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RESEARCH

Isolation, pathotyping and genotyping of Newcastle disease virus from broiler chickens in Egypt

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ABSTRACT

Background: In Egypt, outbreaks of Newcastle disease (ND) have been occurring in vaccinated chickens with great economic losses.
Objective: This work aimed for diagnosis and characterization of Newcastle Disease Virus (NDV) from infected broiler chickens in Egypt.
Methods: Suspected virus isolates on embryonated chicken eggs was detected by Hemagglutination (HA) test and identified using hemagglutination inhibition (HI) test. F protein encoding gene of NDV was amplified using Reverse Transcription-Polymerase Chain Reaction (RT-PCR) then subjected for nucleotide and amino acid sequence detection.
Results: NDV Giza 2014 isolate and NDV Qualubiya 2014 were characterized as velogenic and lentogenic strains, respectively using Mean Death Time (MDT), Intracerebral Pathogenicity Index (ICPI) and studying amino acid motif of the F protein cleavage site. Phylogeny put NDV Giza 2014 in a separate branch independent from other Egyptian isolates, however it is more related to Lasota (genotype II) and Clone 30 vaccinal strains, while NDV Qualubiya 2014 was grouped more related to Ulster strain (genotype I) and Australian isolates originating from the same ancestral node however it is distantly related to other Egyptian strains 2005 and 2006 grouped together with a common ancestral node but on a separate branch. These results proved diversity between the isolated velogenic NDV Giza 2014 strain and other vaccinal and circulating NDV strains.
Conclusion: It is concluded that further studies on the antigenic characters of that variant isolate is required to study antigenic variation between NDV strains circulating in Egypt.

KEYWORDS: HI; ICPI; Lentogenic; MDT; NDV; PCR; Phylogeny; Velogenic.

BACKGROUND

Newcastle disease (ND) is a highly contagious viral disease cause high mortality and severe disease in birds especially chickens as the most susceptible host **(OIE, 2012).** Newcastle Disease Virus (NDV), also named [Avian Paramyxovirus of serotype 1, APMV-1] is classified in the genus Avulavirus, sub-family Paramyxovirinae, family Paramyxoviridae, order Mononegavirales **(ICTV, 2012)**.

Genetically, NDV possesses a single stranded negative-sense RNA genome of approximately 15.2 kb that encodes for six structural proteins. Hemagglutinin-neuraminidase (HN), Fusion (F), and Matrix (M) proteins that are related to the viral envelope. The remaining three proteins, nucleoprotein (NP), the phosphoprotein (P), and the RNA polymerase (L) **(Chambers et al., 1986).** Genetic diversity between NDV strains was proved using DNA sequencing and phylogenetic analysis of either complete or partial nucleotide sequences of the F gene. Genetic analysis of globally isolated NDV has revealed the existence of two main clades, namely classes I and II, with distinct lineages and sub lineages. Class I and II viruses are divided into 9 and 11 genotypes, respectively (**Miller et al., 2010**). Genetic diversity of NDV could be attributed to the long-lasting infections produced in wild birds by NDV that possesses its own

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RNA polymerases and induce cell fusion; conditions that favor recombination and rapid mutation rates **(Han et al., 2008; Qin et al., 2008)**.

Biologically, NDV can be grouped into three pathotypes according to disease severity as Lentogenic strains cause only clinically mild or unapparent respiratory disease, Mesogenic strains produce respiratory and nervous signs with moderate mortality and Velogenic strains cause severe intestinal lesions or neurological disease, resulting in high mortality Assessment of virulence and pathotyping of NDV is based on calculating mean death time (MDT) in embryonated chicken eggs, intracerebral pathogenicity index (ICPI) in day-old chicks and the intravenous pathogenicity index (IVPI) in 6-week-old chickens **(Alexander, 1997).**

The pathogenicity of NDV could also be predicted based on its genetic analysis **(Toyoda et al., 1987; Gould et al., 2003)**. Although virulence of NDV relies on multiple genes, F gene is the critical site for major changes in virulence. Deduced amino acid sequences of the F protein cleavage site showing the motif ^{112}RRQKRF^{117} indicate velogenic isolate, whereas that showing the motif ^{112}GRQGRL^{117} indicating lentogenic isolates (**Glickman et al., 1988**).

In Egypt, NDV outbreaks are still frequently occurring in vaccinated poultry flocks, despite the intensive vaccination programs **(Abdel-Moneim et al., 2006; Mohamed et al., 2009 and 2011; Radwan et al., 2013; Hussein, et al., 2014; Nabila et al., 2014; Awad et al., 2015 and El-Bagoury et al., 2015)**. NDV is best isolated in embryonated chicken eggs, detected with haemagglutination (HA) test and identified using haemagglutination inhibition (HI) **(Alexander, 2009)** and reverse transcription-polymerase chain reaction (RT-PCR) **(Zhang et al., 2010)**.

The main objective of the present work is to study the genetic changes in the F- gene cleavage site of NDV isolated from vaccinated flocks affected by the disease problem, which can have an impact on the antigenic properties of the viral isolates fortified by the isolation, identification, pathotyping and genotyping of NDV isolates from Egypt.

