Research Article

Epidemiological, Phylogenetic Analysis and Pathogenicity of Newcastle Disease Virus Circulating in Poultry Farms, Egypt during 2015-2018

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Abstract | Newcastle disease virus (NDV) is a highly contagious disease in poultry, also considered as a major threat for commercial and traditional poultry industry in Egypt, even in vaccinated flocks. A total of 8121 different samples (organs and swabs) that represented 588 NDV suspected flocks were collected from poultry farms showing respiratory manifestation and/or drop in egg production during 2015-2018. Out of 588 NDV outbreaks 95 flocks were confirmed positive NDV by rRT-PCR using genotyping primers, with a prevalence rate of 16.2%. NDV outbreaks were recorded in 16 governorates, from total of18 investigated governorates and the recorded geo-prevalence of 89 %. Twenty-five samples were selected for further sequencing for the partial fusion protein. Phylogenetic analysis revealed that 20 samples are genotyped as very virulent NDVclass II of genotype VIIb, 4 samples were of high identity (94%-100%) with NDV class II of genotype II (vaccine strain) and 1 sample was phylogenetically related to NDV class II of genotype I with 98% identity. Furthermore, the intracerebral pathogenicity index (ICPI) for selected 5 virulent viruses reveals velogenic features with high pathogenicity index (1.60 to 1.74).

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Introduction

Newcastle disease is a contagious poultry disease affecting many domestic and wild avian species; it is transmissible to humans (Nelson et al., 1952). The significance of ND is reflected in its status as a notifiable disease (OIE, 2016). NDV is a member of the genus *Avulavirus,* family *Paramyxoviridae* within the order *Mononegavirales* which is a single strand non-segmented negative- sense RNA virus is able to infect a wide range of avian species and can be particularly pathogenic in chickens (ICTV, 2015). The epizootics of Newcastle disease in poultry continue to appear in Asia, Africa, Central, and South America while in Europe, sporadic epizootics occur (Naveen et al., 2013). According to mutations in NDV genome, the rate of mortality and morbidity in a flock is changeable (Haque et al., 2010).

The molecular basis for the variation in pathogenicity has been attributed to amino acids at the cleavage site on the fusion protein (Hung et al., 2004). The

precursor fusion protein, F0, must be cleaved by host cell proteases for infection to start via cell fusion and the activation of hemolytic properties; the proteases recognize the specific amino acid motif at the F protein cleavage site (Nagai and Klenk, 1977; Leighton and Heckert, 2007). Most virulent strains display ^{112}(R/K) RQ (R/K) R*F^{117} at the cleavage site, in contrast to ^{112}(G/E) (K/R) Q (G/E) R*L^{117} in a virulent lentogenic viruses (Collins et al., 1993; OIE, 2012). Viruses with a virulent cleavage site can be cleaved by proteases allowing for a systemic infection and extensive viral replication; however, if the cleavage site does not contain that specific amino acid motif, cleavage can only be mediated by trypsin and trypsin-like enzymes found in the respiratory and intestinal tracts which leads to restricted host site replication (Nagai et al., 1976; Toyoda et al., 1987). However, NDV strains with identical cleavage site sequences have shown significant differences in their virulence (Diel et al., 2012). Genomic diversity of NDV increases the possibility of diagnostic failures, resulting in unidentified infections and vaccination failure (Miller et al., 2010).

Isolates of NDV are designated into five pathotypes, named: a- apathogenic b- Lentogenic or avirulent forms; c- Mesogenic isolates: of moderate virulence but not fatal; d- Velogenic viscerotropic isolates of high virulence causing fatal enteric disease. e- Velogenic neurotropic isolates of high virulence causing high mortalities and nervous signs (Hanson and Brandly, 1955). Genotypically, APMV-1 is categorized into two classes entitled class I and class II. The majority of APMV-1 class II strains, including both virulent and nonpathogenic strains, which includes viruses of economic importance to chickens, while class I isolates mainly circulate in wild waterfowl (Aldous et al., 2003). The class II genotypes I and II include mainly avirulent vaccine strains of NDV (Miller et al., 2010), while virulent strains belong to class II genotypes III to IX and XI to XVI (Courtney et al., 2013). Virulent viruses belong to genotypes V, VI, and VII are highly mobile, spread worldwide and are responsible for the majority of recent outbreaks in poultry and wild birds worldwide (Dimitrov et al., 2016).

