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Molecular Characterization of the Newcastle Disease Virus detected in some provinces of The West Delta in Egypt

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ABSTRACT

In the last couples of years, Egyptian poultry farms have faced many outbreak mortalities. Newcastle disease virus (NDV) was suggested as one leading cause of these mortalities. In the current study, we characterized the NDV from outbreaks affecting poultry farms in Egypt between 2012 and 2014. Different organ tissues were collected from twenty different chicken farms showing clinical signs of NDV infection in El-Behira and Kafr-Elshiekh provinces. Inoculation of the processed samples in 10-day-old embryonated chicken specific-pathogen-free eggs indicated presence of haemagglutinating agent in 12 samples. These haemagglutinating agents were confirmed as NDV by real-time reverse transcription polymerase chain reaction (rRT-PCR). In the employed rRT-PCR assay, two sets of primers and probes were used. A first primer- probe set was designed to detect sequences from a conserved region of the Matrix gene that recognizes a diverse set of virus isolates. A second primer-probe set was targeted to comprise the cleavage site of the F gene and to detect potentially virulent NDV isolates. The rRT-PCR results revealed that seven detected ND viruses were of velogenic type, whereas the other five detected ND viruses were of lentogenic nature. Sequencing of two suspected ND velogenic viruses was carried out. The phylogenetic analysis based on the partial sequence of F protein gene showed that the detected viruses were closely related to viruses from genotype VII subgroup D. Furthermore, the nucleotide and amino acid analysis approved the previous finding. Overall, the study confirmed circulating of NDV genotype VII among Egyptian poultry farms.

Keywords: NDV; genotype VII; Real time RT-PCR; Phylogenetic analysis

INTRODUCTION

Newcastle disease (ND) is one of the most devastating disease that generally affects majority of poultry species, causing a high morbidity and death up to 100% (Alexander *et al.*, 1992). ND virus (NDV), the causative agent of ND, is a member of the genus Avulavirus (Avian Avulavirus 1) of the family Paramyxoviridae, within the order Mononegavirales, (ICTV, 2015). It is an enveloped virus that contains a linear, non-segmented, single stranded negative sense RNA genome of nearly 15.2 kb that encodes at least six structural proteins; the hemagglutinin-neuraminidase protein, the fusion protein (F), the matrix protein, the nucleoprotein, the phosphoprotein and the RNA polymerase, (Lamb and Kolakofsky, 1996)

The F protein of NDV is of a major importance in virus virulence as it mediates virus-cell membrane fusion (Aldous *et al.,* 2003; de Leeuw *et al.,* 2005, Panda *et al.,* 2004). The cleavage of F glycoprotein precursor (F0) by host cell proteases into two di-sulfide linked subunits, F1 and F2 is essential to make the protein functional (Aldous *et al.,* 2003; de Leeuw *et al.,* 2005, Panda *et al.,* 2004) Therefore the cleavage site sequence of F protein denotes the key molecular element of NDV virulence (Glickman *et al.,* 1988). The cleavage site sequence differs in different NDV strains as it is 112R/K-R-Q/K/R-K/R-R116, with a phenylalanine (F) at residue 117 in highly virulent strains, while in the low virulent strains, the sequence is 112G/E-K/R-Q-G/E-R116, with a leucine (L) residue at position 117 (Aldous *et al.,* 2003; de Leeuw *et al.,* 2005, Panda *et al.,* 2004). Consequently, the cleavage site and full length of F protein can be used as a useful tool for the identification of NDV virulence and phylogenetic virus classification (Diel *et al.,* 2012; Meng *et al.,* 2012). Two schemes have been used to categorize NDV isolates based on F protein sequences. The first one classified NDV isolates into 7 lineages and 20 sub-lineages (Aldous *et al.,* 2003; Snoeck *et al.,* 2009; Cattoli et al 2010). The second one classifies NDV isolates into two classes. Class I includes avirulent strains that affect mainly wild birds and they are categorized into 9 genotypes. Class II includes both virulent and avirulent stains that affect wild and domestic birds and they are categorized into 11 genotypes (Czegledi *et al.,* 2006; Miller *et al.,* 2010; Kim *et al.,* 2007a; Kim *et al.,* 2007b). Some scientists suggested that class I has only a single genotype and class II has 18 genotypes (Diel *et al.,* 2012; Courtney *et al.,* 2013; Snoeck *et al.,* 2013). The class II genotypes one and two include avirulent strains and NDV vaccine strains (Miller *et al.,* 2010) whereas virulent strains belong to class II genotypes 3 to 9 and 11 to 16 (Courtney *et al.,* 2013). Genotypes 5, 6 and 7 are the most common circulating genotypes worldwide and are accountable for the majority of latest epidemics in poultry and wild birds (Dimitrov *et al.,* 2016).