MATERIALS AND METHODS

Virological Samples:

Proventriculus, lung, kidneys, intestine, cecal tonsils, spleen and liver were obtained from ND suspected broiler chicken flocks of different ages showing eye closure, respiration difficulties, green diarrhea, ruffled feathers and high mortality from Giza and Qualubiya governorates, Egypt at November 2012 to April 2014. These samples were labeled, transported immediately on the ice to the laboratory and stored at –80°C for processing and isolation.

Table (1): Suspected samples collected from broiler chicken flocks in Giza and Qualubiya governorates, Egypt.

Sample Code	Governorate	Flock Capacity	Age
# 1 G	Giza	4500	24 days
# 2 G	Giza	10000	22 days
# 3 G	Giza	14000	30 days
# 4 G	Giza	8000	25 days
# 5 G	Giza	10000	20 days
# 6 K	Qualubiya	10000	30 day
# 7 K	Qualubiya	10000	28 day

G: samples collected from Giza, K: samples collected from Qualubiya

Experimental hosts

Specific Pathogen Free Embryonated Chicken Eggs (SPF-ECE)

One-day old SPF-ECE were obtained from the SPF production farm, Koum Oshiem, Fayoum, Egypt. It was kept in the egg incubator at 37ºC with humidity 70% till the age of 10 days old and

was used for isolation, infectivity titration and pathotyping of NDV by calculation of the mean death time (MDT) of egg embryos (**OIE, 2012**).

One-day old SPF chicks

A total number of 30 one-day old SPF chicks were purchased from SPF farm Koum Oushim El-Fayoum, reared in separated cages and kept in a strictly isolated mosquito proof room. The room was previously cleaned, thoroughly disinfected and were provided with water and feed, and used for pathotyping of NDV isolates.

Newcastle Disease Virus (NDV) reference antiserum

Reference Antiserum against NDV (Anti-NDV) was supplied by CLEVB. It is raised in chickens and has a titer of 12 log2 using HI test and used for identification of viral isolates using HI test.

Isolation of NDV on SPF - ECE

Virus isolation was performed for four serial passages according to the protocol adopted by (**OIE, 2012**). Harvested allantoic fluid (AF) was collected with a sterile syringe and centrifuged at 3000 rpm for 5 minutes to remove mixed blood and tissues, divided into aliquots and stored in sterile screw-capped vials at – 80°C till further use. Presence of virus was confirmed by spot-HA test.

Haemagglutination (HA) and haemagglutination inhibition (HI) tests

HA and HI tests were carried out according to (**OIE, 2012**) using 1% freshly prepared chicken RBCs suspension. HI test was used for identification of NDV in HA positive samples using ND antiserum.

Pathogenicity test

Pathotyping of the NDV isolates were carried out using calculation of Mean Death Time (MDT) and Intracerebral Pathogenicity Index (ICPI) according to (**OIE, 2012**).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Extraction of viral RNA

The genomic viral RNA was extracted from AF by using the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacture's protocol and was stored at –86°C until use.

Oligonucleotide primers

The choice of primers was made according to **Jestin and Jestin (1991),** synthetized by Appligene with the assistance of a microcomputer: PC/gene program (Intelligenetics Inc and Genofit SA), along the F gene sequences. Primer 1 was a 19-mer oligonucleotide 5'CTTTGCTCACCCCCCTTGG3' localized at position 315 to 333 of the cDNA; Primer 2 was a 18-mer oligonucleotide, 5'CTTCCCAACTGCCACTGC3' localized at position 572 to 589 of the cDNA. They were used for the amplification of 275 bp amplicons corresponding the cleavage activation site of F gene of NDV.

cDNA amplification using the polymerase chain reaction (PCR):

The protocol was adapted from that supplied with the Gene Amp Kit (Perkin-Elmer Cetus) and **Jestin and Jestin (1991)**, the test was calibrated in such a way that dNTP was 1.25 mM each, PCR primers were 100 ng each, Taq polymerase was adjusted to 1 U in a total volume

of 20 gl. Several dilutions of each cDNA obtained (previously denatured at 100°C for 10min) were tested as detailed below, cDNA was amplified with a programmable thermal cycler (Techne PHC-1). The PCR program was defined as denaturation at 94°C for 1.5 min, annealing at 51 °C for 2.5min, elongation at75 °C for 1 min for 35 cycles. After the 35th cycle, the time of extension at 75 °C was 2min.

Agarose gel electrophoresis:

The PCR products were separated in 1.5% agarose gel in TAE buffer stained with ethidium bromide and compared with molecular mass marker (50 bp DNA markers) and visualized by ultraviolet (UV) transillumination.