In Egypt, velogenic and avelogenic strains of NDV have been frequently identified and clustered as class II genotype II, VI and VII since the first isolation in 1947 (Daubney and Mansy, 1948; Hussein et al., 2005; Mohamed et al., 2009; Radwan et al., 2013). However, class II genotype VII is suggested to be predominant NDV isolates circulating among chickens and associated with outbreaks in commercial poultry farms and backyard chickens in Egypt and phylogenetically related to the NDV strains isolated previously in China (Mohamed et al., 2009; Hussein et al., 2014; Saad et al., 2017).

In the current work, we aimed to determine the circulating NDV genotypes that causing severe outbreaks in poultry farms and to evaluate the pathogenicity of the isolated viruses in SPF chicks.

Materials and Methods

Sample collection

A total of 8121 different samples; organs (Brain, trachea, lungs, proventriculus, small intestine, and pancreas) and tracheal swabs (10 for each sample) that represented 588 suspected flocks including different Egyptian governorates showed respiratory, nervous manifestation and diarrhea, sudden onset high mortality and/or drop in egg production (Table 1). These samples were coded and transported immediately on ice to the reference laboratory for quality control on poultry production (RLQP) to be processed immediately for rRT-PCR.

Detection of NDV by rRT-PCR

Viral RNA of 588 sample were extracted from the infected tracheal swabs and organs using QiAmp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, rRT-PCR was carried out using Quantitect probe RT-PCR kit (Qiagen, Inc. Valencia CA). Primers used were described by Wise et al. (2004). rRT-PCR was conducted in the Stratagene 3005P MXpro Real-Time PCR System (Stratagene, USA) according to manufacturer instructions.

Virus isolation

The clarified supernatant from the swab pools (0. 1 ml/egg) was inoculated into the allantoic cavity of 10 day-old embryonated specific pathogen free (SPF) eggs (SPF farm, KoamOsheim, El-Fayoum, Egypt). The inoculated eggs were incubated at 37°C and candled 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).

Table 1: *Epidemiological, genetic and pathogenicity data of the isolated viruses.*

