Research Article

Genotyping of Recent Virulent Newcastle Disease Virus Strains Isolated from Menofia Governorate, Egypt

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Abstract | Velogenic strains of Newcastle disease virus (NDV) cause Newcastle disease (ND), a devastating disease of poultry and wild birds. Phylogenetic analysis of recent Egyptian isolates in Menofia governorate, Egypt was constructed, and showed to be of genotype VII. Eight NDV viruses were isolated from vaccinated commercial broiler flocks showing respiratory manifestation in Menofia governorate, Egypt during 2019. Those viruses showing deaths and haemorages of inoculated SPF ECE eggs, harvested allantoic fluids showed haemagglutination activity by using 1% RBCs and also haemagglutination inhibition in case of using NDV reference antiserum, As well as realtime polymerase chain reaction by using standardized NDV specific primers and finally eight viruses were selected for further sequencing for the partial fusion protein, The eight NDVs isolates of velogenic genotype VII and contain the unique cleavage site motif 112RRQKRF117 with high relation to very virulent NDV Chinese strain Chicken /China/SDWF07/2011 strain with nucleotide identity percentage (99.3% -100%). The main causative agent of recent ND outbreaks in vaccinated broiler flocks in Menofia governorate, Egypt was found to belong to very virulent genotype VII. This strain was genetically identical to Egyptian genotype VII isolates isolated in the last period.

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Introduction

Newcastle disease (ND), caused by the virulent Newcastle disease virus (NDV), is a highly contagious disease that can cause significant losses in poultry worldwide. NDV is a member of the genus Avulavirus of the family Paramyxoviridae (Mayo, 2002). NDVs are single-stranded, negative sense RNA genome of 15,186, 15,192, or 15,198 nucleotides (nt) in length (Czeglédi *et al*., 2006; Kim *et al*., 2012). NDV strains divided into two clusters based on Phylogenetic analysis of the fusion gene (Liu *et al*., 2011). Class I and class II viruses have been recently classified into 1 and 15 genotypes, respectively (Diel *et al*., 2012). Four ND panzootics were existed since the first recognition of the disease in 1926. Genotype VII NDV strains represent the predominant genotype involved during the fourth panzootic since the late 1980s (Liu *et al*., 2008). Genotype VII NDV strains represent the predominant genotype in Egypt (Saad *et al*., 2017; Selim *et al*., 2018; Zanaty *et al*., 2019; Mahmoud *et al*., 2019). Complete sequences of the F genes of eight isolates from Egypt Menofia district were determined by reverse transcription (RT)-PCR

and direct sequencing. 139 amino acid sequence identities of F fusion protein including cleavage site among these eight isolates ranged from 98.6 to 100% and 99.1% to 99.6% respectively. The predicted amino acid sequences surrounding the cleavage site of F protein in all 8 isolates displayed the motif 112RRQKR*F117, which is typical of virulent NDV isolates. Phylogenetic analysis based on 139 amino acid sequences of the F genes classified these isolates into genotype XII, together with strains isolated from Egypt in 2010 till 2019, which becoming the predominant genotype responsible for most outbreaks of ND in Egypt during recent years. Although these strains belonged to the same genotype as the Egyptian strains. In the current work, we aimed to determine the circulating NDV genotypes that causing severe outbreaks in poultry flocks.

Materials and Methods

Samples

Tracheal swabs were collected from 20 vaccinated broiler flocks with severe respiratory signs and PM suspected Newcastle disease (ND), located in Menofia governorates (Table 1). Swabs were collected from twenty birds per flock and pooled, as one sample for each flock, in buffered saline solution with antibiotic (10,000IU/ml.penicillin, 10mg/ml streptomycin, 0.25 mg/ml gentamicin and 5,000 IU/ ml nystatin), adjusted to pH 7.0-7.4 OIE (2012). All samples stored in Reference Laboratory of Veterinary Quality Control on Poultry Production (RLQP), Animal Health Research Institute (AHRI). All tests applied in (RLQP).