In Egypt, NDV class II genotype 2 (Mohamed *et al.,* 2009; Mohamed *et al.,* 2011; Radwan *et al.,* 2013) and 6 (Hussein *et al.,* 2005) have been frequently identified and currently 7D (AboElkhair *et al.,* 2012; Radwan *et al.,* 2013; Hussein *et al.,* 2014). Genotype 7 is claimed to be the reason for the latest NDV outbreaks in Egypt (Hussein *et al.,* 2014; Radwan *et al.,* 2013).

Mutational changes of the NDV genome have been associated with the failure of new genetic variants of the virus and impairment of disease control (Cattoli *et al.,* 2010; Diel *et al.,* 2012; Khan *et al.,* 2010). Thus, the continuous epidemiologic and molecular surveillance for NDV is so important. The obtained data will provide significant insights on the possible origins and genetic characterization of these viruses which may help in articulating more effective ND prevention and control plans. Therefore in the present study, field strains of NDVs with different geographical locations isolated from commercial chicken flocks were analysed. Sequence data of F gene were compared with NDV strains from different parts of the world during different time periods.

MATERIALS AND METHODS:

Samples collection and virus propagation

Twenty pools of tissue specimens (proventriculus, lung, trachea, kidneys, cecal tonsils, spleen, brain and liver) were collected from twenty broiler flocks raised in Kafr-Elsheikh and El-Behera governorates, Delta Nile River, Egypt during the period between April 2012 and February 2014 (Table 1). The flocks aged between 15 and 46 days and had a history of vaccination against ND, however, they exhibited nervous and respiratory signs, diarrhoea and increased mortality (10-15%). The tissues were aseptically collected from dead and moribund birds, transported on ice and maintained at -80 °C till processed. All processed samples and applied test were carried out in National Laboratory of veterinary quality control on poultry production (NLQP) Animal Health Research Institute (AHRI, Dokki, Giza, Egypt).

Isolation of virus was carried out using the method described by (OIE, 2012). Each tissue pool was homogenized as 1:10 (w/v) suspension in phosphate buffer saline pH 7.4 containing 10 mg/mL streptomycin, 10000 IU/mL penicillin, 250 µg gentamycin sulphate/ml and 5000 IU nystatin. Following centrifugation at 2,000 rpm for 10 min, a volume of 0.2 ml from each clarified supernatant was injected into the allantoic cavities of two 9-day-old embryonated chicken eggs (ECE). The eggs were maintained at 37°C up to 5 days with daily observation for embryo viability. Every tissue pool was passaged twice in ECE. Following egg chilling on day 5, the allantoic fluids (AF) was harvested and tested for HA activity as described before (OIE, 2012). Briefly, two-fold dilutions of AF in PBS were mixed with an equal volume of a 1 % (v/v) red blood cells in a V bottomed 96-well micro-titer plate. All HA-positive samples were further assayed by real-time RT-PCR in order to exclude other hemagglutinating pathogens and determine identity of suspected NDV strains.