	Sample code	Governorates	Production	Collection date	F-protein cleavage site	ICPI	Geno-type	Accession number
1	NDV-EGYPT/1516F-2015	Al Fayoum	Broiler	4-2015	^{112}RRQKRF^{117}		VII	MK604197
2	NDV-EGYPT/151309F-2015	Ismailia	Layer	8-2015	^{112}RRQKRF^{117}	1.73	VII	MK604198
3	NDV-EGYPT/15492F-2015	Al Monofiya	Broiler	9-2015	^{112}RRQKRF^{117}		VII	MK604199
4	NDV-EGYPT/15986F-2015	Al Behera	Broiler	4-2015	^{112}RRQKRF^{117}		VII	MK604200
5	NDV-EGYPT/15158F-2015	Al Daqahlia	Layer	6-2015	^{112}RRQKRF^{117}		VII	MK604201
6	NDV-EGYPT/16280F-2016	Al Daqahlia	Broiler	3-2016	^{112}RRQKRF^{117}		VII	MK604202
7	NDV-EGYPT/16261F-2016	Al Fayoum	Breeder	8-2016	^{112}RRQKRF^{117}		VII	MK604203
8	NDV-EGYPT/16553F-2016	Al Gharbia	Breeder	6-2016	^{112}RRQKRF^{117}	1.70	VII	MK604204
9	NDV-EGYPT/1610CV-2016	Al Giza	Broiler	8-2016	^{112}GRRGRL^{117}		II	MK604205
10	NDV-EGYPT/161158F-2016	Al Fayoum	Broiler	9-2016	^{112}RRQKRF^{117}		VII	MK604206
11	NDV-EGYPT/17295F-2017	Al Monofiya	Broiler	5-2017	^{112}RRQKRF^{117}		VII	MK604207
12	NDV-EGYPT/17273F-2017	Cairo	Breeder	10-2017	^{112}RRQKRF^{117}	1.60	VII	MK604208
13	NDV-EGYPT/171014F-14-2017	Al Qaliobia	Broiler	11-2017	^{112}RRQKRF^{117}		VII	MK604209
14	NDV-EGYPT/171014F-15-2017	Al Sharqiya	Breeder	2-2017	^{112}RRQKRF^{117}		VII	MK604210
15	NDV-EGYPT/171014F-16-2017	Alexandria	Broiler	4-2017	^{112}RRQKRF^{117}	1.69	VII	MK604211
16	NDV-EGYPT/17140F/S-2017	Beni-Sueif	Broiler	1-2017	^{112}GRRGRL^{117}		II	MK604212
17	NDV-EGYPT/17151F/S-2017	Al Fayoum	Broiler	5-2017	^{112}GRRGRL^{117}		II	MK604213
18	NDV-EGYPT/18156F/S-2018	Ismailia	Broiler	8-2018	^{112}GRRGRL^{117}		II	MK604214
19	NDV-EGYPT/18246F/S-2018	Kafer Al-sheikh	Layer	5-2018	^{112}GKRGRL^{117}		I	MK604215
20	NDV-EGYPT/181210F-2018	Al Fayioum	Broiler	6-2018	^{112}RRQKRF^{117}		VII	MK604216
21	NDV-EGYPT/18734F-2018	North Sinai	Broiler	11-2018	^{112}RRQKRF^{117}		VII	MK604217
22	NDV-EGYPT/18403F-2018	Port Said	Broiler	1-2018	^{112}KRQKRF^{117}	1.74	VII	MK604218
23	NDV-EGYPT/18629F-2018	Al Daqahlia	Broiler	4-2018	^{112}RRQKRF^{117}		VII	MK604219
24	NDV-EGYPT/18238F-2018	Al Behera	Broiler	12-2018	^{112}RRQKRF^{117}		VII	MK604220
25	NDV-EGYPT/1890FI-2018	Al Daqahlia	Broiler	3-2018	^{112}RRQKRF^{117}		VII	MK604221

Intracerebral pathogenicity index (ICPI)

The egg infective dose 50 (EID_{50}) was calculated, according to Reed and Muench, (1938). ICPI for five selected isolates were determined using standard procedures. Briefly, 1-day-old chicks were inoculated intracerebrally with 0.1 ml of a 1:10 dilution of infective allantoic fluid. Chicks were monitored during 8 day observation period and scored as normal (0), sick or paralyze (1), and dead (2). Total scores were recorded, and the mean daily score was estimated to obtain the ICPI. The ICPI values 0.7 or greater are identified as virulent according to OIE instructions (OIE, 2016).

The sequence of the fusion gene and phylogenetic analysis

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 and relationships of the partial (25 sequences) obtained in this study were compared with previously published NDV vaccine and references strains available in the public database (BLAST, NCBI, USA). Amino acids phylogenetic relationship was constructed using MEGA version 6 (Tamura et al., 2013). A comparative analysis of 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).

Results and Discussion

NDV incidence and geo-prevalence

Out of 588 NDV outbreaks, 95 were confirmed in different chicken farms, with a prevalence rate of 16.2% (Table 2). The NDV outbreaks were recorded in following 16 governorates (Cairo, Kafr El Sheikh, Alexandria, Al Giza, Al Monofiya, Al Behera, Al Fayioum, Ismaliya, BeniSueif, Al Daqahlia, Al Gharbia, Al Sharqiya, Port Said, Matrouh, Al Minia and North Sinai) from total 18 investigated governorates and the recorded geo-prevalence of 89% (Figure 2).

Sequence, ICPI and phylogenetic analysis of partial F gene

Genetic characterization of 400 bp amplified PCR products of the fusion gene including the cleavage site were obtained from the 25 selected positive samples, then the phylogenetic and sequence analysis was conducted. A phylogenetic tree was constructed from the nucleotide sequences of the F gene (Figure 1). Results indicated that Egyptian NDV viruses in this study were divided into three distinct genotypes (VII, II and I). Twenty isolates out of 25 obtained in this study, were found to be closely related to the very virulent genotype VIIb and 4 isolates, were genetically related to the genotype II of NDV virus with high identity (94 to 100%) with LaSota vaccine strain (Table 1). NDV-EGYPT/18246F/S-2018 was characterized as lentogenic NDV genotype I sharing high identity (98%) with Vectormune ND strain (D26 strain). The ICPI for the five tested isolates of genotype VII was between 1.60 and 1.74 which indicated their velogenic nature (Table 1).

