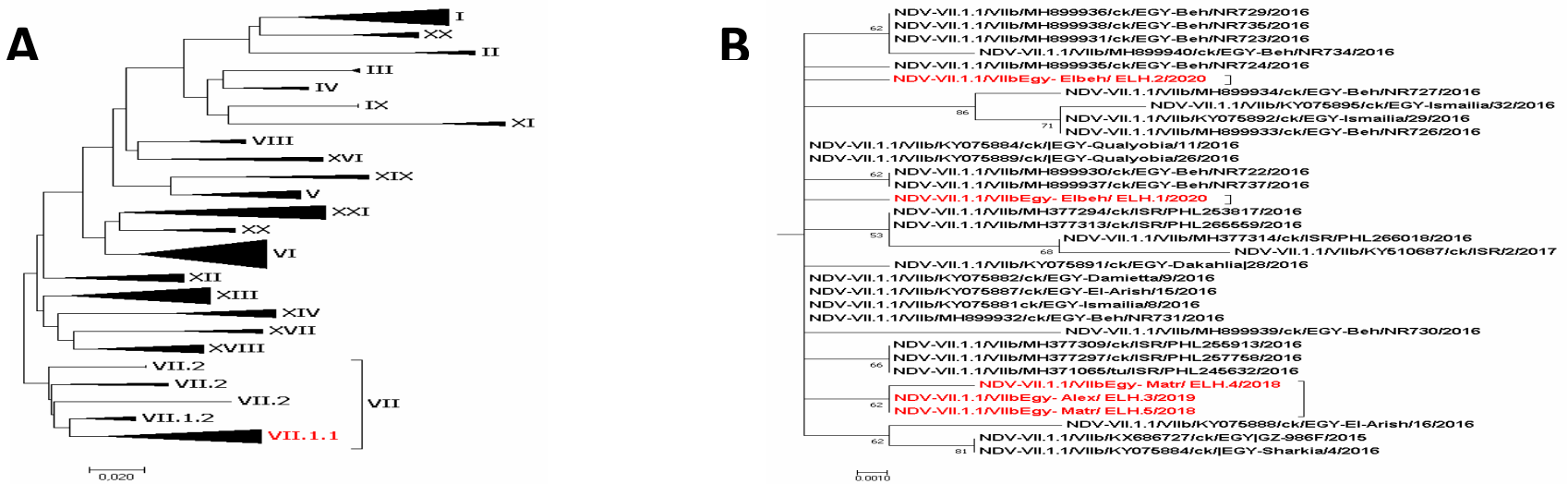
Number of samples	Result of M- gene	CT value
1	Positive	28
2	Positive	27
3	Positive	28
4	Positive	31
5	Positive	17
6	Negative	No CT
7	Positive	31
8	Positive	35
9	Negative	No CT
10	Positive	31.4
11	Positive	29.7
12	Negative	No CT
13	Negative	No CT
14	Positive	35
15	Positive	32
16	Positive	33
17	Positive	35
18	Positive	30
19	Positive	35
20	Positive	29
21	Positive	+ CT
22	Negative	No CT
23	Negative	No CT

CT: threshold cycle.

Table 3: Evolutionary distances of Study isolates and other sub-genotype VII.1.1 and vaccines viruses estimated using the partial fusion gene coding sequences:

1	**NDV-VII.1.1/VIIb/ck/Egy-_Elbeh/_ELH.1/2020**																								
2	**NDV-VII.1.1/VIIb/ck/Egy-_Elbeh/_ELH.2/2020**	0,01																							
3	**NDV-VII.1.1/VIIb/ck/Egy-_Alex/_ELH.3/2019**	0,01	0,01																						
4	**NDV-VII.1.1/VIIb/ck/Egy-_Matr/_ELH.4/2018**	0,01	0,01	0,00																					
5	**NDV-VII.1.1/VIIb/ck/Egy-_Matr/_ELH.5/2018**	0,01	0,01	0,00	0,00																				
6	**NDV-VII.1.1/VIIb/KX686723/ck/EGY/KFR-B7/2012**	0,01	0,01	0,01	0,01	0,01																			
7	**NDV-VII.1.1/VIIb/MH001441/ck/EGY-Qena/104/2012**	0,02	0,02	0,02	0,02	0,02	0,01																		
8	**NDV-VII.1.1/VIIb/|KX686726/ck/EGY-BEH/261F/2014**	0,01	0,01	0,01	0,02	0,01	0,01	0,01																	
9	**NDV-VII.1.1/VIIb/KX686724/ck/EGY/35/2014**	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01																
10	**NDV-VII.1.1/VIIb/KU365652/ck/EGY/FU13-NLQP/2014**	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,00															
11	**NDV-VII.1.1/VIIb/MH392219/ck/EGY-Soh/2014**	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01														
12	**NDV-VII.1.1/VIIb/KX686727/ck/EGY|GZ-986F/2015**	0,01	0,01	0,01	0,01	0,01	0,01	0,02	0,02	0,01	0,01	0,01													
13	**NDV-VII.1.1/VIIb/KY075892/ck/EGY-Ismailia/29/2016**	0,01	0,01	0,01	0,01	0,01	0,02	0,02	0,02	0,01	0,01	0,02	0,01												
14	**NDV-VII.1.1/VIIb/KY075895/ck/EGY-Ismailia/32/2016**	0,01	0,01	0,01	0,02	0,01	0,02	0,03	0,02	0,01	0,02	0,02	0,02	0,00											
15	**NDV-VII.1.1/VIIb/KY075881ck/EGY-Ismailia/8/2016**	0,00	0,00	0,00	0,01	0,00	0,01	0,01	0,01	0,00	0,01	0,01	0,01	0,01	0,01										
16	**NDV-VII.1.1/VIIb/KY510687/ck/ISR/2/2017**	0,01	0,01	0,01	0,01	0,01	0,02	0,02	0,02	0,01	0,01	0,02	0,01	0,02	0,02	0,01									
17	**NDV-VII.1.1/VIIb/MH899939/ck/EGY-Beh/NR730/2016**	0,01	0,01	0,01	0,01	0,01	0,02	0,02	0,02	0,01	0,01	0,02	0,01	0,02	0,02	0,01	0,02								
18	**NDV-VII.1.1/VIIb/MH899931/ck/EGY-Beh/NR723/2016**	0,01	0,01	0,01	0,01	0,01	0,01	0,02	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,00	0,01	0,01							
19	**NDV-VII.1.1/VIIb/KY510685/ck/ISR/428/2016**	0,02	0,02	0,02	0,03	0,02	0,02	0,02	0,02	0,02	0,02	0,02	0,03	0,03	0,03	0,02	0,03	0,03	0,02						
20	**NDV-VII.1.1/VIIb/MH377294/ck/ISR/PHL253817/2016**	0,01	0,01	0,01	0,01	0,01	0,01	0,02	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,00	0,01	0,01	0,01	0,02					
21	**NDV-VII.1.1/VIIb/MH377309/ck/ISR/PHL255913/2016**	0,01	0,01	0,01	0,01	0,01	0,01	0,02	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,00	0,01	0,01	0,01	0,02	0,01				
22	**NDV-VII.1.1/VIIb/MH377314/ck/ISR/PHL266018/2016**	0,01	0,01	0,01	0,01	0,01	0,02	0,02	0,02	0,01	0,01	0,02	0,01	0,02	0,02	0,01	0,01	0,02	0,01	0,03	0,01	0,01			
23	**NDV-II/La_Sota/AJ629062**	0,26	0,26	0,26	0,26	0,26	0,25	0,26	0,25	0,25	0,25	0,25	0,26	0,26	0,27	0,26	0,27	0,26	0,26	0,25	0,25	0,26	0,26		
24	**NDV-II//Hitchner/JN872151**	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,0

		6	6	6	6	6	5	6	5	5	5	5	6	6	7	6	7	6	6	5	5	6	6	1		
25	NDV-II/clone_30/Y18898	0,26	0,26	0,26	0,26	0,26	0,25	0,26	0,25	0,25	0,25	0,25	0,26	0,26	0,27	0,26	0,27	0,26	0,26	0,25	0,25	0,26	0,26	0,00	0,01	
26	NDVI/VG/GA-AVINEW/KC906188	0,22	0,22	0,22	0,22	0,22	0,21	0,22	0,21	0,21	0,21	0,21	0,22	0,22	0,23	0,22	0,23	0,21	0,22	0,21	0,22	0,22	0,23	0,14	0,14	0,14



C

	Isolate	1	2	3	4
1	NDV.VII.1.1/_Egy-_Elbeh/_ELH.1/2020				
2	NDV.VII.1.1/_Egy-_Elbeh/_ELH.2/2020	0,01			
3	NDV.VII.1.1/_Egy-_Alex/_ELH.3/2019	0,01	0,01		
4	NDV.VII.1.1/_Egy-_Matr/_ELH.4/2018	0,01	0,01	0,00	
5	NDV.VII.1.1/_Egy-_Matr/_ELH.5/2018	0,01	0,01	0,00	0,00