Real time reverse transcription polymerase chain reaction (rRT-PCR):

RNA extraction and rRT-PCR was carried out according to (Wise *et al.,* 2004, Saad *et al* 2017).

Sequence and phylogenetic analysis:

Partial fragments of F gene of two suspected virulent isolates were amplified by RT-PCR according to (Mase *et al.,* 2002). The PCR products were sequenced as previously described in (Saad et al 2017). The obtained sequences were assembled and analysed using BioEdit® software version 7.1.3.0 (Hall, 1999). The identification and homology of the gotten nucleotide sequences were determined using BLAST http://www.ncbi.nlm.nih.gov. The obtained sequences were compared with some NDV reference strains that represent the different NDV genotypes and subgenotypes. Phylogenetic analysis was performed using MEGA version 4 (Tamura *et al.,* 2007). The phylogenetic tree was constructed by the neighbour joining method with the maximum composite likelihood substitution model at 1000 bootstrap replicates.

RESULTS:

Virus isolation and identification:

Following inoculation in SPF eggs an HA assay, twelve samples showed haemagglutinating activity. (Table 2) represents the obtained results after the 2 passage in SPF eggs. Some samples caused death of embryos of ECE within 24 - 96 hrs post-inoculation. The dead embryos showed congestion and haemorrhage in the whole bodies. Subcutaneous tissues of the heads of the embryos were filled with blood. The blood vessels over the bodies were prominent.

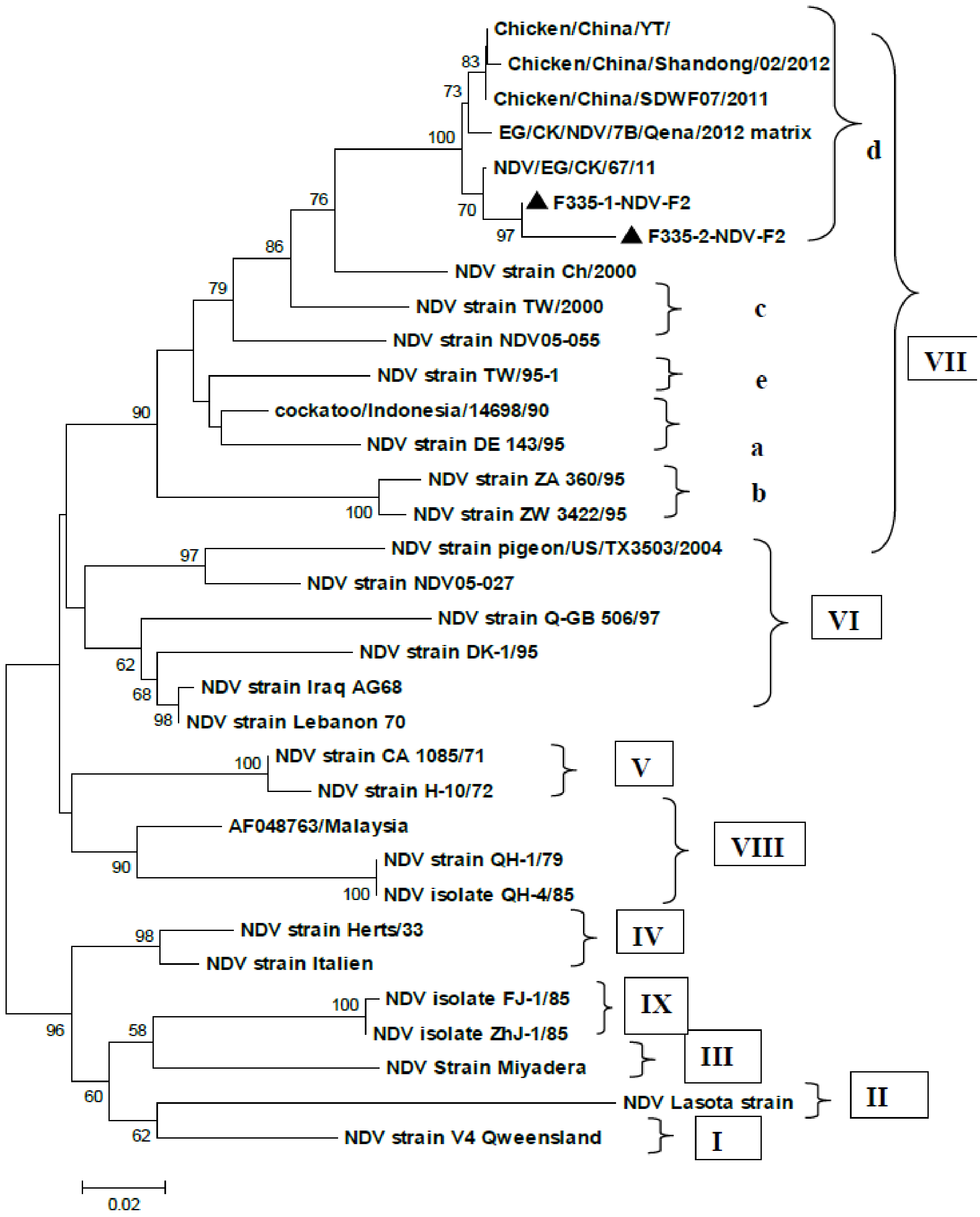
Real time RT-PCR:

All the twelve suspected NDV isolates were positive for M gene. For F gene, seven samples were positive. Ct values of some samples were detected from 26 cycles. This confirmed that all isolates belong to NDV.

Genetic and phylogenetic characterizations:

Analysis of partial deduced amino acid sequence (positions 11 to 101) of F-gene revealed that two isolates were closely related to VII NDV genotype subtype D (Tables 3 and 4). The phylogenetic analysis showed that the two ND viruses appeared to be closely related to viruses from genotype VII sub genotype D (Figure 1).

Figure (1) Phylogenetic analysis of detected NDVs in the current study



▲ F335-1-NDV-F2and F335-1-NDV-F2: Two identified viruses of the current study

DISCUSSION:

In Egypt, despite intensive vaccination programs with live and inactivated vaccines, NDV remains a constant threat to the commercial poultry (AboElkhair *et al.*, 2012; Radwan *et al.*, 2013; Hussein *et al.*, 2014; Saad *et al.*, 2017). Moreover, the occurrence of mixed infection of NDV and other poultry viral diseases, e.g., avian influenza and infectious bronchitis in the last couple of years resulted in major serious problems in the Egyptian poultry flocks especially broilers. Therefore, the investigation of the genetic diversity of NDV field strains in different locations in Egypt is crucial to comprehend the epidemiology of the NDV and determine the genetic relatedness among detected virus strains.

In this study, NDV was searched in some provinces of West Delta of River Nile in Egypt using different assays. Nucleotide sequence analysis of the F-gene cleavage site is used for determining NDV pathotypes (Gould *et al.,* 2001). According to OIE, the virulence of NDV can be established if at least three basic amino acids (arginine or lysine) are detected between 113 and 116 residues either directly or by deduction at F protein and phenylalanine at residue 117 which is the N-terminus of F1 protein (OIE, 2012). Therefore, F gene cleavage site was targeted in this study by rRT-PCR and nucleotide sequence analyses. In our study, the deduced amino acid analysis of the obtained sequences of the ND viruses combined with rRT-PCR results proved that seven obtained ND viruses were velogenic viruses while the other five obtained ND viruses were lentogenic viruses. Although virulence of NDV depends on multiple genes, the amino acid sequence at the cleavage site of F-protein has been assumed to be a principal molecular determinant of NDV virulence (Panda *et al.,* 2004). Viruses with a two basic motif are considered virulent because they can be activated by ubiquitous intracellular proteolytic enzymes, leading to systemic infections (Czegledi *et al.,* 2006). On the other hand, the avirulent NDVs that contain a monobasic amino acid motif at the F0 cleavage site, which is cleaved only by extracellular proteolytic enzymes, result in localized and/or asymptomatic infections (Czegledi *et al.,* 2006).