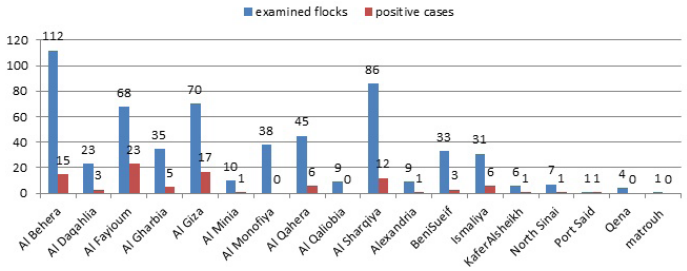

Figure 1: *Positive cases of NDV examined from different governorates during 2015-2018 in Egypt.*

The isolates of very virulent genotype VII contain the unique cleavage site motif ^{112}RRQKRF^{117} which is indicative of velogenic character with high relation to Chicken /China/SDWF07/2011 strain with identity (96%-100%). Only sample NDV-EGYPT/18403F-2018 show ^{112}KRQKRF^{117} instead (Table 1).

Table 2: *Positivity rate of NDV examined from different governorates during 2015-2018 in Egypt.*

Governorates	Number of examined flocks	No. of positive cases	Percent of positive sample
Al Behera	112	15	13.4%
Al Daqahlia	23	3	13.0%
Al Fayioum	68	23	33.8%
Al Gharbia	35	5	14.3%
Al Giza	70	17	24.3%
Al Minia	10	1	10.0%
Al Monofiya	38	0	0.0%
Cairo	45	6	13.3%
Al Qaliobia	9	0	0.0%
Al Sharqia	86	12	14.0%
Alexandria	9	1	11.1%
Beni-Sueif	33	3	9.1%
Ismaliya	31	6	19.4%
Kafer Al-sheikh	6	1	16.7%
North Sinai	7	1	14.3%
Port Said	1	1	100.0%
Qena	4	0	0.0%
Matrouh	1	0	0.0%
Total	588	95	16.2%

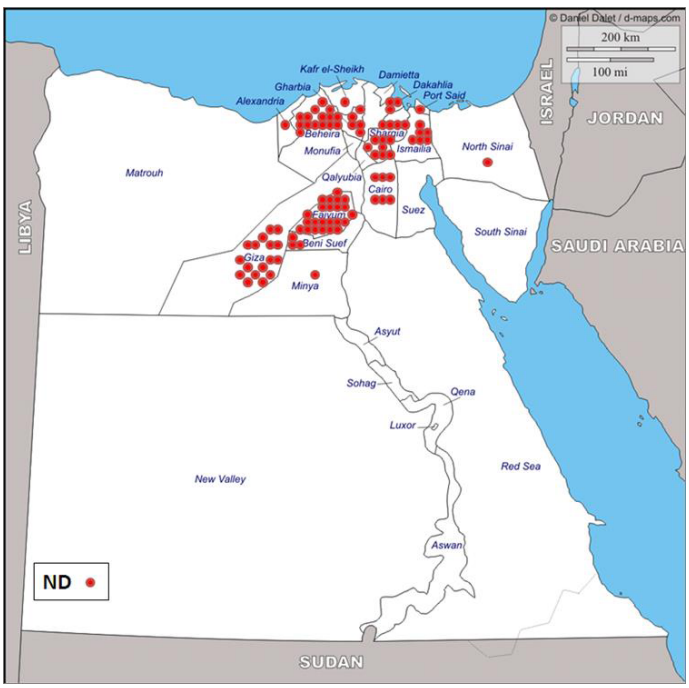

Figure 2: *Geo-prevalence of NDV during 2015-2018 in Egypt.*

In relation to vaccinal NDV strains (Lasota, Hitchner, clone 30 and Avinew) used in Egypt which genetically genotyped as lentogenic strains of NDVs (class II