D

	Isolate	1	2	3	4
1	NDV.VII.1.1/_Egy-_Elbeh/_ELH.1/2020				
2	NDV.VII.1.1/_Egy-_Elbeh/_ELH.2/2020	0,01			
3	NDV.VII.1.1/_Egy-_Alex/_ELH.3/2019	0,01	0,02		
4	NDV.VII.1.1/_Egy-_Matr/_ELH.4/2018	0,01	0,02	0,00	
5	NDV.VII.1.1/_Egy-_Matr/_ELH.5/2018	0,01	0,02	0,00	0,00

Figure (1): Phylogenetic analysis and distances for the study isolates, A: showing the clustering of the study isolates in velogenic NDV VII.1.1 subgroup, B: showing the clustering of the study isolates with other VII.1.1 viruses isolated from Egypt and Middle East, C: the nucleotide distance between the study isolates. D. the amino acids distances between the study isolates.

DISCUSSION

The Egyptian poultry has suffered significant economic losses because of persistent velogenic ND outbreaks in several areas of the commercial poultry. Also, the occurrence of mixed infection of NDV and other poultry viral diseases, e.g., avian influenza and infectious bronchitis in Egyptian poultry flocks in the last years may play a major role in the increased mortalities in poultry flocks, especially broilers. Therefore, the extensive surveillance of the Egyptian poultry flocks for NDV is a fundamental requirement for understanding the epidemiology of this virus. Since 2010, recombinant and/or inactivated vaccines based on genotype VII have been included in the immunization schedule in ND-endemic countries, including Egypt, due to an increase in ND outbreaks (El Naggar et al., 2018). Because there is only one serotype of Orthoavulavirus 1 (AOaV-1) serotype, lentogenic strains (e.g., LaSota) are primarily used to protect birds from velogenic NDVs as live-attenuated vaccines that can prevent disease but not infection, even though virus replication and shedding still occur in vaccinated birds (El Naggar et al., 2018).

Studying of genetic diversity of NDV field strains in different geographic regions of Egypt is the goal of this work. The early embryonic deaths within 36 hours post-inoculation of all inoculated isolates indicates the velogenic nature of these isolates (Gopinath VP et al., 2011). Detection of seventeen NDV-positive samples out of twenty-three (74%) by qRT-PCR suggests high sensitivity, high specificity, efficiency, and mostly its capacity of the assay for detecting the virus. Also, the high detection level of NDV among Egyptian poultry flocks from different provinces suggests endemicity and circulation of the NDV in Egypt. The amplification of matrix gene from isolate samples confirmed the chickens were exposed to Newcastle disease. This finding agreed with (Worku et al., 2022) who isolated and identified the virus from suspected Newcastle disease by qRT-PCR. Moreover, results of partial F-gene sequencing confirmed that all the five isolates (NDV.VII.1.1/Egy-Elbeh/ELH.1/2020., NDV.VII.1.1/Egy-Elbeh/ELH.2/2020, NDV.VII.1.1/Egy-Alex/ELH.3/2019, NDV.VII.1.1/Egy-Matr/ELH.4/2018 and NDV.VII.1.1/Egy-Matr/ELH.5/2018) shared the cleavage site motif 112RRQKR ↓F117, which confirmed the virulent nature of these isolates (Nagai et al., 1976). Meanwhile, phylogenetic analysis of the partial-length F gene (350 bases) revealed that the isolated strains belong to class II/genotype VII; sub genotype VII.1.1, formerly classified as VII.b. This finding agreed with (Selim et al., 2018). When the five isolates were compared to the vaccine strains, there was a distance ranging from 14% to 26 %. Increased genetic divergence between field strains and vaccinal strains has been hypothesized as a possible cause of ND outbreaks in vaccinated flocks due to vaccine strains' lack of immunological protection (Munir et al., 2012).

Several studies have identified velogenic NDV strains with genetic diversity in the Middle East. Yanhong et al. (2017) had a comparative study of

the F protein, the studied isolates indicated a greater evolutionary restriction on the F proteins. The changes in the F-protein explain the immune escape ability of these isolates and the lack of sterilization immunity offered by vaccines that are available.

CONCLUSION

Newcastle disease virus is known to be the most prevalent poultry virus disease in Egypt causing high economic losses, even with extensive vaccination programs. This might result in field outbreaks or the emergence of new pathotypes/genotypes leading to severe infections due to genetic diversity among NDV strains during the past decade. Therefore, isolation and characterization of NDV is important for NDV control and for the assessment of vaccination. The transmissible nature of the virus requires high biosecurity measures to prevent the introduction of NDV to Egyptian poultry flocks.

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