Virus propagation

The viruses were propagated in 10 days old specific pathogen free (SPF) embryonated chicken eggs (SPF farm, Koam Osheim, El-Fayoum, Egypt). Centrifuged supernatant of the swab pools (100 ul/ egg) was inoculated intra allantoic in 10 days old embryonated eggs (SPF). Eggs were incubated at 37°C and examined daily for 5 days. Allantoic fluid was collected from dead embryos after the first 24 hours and examined for hemagglutination (HA) and hemagglutination inhibition (HI) activity using four HA units, according to OIE guidelines OIE (2012).

RNA isolation and PCR amplification and sequencing

RNAs from each isolate were extracted from allantoic fluids using QiAmp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, rRTPCR was carried out using Quantitect probe RTPCR kit (Qiagen, Inc. Valencia CA). Primers used were described by (Wise *et al*., 2004). rRT-PCR was conducted in the Stratagene3005P MXpro RealTime PCR System (Stratagene, USA) according to manufacturer instructions.

PCR amplification was performed by using Qiagen One-Step RT-kit according to the manufacturer's instructions, using primer sets designed by (Selim *et al*., 2018). Gel containing DNA bands (1.5%) of the expected size (400 bp) was excised and purified with QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer instruction. Purified RT-PCR products were sequenced using Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA) and Applied Biosystems 3130 genetic analyzer (ABI, USA). Sequences identities obtained in this study were compared with previously published NDV vaccine and references strains available in the public database (BLAST, NCBI, USA). 139 Amino acids phylogenetic relationship was constructed using MEGA version 6 (Tamura *et al*., 2013). A comparative analysis of 139 deduced amino acids and nucleotides sequences of the sequenced fusion gene was created using the CLUSTALW Multiple Sequence Alignment Program, version 1.83 of the MegAlign module of Lasergene DNAStar software. Sequences generated in the frame of this study were submitted to the GenBank database with accession numbers as showed in (Table 1).

Nucleotide sequence accession numbers: Partial fragment F gene sequences (n= 8) of virulent NDV obtained in this study were submitted to GenBank and are available under the accession numbers MW269675 to MW269682.

Results and Discussion

NDV detection

Eight samples, each representing one NDV farm, were positive for NDV by real-time PCR and were confirmed by HA and HI results (Table 1).

Phylogenetic analysis

Sequence analysis of F gene protein cleavage site revealed that the eight NDV isolates contain the typical sequence of velogenic NDV strains where multiple basic amino acids were observed showing the pattern RRQKR*F (Table 1).

Table 1: *History of samples No., Species, Governorate, Year, CPE Inoculated eggs, HI NDV, PCR , F- Protein cleavage site sequence, Virulence, Genotype, Isolate designation and Access number of GENBANK.*

Samples	species	Governorate	Year	PCR NDV	CPE eggs	HI NDV	F- protein cleavage site sequence	Virulence	Genotype	Isolate designation	Access number GENBANK
1	Chicken	Menofia	2019	+	+	+	RRQKR*F	Viru.	VII	NDV-EG/MENO/1-019	MW269675
2	Chicken	Menofia	2019	+	+	+	RRQKR*F	Viru.	VII	NDV-EG/MENO/2-019	MW269676
3	Chicken	Menofia	2019	+	+	+	RRQKR*F	Viru.	VII	NDV-EG/MENO/3-019	MW269677
4	Chicken	Menofia	2019	+	+	+	RRQKR*F	Viru.	VII	NDV-EG/MENO/4-019	MW269678
5	Chicken	Menofia	2019	+	+	+	RRQKR*F	Viru.	VII	NDV-EG/MENO/5-019	MW269679
6	Chicken	Menofia	2019	+	+	+	RRQKR*F	Viru.	VII	NDV-EG/MENO/6-019	MW269680
7	Chicken	Menofia	2019	+	+	+	RRQKR*F	Viru.	VII	NDV-EG/MENO/7-019	MW269681
8	Chicken	Menofia	2019	+	+	+	RRQKR*F	Viru.	VII	NDV-EG/MENO/8-019	MW269682