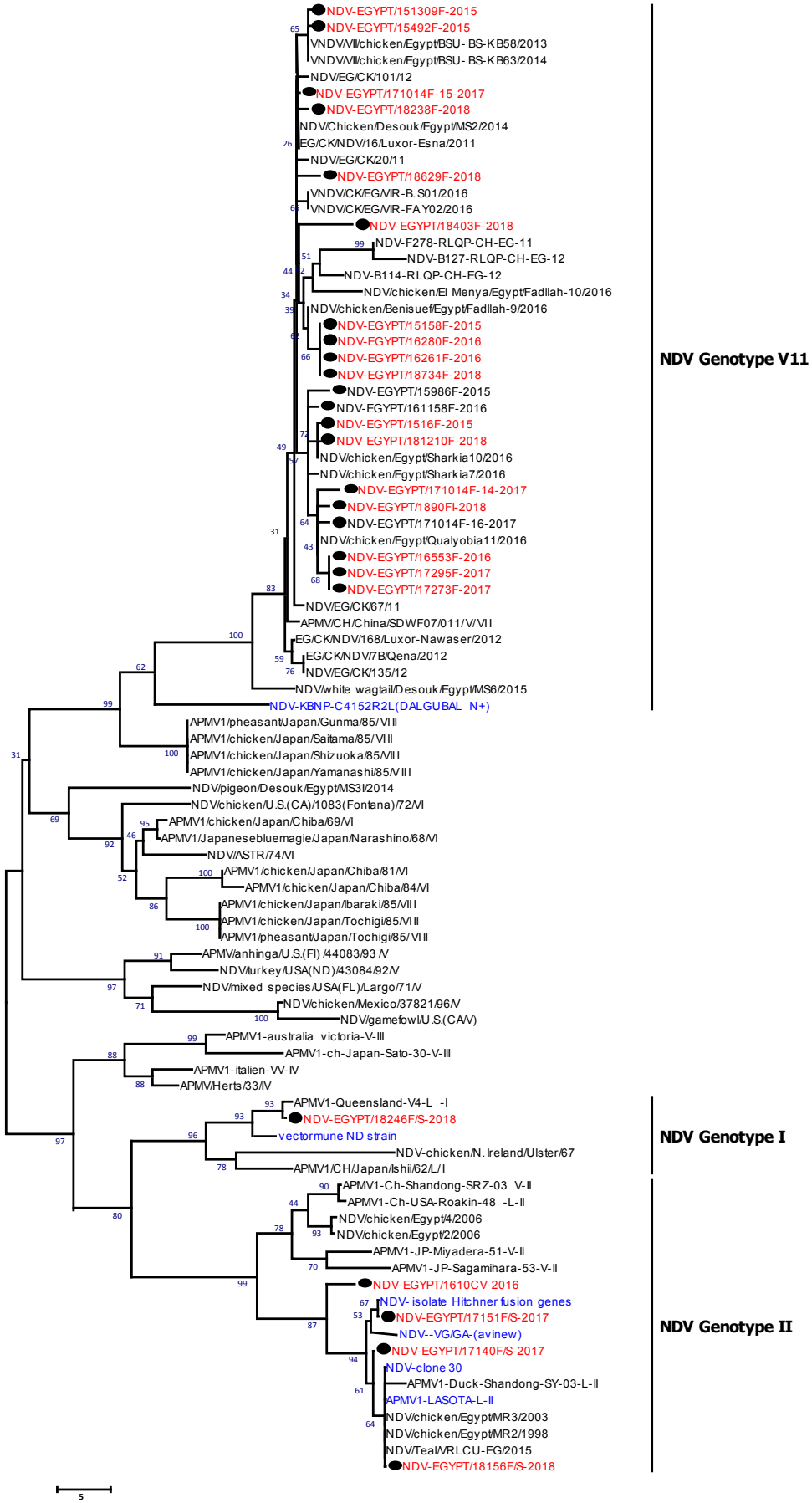

Figure 3: *Phylogenetic tree of nucleotide sequences of the partial fusion gene fragment of Egyptian isolates (marked with a solid black circle) and the references strains from GenBank using neighbor-joining methods of Mega6 software.*