The phylogenetic analysis showed that two ND viruses detected in the current study appeared to be closely related to viruses from genotype VII sub genotype D (Figure 1). It was reported that genotype VII NDV has been associated with many of the most recent outbreaks in Asia, Africa and the Middle East (Khan *et al.,* 2010; Kim *et al.,* 2007a). One of the characteristics of genotype VII D NDV isolates that they can induce more severe damage to lymphoid tissues, especially to spleen when compared to virulent viruses of other genotypes isolates (Hu *et al.,* 2011). This could explain that the important role of NDV infections that could have in recent mortalities in Egyptian poultry flocks. Previous studies also reported detection of NDV genotype VII in Egyptian poultry flocks (AboElkhair *et al.,* 2012; Radwan *et al.,* 2013; Hussein *et al.,* 2014, Saad *et al.,* 2017). The isolation of NDV genotype VII was first reported in Egypt since 2012 (Radwan *et al.,* 2013). The previous dominant NDV strains in Egypt were identified to be class II, genotype II and genotype VI (Hussein *et al.,* 2005, Mohamed *et al.,* 2011). It was suggested that genotype VII could reach Egypt through two possible ways. The first possible way could be through trading of poultry and poultry products with Middle Eastern countries and China. The second possible way could be through the transfer of the virus through migratory birds (Radwan *et al.,* 2013).

The detection of non-virulent ND viruses might suggest that these viruses may be generated from vaccinal strains. Although only two viruses of the current study have been partially nucleotide sequenced, the identity of the two viruses as shown in (tables 3 and 4) might suggest that all detected velogenic viruses could be genotype VII.

In conclusion, the finding of this study confirms circulation of NDV in Egyptian poultry flocks and points out the importance of continuous surveillance for NDV in poultry flocks. In additions, the presence of multiple NDV strains in Far East and highly transmissible nature of the virus require high biosecurity measures to prevent the introduction of NDV to Egyptian poultry flocks.

Table (1) Data of the collected samples from different Egyptian broiler flocks during 2012-2014 for NDV detection

Sample ID	Type of Bird	Age of bird	Flock number	Mortalities	Province	Collection date	Signs & pm
1	Sassou	46d	1000	300	Elbehira	20/1/2014	Coughing & torticollis
2	Cobb	21d	10000	600/4days	Elbehira	23/1/2013	Torticollis and respiratory signs
3	Cobb	28d	10000	700/day	Elbehira	11/4/2013	Ruffled feathers, depression & greenish diarrhea
4	Cobb	26d	800	100/3days	Elbehira	7/4/2012	Ruffled feathers, depression &sneezing
5	Ross	38d	5000	100/day	Elbehira	18/4/2013	Ruffled feathers, depression & sneezing
6	Cobb	24d		300/day	Elbehira	2012	Coughing & torticollis
7	Cobb	15d	50	50	Elbehira	15/9/2013	Edema of the head and wattles, nervous and respiratory signs
8	Cobb	30d	900	200/3days	Elbehira	2/5/2013	Greenish diarrhea. petechial hemorrhage of proventriculus
9	Cobb	36d	3000	50	Elbehira	3/6/2013	Sneezing, coughing & torticollis
10	Cobb	15d	50	50	Elbehira	15/9/2013	Depression, nervous signs
11	Cobb	27d	13000	50/3days	Kafrelshikh	27/1/2014	Ruffled feathers, depression
12	Avian		3000	15/3days	Elbehira	8/7/2013	Diarrhea, depression
13	Cobb	29d	1000	13	Elbehira	8/2/2014	Ruffled feathers, depression
14	Cobb	22d	9000	10	Elbehira	17/10/2013	Greenish diarrhea &petechial hemorrhage of proventriculus
15	Cobb	27d	5000	30	Elbehira	10/11/2013	Greenish diarrhea, petechial hemorrhage of proventriculus
16	Cobb	29d		4/day	Elbehira	8/3/2013	Ruffled feathers, depression & Greenish diarrhea
17	Cobb	33d	4500	480	Elbehira	6/11/2012	Diarrhea, depression
18	Cobb	35d	2500	50/3days	Elbehira	5/10/2012	Ruffled feathers, depression
19	Cobb	35d	3000	30	Elbehira`	2012	Nervous signs
20	Cobb	30d	3500	300	Kafrelshikh	2013	Edema of the head and wattles, nervous signs