**: Point of cleavage (Alexander, 2003);Viru.: Virulent. +: CPE cytopathic effect, deaths, haemorages, and Haemagglutination positive.*

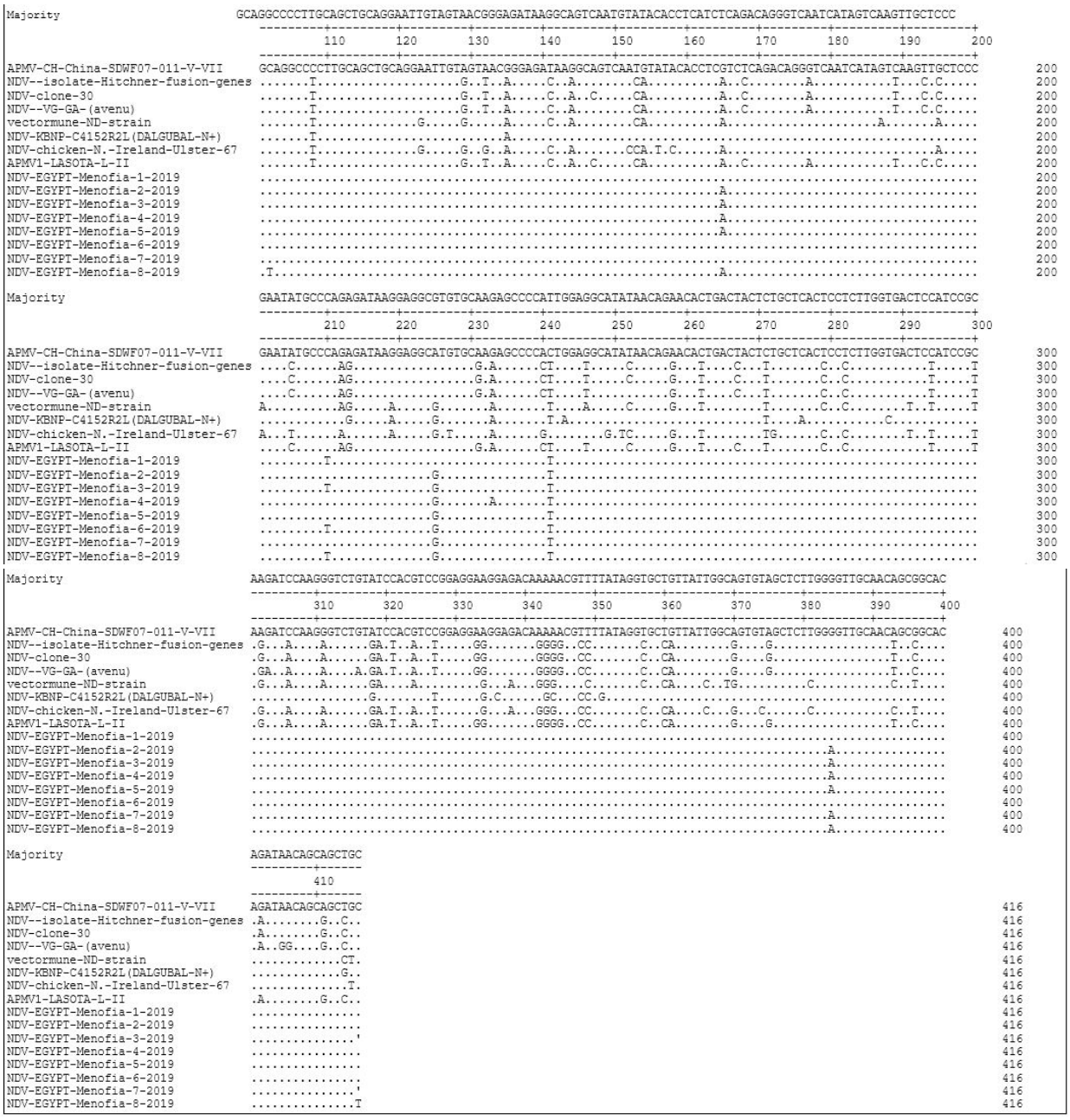
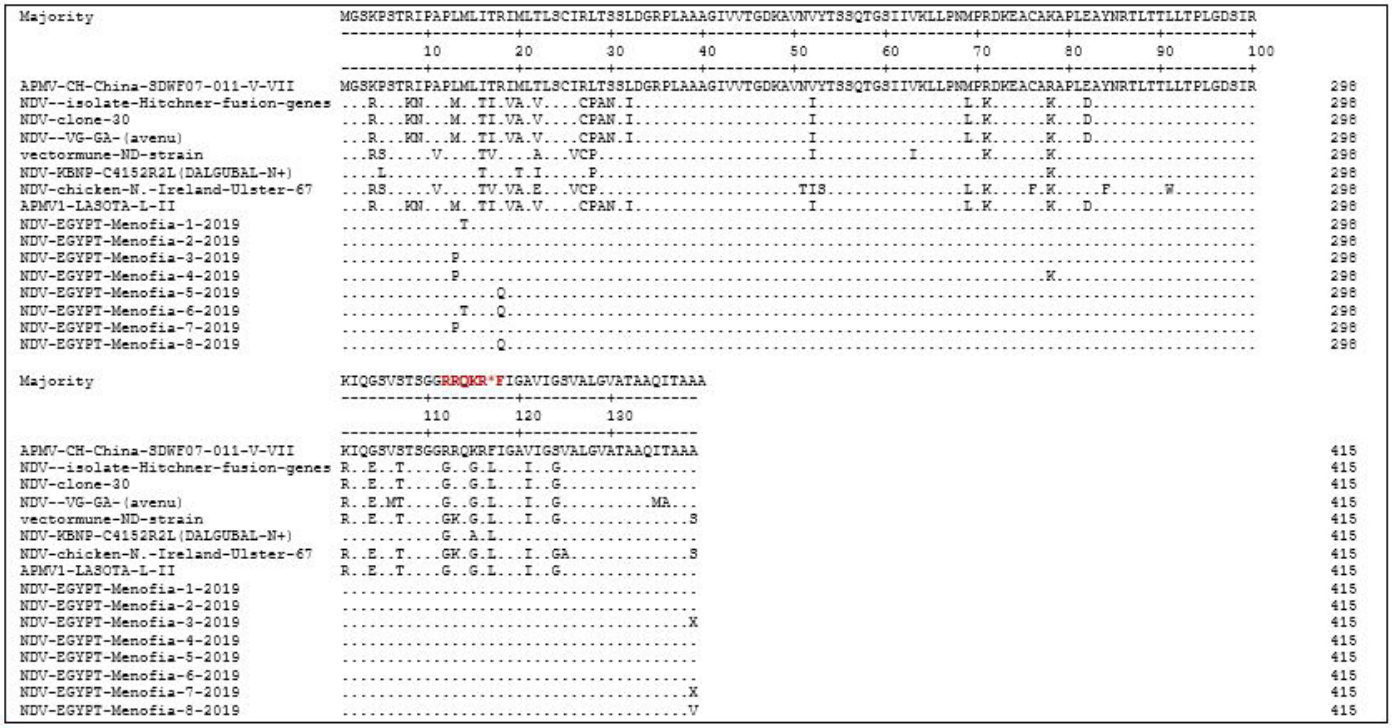