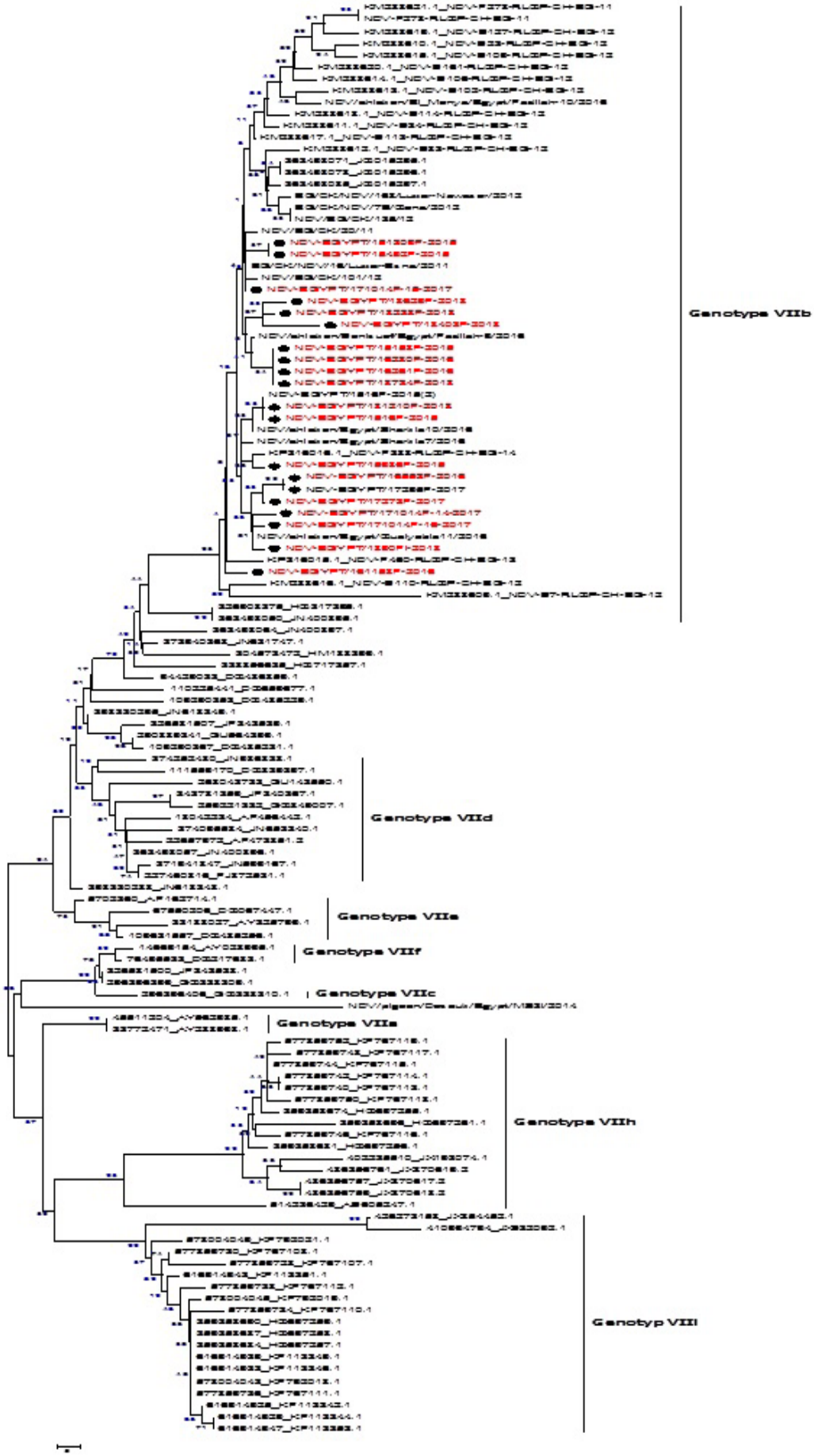

Figure 4: *Detailed phylogenetic tree of NDV genotype VII using partial nucleotide sequences of the NDV-F gene. Sequences obtained in this study are marked with solid black circles. The phylogenetic tree was computed in the MEGA 6 software using neighbor-joining method with 1000 bootstrap replicates using Kimura-2 parameter and nucleotide substitution model.*

genotype II), the strains obtained in this study (class II Genotype VIIb) show distant degree of amino acids identity ranged from 77% to 81%. While for the Vectormune ND vaccine (D26 strain) (class II genotype I) the amino acid identity with genotype VIIb ranged from 82%-84%, according to the partial fusion amino acid sequences. NDV genotype VII vaccine named; NDV-KBNP-C4152R2L show 90% to 93% amino acids homology with the strains obtained in the current study (class II Genotype VIIb).