Table (2): Results of HA test detected in the harvested allantoic fluids

Sample NO	1st passage	2nd passage
1	Positive (2^5)	Positive (2^7)
2	Positive (2^6)	Positive (2^8)
3	Positive (2^5)	Positive (2^6)
4	Negative	Negative
5	Negative	Negative
6	Positive (2^9)	Positive (2^{10})
7	Positive (2^8)	Positive (2^{10})
8	Positive(2^8)	Positive (2^{10})
9	Positive (2^5)	Positive (2^8)
10	Negative	Negative
11	Negative	Negative
12	Negative	Negative
13	Negative	Negative
14	Positive (2^6)	Positive (2^4)
15	Positive (2^8)	Positive (2^7)
16	Positive (2^2)	Positive (2^5)
17	Negative	Negative
18	Negative	Negative
19	Positive (2^6)	Positive (2^7)
20	Positive (2^7)	Positive (2^8)

Table (3) Amino acids comparison of F-protein of the isolates of the current study with other NDV genotypes

Isolate/genotype/subgenotype	11	12	13	14	16	17	18	19	20	22	25	26	28	29	30
Consensus	V	P	L	M	I	T	R	I	M	I	C	I	L	T	S
F335-1-NDV-F2 VII-d	A	.	L	.	.	.	.	.	.	T	.	.	.	.	.
F335-2-NDV-F2 VII-d	A	.	L	.	.	.	.	.	.	T	.	.	.	.	.
CK/Ch/SDWF07/2011 VII-d	A	.	L	.	.	.	.	.	.	T	.	.	.	.	.
CK/Ch/YT VII-d	A	.	L	.	.	.	.	.	.	T	.	.	.	.	.
EG/CK/7B/Qena/2012 VII-d	A	.	L	.	.	.	.	.	.	T	.	.	.	.	.
NDV strain NDV05-055 VII-c	A	.	L	I	.	.	.	.	.	.	.	.	.	A	.
NDV strain TW/2000 VII-c	A	.	L	T	.	.	.	.	.	.	.	.	.	.	.
NDV strain ZA 360/95 VII-b	.	.	L	.	.	.	.	V	.	.	.	.	.	.	G
NDV strain ZW 3422/95 VII-b	.	.	L	.	.	.	.	V	.	.	.	.	.	.	G
NDV strain DE 143/95 VII-a	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.
cockatoo/14698/90 VII-a	.	.	L	.	.	.	.	.	.	.	.	.	S	.	.
NDV strain TW/95-1 VII-e	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.
NDV strain QH-1/79 VIII	T	.	S	.	.	.	.	.	.	.	.	.	.	.	G
NDV isolate QH-4/85 VIII	T	.	S	.	.	.	.	.	.	.	.	.	.	.	G
NDV isolate ZhJ-1/85 IX	A	.	L	.	T	A	.	.	A	A	.	V	.	.	N
NDV isolate FJ-1/85 IX	A	.	L	.	T	V	.	.	A	A	.	V	.	.	N
NDV strain Iraq AG68 VI	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.
NDV strain Lebanon 70 VI	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.
NDV pigeon/TX3503/2004 VI	A	.	L	.	.	.	.	.	T	.	.	.	.	.	.
NDV strain NDV05-027 VI	A	.	L	.	.	.	.	.	T	V	.	.	.	.	.
NDV strain Q-GB 506/97 VI	P	.	L	.	.	.	.	.	V	.	S	V	.	.	.
NDV strain DK-1/95 VI	.	.	P	.	.	.	.	.	T	.	.	.	.	.	.
NDV strain H-10/72 V	.	.	L	.	.	.	.	.	T	.	.	.	.	.	.
NDV strain CA 1085/71 V	.	.	L	.	.	.	.	.	T	.	.	.	.	.	.
NDV strain Italien IV	.	.	L	.	.	I	.	.	A	T	.	.	.	.	.
NDV strain Herts/33 IV	A	.	P	.	.	I	.	.	V	T	.	.	.	.	.
NDV Strain Miyadera III	A	.	L	.	T	I	W	.	A	A	.	V	.	.	.
Lasota II	A	.	M	T	T	I	.	V	A	V	.	.	P	A	N
NDV strain V4 Qweensland I	.	.	L	.	T	V	.	V	.	A	.	V	P	.	.
Ulster/67 I	.	.	L	.	T	V	.	V	A	E	.	V	P	.	.