Figure 1: *Nucleotide Sequence alignment of NDV isolates for 416 nt of F-gene in comparison to Chinese VII strain APMV-CH-China-SDWF07-011.*



* F Protein Cleavage site indicated in bold & red coloured.

* Amino acid abbreviations A: Alanine, C: Cysteine, D: Aspartic acid, E: Glutamic acid F: Pheny-lalanine, G: Glycine, H: Histidine, I: Isoleucine, K: Lysine, L: Leucine, M: Methionine, N: Asparagine, P: Proline, Q: Glutamine, R: Arginine, S: Serine, T: Threonine, V: Valine, W: Tryptophan, Y: tyrosine.

Figure 2: *Amino Acid Sequence alignment of NDV isolates (139 AA) from AA No. 1 till AA No. 139 from F- protein in comparison to Chinese VII strain APMV-CH-China-SDWF07-011.*

Phylogenetic analysis results revealed that eight isolates were belong to class II genotype VII, the sequences of the eight isolates were investigated based on: the criteria for identification of NDV genotypes proposed by (Diel *et al.*, 2012; Saad *et al.*, 2017; Selim *et al.*, 2018; Mahmoud *et al.*, 2019). The genetic characterization of virulent viruses circulating in west and central Africa investigated by (Snoeck *et al.*, 2013).

Newcastle disease (ND) is one of the most respiratory viral serious diseases affecting poultry as well as highly pathogenic avian influenza, infectious bronchitis and especially in broiler production and infectious Laryngeotrachitis in layers (Alexander *et al.*, 2003). Egypt is endemic for Newcastle disease virus (NDV) with continuous evoluting outbreaks causing great economic losses in broiler chicken industry due to high mortality which may reach 100% in velogenic strains of NDV, despite the intensive vaccination programs (Mohamed *et al.*, 2009; Radwan *et al.*, 2013), therefore there is a need for updating vaccine strategies (Palya *et al.*, 2012; Ehud *et al.*, 2018; Ya-wen *et al.*, 2019).

The eight NDVs isolates of velogenic genotype VII and contain the unique cleavage site motif 112RRQKRF117 with high relation to very virulent NDV Chinese strain Chicken/China/SDWF07/2011 strain with nucleotide identity percentage (99.3%-100%) (Figures 1, 2 and 3).

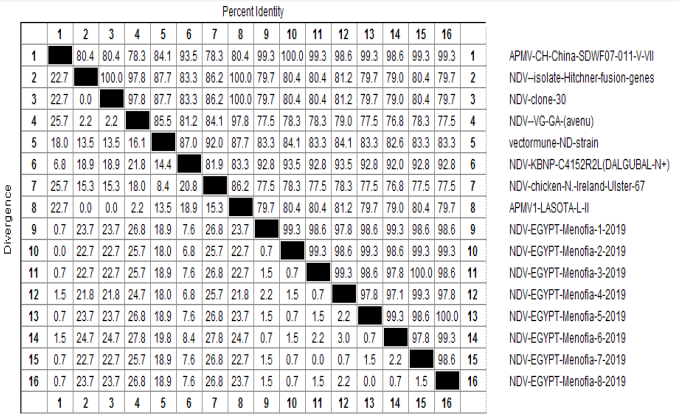


Figure 3: *Nucleotide identity and divergence of NDV isolates in the study and reference VII Chinese strain and Lasota strain.*

Results for deduced amino acid phylogeny (Figures 2 and 4) express that the analyzed sequences grouped within genotype VII, which are one of the predominant virulent viruses circulating globally (Diel *et al.*, 2012).

In this study, Eight Egyptian NDV isolates belonged to genotype VII by phylogenetic analysis of partial F protein amino acid sequence including cleavage site

sequence and according to classification system of NDV proposed by (Diel *et al*., 2012). Also, VII was previously described as the predominant genotype VII circulating with severe outbreaks in Egypt (Radwan *et al*., 2013; Abdel-Glil *et al*., 2014; Saad *et al*., 2017; Selim *et al*., 2018; Zanaty *et al*., 2019).

In conclusion, the main causative agent of recent ND outbreaks in vaccinated broiler flocks under field conditions have been reported in Menofia governorate, Egypt was found to belong to very virulent genotype VII. This strain was genetically identical to Egyptian genotype VII isolates isolated in the last decade, this indicating the limited efficacy of the current vaccines and the need for the vaccine strategies update.

Figure 4: *Phylogeny of 139 F protein amino acids NDV isolates. Blue colour isolates of the study; Red colour live vaccine strains; Black colour genbank reference strains of different genotypes.*

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Author's Contribution

All authors contribute equally in preparation of manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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