Newcastle disease (ND) is one of the most divesting diseases affecting poultry especially in extensive production (Alexander et al., 2003). Egypt is endemic for Newcastle disease virus (NDV) with continuous long-lasting outbreaks causing significant economic losses in the poultry industrydue to high mortality which may reach 100% in very virulent strains of NDV, despite the intensive vaccination programs (Mohamed et al., 2009; Radwan et al., 2013).

ICPI is a rapid and reliable method for NDV pathotyping in comparison to the MDT and the IVPI tests (De Battisti et al., 2013; Ganar et al., 2014). NDVs with an ICPI ≥ 0.7, multiple basic amino acids within the cleavage site of the fusion protein (OIE, 2012) are designated as velogenic viruses. For that, 20 out of the 25 samples tested in the present work were velogenic viruses. The ICPI for the five tested isolates was between 1.60 and 1.74. Furthermore, the presence of polybasic amino acid motifs ^{112}RRQKRF^{117} was recorded in all twenty isolates except NDV-EGYPT/18403F-2018 (^{112}RKQKRF^{117}) that show the highest ICPI (1.74). This two cleavage site pattern has previously been reported in European and Chinese genotype VII isolates and previously identified as highly virulent NDV (Liu et al., 2003; Abolnik et al., 2004).

The results for the phylogenetic analysis (Figure 3, 4) clearly showed that the sequences analyzed grouped with genotype VII, which are one of the predominant virulent viruses circulating globally (Diel et al., 2012). That was firstly categorized into two subgenotype: VIIa, which represents viruses that emerged in the 1990s in the Far East and spread to Europe and Asia; and VIIb, which represents viruses that emerged in the Far East and extended to South Africa. Later on, VII further divided into 10 sub-genotypes (e.g. VIIa, VIIb, VIId, VIIe, VIIf, VIIg, VIIh, VIIi, VIIj and VIIk), that have been identified in several, countries including China, Ethiopia, Indonesia, Israel, Japan, Malaysia, Mozambique, Pakistan, Taiwan, Vietnam and Namibia (Fentie et al., 2014; Miller et al., 2015; Mapaco et al., 2016; Molini et al., 2017; Xue et al., 2017). VIIb along with VIId are the predominant genotypes and still continue to spread in the world (Miller et al., 2015).

In the context of phylogenetic study and sequence analysis, comparison of the partial fusion gene sequence of the Egyptian viruses with different NDV reference strains and available vaccines in Egyptian market revealed that all isolates of genotype VII have nucleotide similarity reached up to 98% with each other and with Chicken/China/SDWF07/2011. Furthermore, all isolates of genotype VII show a distant degree of amino acids identity with NDV vaccine strains used in Egypt (Genotype I and II). This finding supported by the Selim et al. (2018).

To determine whether the NDVs from Egypt belonged to a new subgenotype, a detailed phylogenetic analysis of genotype VII revealed that all the virulent viruses belonged to genotype VIIb, according to the unified nomenclature and classification system of NDV proposed by Diel et al. (2012). Also, VIIb was previously described as the predominant sub-genotype of genotype VII circulating with severe outbreaks in Egypt (Saad et al., 2017). In conclusion, the causative agent of recent ND outbreaks in vaccinated broiler flocks from Egypt was found to belong to velogenic genotype VIIb. This strain was genetically close to other Egyptian genotype VII isolates obtained during the last decade with high pathotypic features.

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Author's Contribution

All authors contribute equally in preparation of manuscript.

Ethical Declaration

Animals used in experiment were approval from

appropriate Ethical committee in accordance with "Principles of Laboratory Animal Care".

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