Table (4) Amino acids comparison of F-protein of the isolates of the current study with other NDV genotypes

Isolate/genotype/subgenotype	36	52	71	78	79	82	101
Consensus	P	I	K	K	A	E	R
F335-1-NDV-F2 VII-d	.	V	R	R	.	.	K
F335-2-NDV-F2 VII-d	.	V	R	R	.	.	K
CK/Ch/SDWF07/2011 VII-d	.	V	R	R	.	.	K
CK/Ch/YT VII-d	.	V	R	R	.	.	K
EG/CK/7B/Qena/2012 VII-d	.	V	R	R	.	.	K
NDV strain NDV05-055 VII-c	.	.	.	.	.	.	K
NDV strain TW/2000 VII-c	.	V	.	.	.	.	K
NDV strain ZA 360/95 VII-b	.	.	.	.	.	.	.
NDV strain ZW 3422/95 VII-b	.	.	.	.	.	.	.
NDV strain DE 143/95 VII-a	.	.	.	.	.	.	K
cockatoo/14698/90 VII-a	.	.	.	.	.	.	K
NDV strain TW/95-1 VII-e	.	.	.	.	T	.	K
NDV strain QH-1/79 VIII	.	.	.	R	T	.	.
NDV isolate QH-4/85 VIII	.	.	.	R	T	.	.
NDV isolate ZhJ-1/85 IX	.	.	.	.	.	.	.
NDV isolate FJ-1/85 IX	.	.	.	.	.	.	.
NDV strain Iraq AG68 VI	.	.	.	.	.	.	.
NDV strain Lebanon 70 VI	.	.	.	.	.	.	.
NDV pigeon/TX3503/2004 VI	.	.	.	.	.	.	.
NDV strain NDV05-027 VI	.	.	.	.	.	.	.
NDV strain Q-GB 506/97 VI	.	.	R	.	.	.	.
NDV strain DK-1/95 VI	.	.	.	.	.	.	.
NDV strain H-10/72 V	.	.	.	.	.	.	.
NDV strain CA 1085/71 V	.	.	.	.	.	.	.
NDV strain Italien IV	.	.	.	.	.	.	.
NDV strain Herts/33 IV	.	.	.	.	.	.	.
NDV Strain Miyadera III	.	.	.	.	.	.	.
Lasota II	.	.	.	.	.	D	.
NDV strain V4 Qweensland I	.	.	.	.	.	.	.
Ulster/67 I	.	.	.	.	.	.	.

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