Purification of PCR Products from the gel, sequencing and bioinformatics study:

The specific bands were excised from gel and purified using QIAquick PCR Product extraction kit (Qiagen Inc. Valencia CA) according to the kit protocol. The purified DNA was sequenced in an automated ABI 3730 DNA sequence (Applied Biosystems, USA). RT-PCR products purification and sequencing were done in the Gene sequencing unit of the National Laboratory for Quality Control of Poultry Production (NLQP), Dokki, Egypt. The obtained sequences were aligned by the Clustal W method using MEGA V5.05 software. The nucleotide sequences were compared with NDV sequences available in GenBank (Table 2). A phylogenetic tree of aligned sequences was constructed by Maximum Likelihood method. The deduced amino acid sequences were determined to detect the pathotype of isolated NDV.

Table (2): Published Fusion protein gene sequences of some selected local, vaccinal and reference NDV on the gene bank.

Reference Strain	Accession Number	Pathotype/Genotype
NDV01/China	FJ386392	Virulent
NDV02/China	FJ386393	Virulent
NDV03/China	FJ386394	Virulent
NDV05/China	FJ386396	Virulent
NDV/Chicken/Egypt/4/2006	FJ969395	Virulent
NDV/Chicken/Egypt/2/2006	FJ969393	Virulent
NDV/Chicken/Egypt/3/2006	FJ969394	Virulent
NDV/Chicken/Egypt/1/2005	FJ939313	Virulent
APMV-1/Chicken/US(TX)GB/1948	GU978777	Virulent
NDV/LaSota	AY845400	Lentogenic/Genotype 2
NDV/B1	NC_002617	Lentogenic
Clone30	AF099661	Lentogenic
NDV/Chicken/N.Ireland/Ulster/67	AY562991	Lentogenic /Genotype 1
NDV/99/0655/1999/Australia	AY935494	Virulent
NDV/99/PR32/1997/Australia	AY935497	Virulent
NDV/Australia/Victoria	M21881	Virulent
NDV/Italien	EU293914	Virulent
NDV/rAnhinga	EF065682	Virulent
NDV/Ulster	D00243	Lentogenic
NDV/Mukteswar	EF201805	Virulent/Genotype 3
NDV/US/Largo/71	AY562990	Virulent/Genotype 5
Pigeon paramyxovirus-1/IT-227/82	AJ880277	Virulent/Genotype 6
NDV/strain NA-1	DQ659677	Virulent/Genotype 7

Sequence Submission to Gene Bank:

Sequence of the isolated NDV strains in this study was submitted to the Gene Bank by following instructions of the BankIt tool of the Gene Bank http://www.ncbi.nlm.nih.gov/WebSub/tool=genbank. The submitted sequences to the gene bank were published as verified NDV isolates with accession numbers of KR535623 and KR535624 for the local NDV isolates NDV/Ch/Qualubiya2014 and NDV/Ch/Giza2014, respectively.

RESULTS

Trials for virus isolation followed by detection of the isolates in the collected allantoic fluid of inoculated ECE showed that 5 out of 7 samples gave positive results (3 out of 5 samples from Giza governorate and 2 out of 2 samples from Qualubiya governorate), after the third passage.

Titration of the suspected NDV isolates using HA test showed titers ranged between 4 and 10 $\log_2$ HA units/ml for positive samples. Identification of suspected NDV isolates using HI test showed positive results in samples #1G, #3G, #5G, #6K and #7K.

Pathotyping of NDV isolates positive with HI test (highest serum titers 6 $\log_2$) showed that NDV isolate from Giza governorate (#1G) was velogenic with 48 hours and 1.66 for MDT and ICPI, respectively while NDV isolate from Qualubiya governorate (#6K) was lentogenic with 96 hours and 0.44 for MDT and ICPI, respectively. These results were shown in table (2).

Table (3): Isolation, identification and pathotyping of suspected NDV.

Sample Code	Isolation	HA test (Log_2 HA unit/ml)	HI test (Log_2 serum titer)	Pathotyping		
				*MDT	*ICIP	Pathotype
# 1 G	Positive	9	6	48	1.66	Velogenic
# 2 G	Negative	0	0	ND	ND	-
# 3 G	Positive	6	4	ND	ND	-
# 4 G	Negative	0	0	ND	ND	-
# 5 G	Positive	4	2	ND	ND	-
# 6 K	Positive	10	6	96	0.44	Lentogenic
# 7 K	Positive	4	2	ND	ND	-

MDT values is less than 50 hours for velogenic strains, 50–90 hours for mesogenic strains and more than 90 hours for lentogenic strains while ICIP values more than 1.5 for velogenic strains, 0.5-1.5 for mesogenic strains and less than 0.5 for lentogenic strains (OIE-Manual, 2012).

Local NDV isolates from Giza (#1G) and Qualubiya (#6K) were detected using RT-PCR for amplification of the F protein encoding gene using specific primers presenting specific PCR product with the specific size at 275 bp for both isolates as shown in Figure (1).

The nucleotide sequence (240 nucleotides) and deduced amino acid sequences of the fusion protein for local NDV isolates from Giza and Qualubiya governorates were aligned and compared with other reference and vaccinal strains of NDV published on the gene bank (Table-2).

The NDV local isolates from Giza 2014 showed a pattern of ^{112}R/K-R-Q-R/K-R↓F^{117} F protein cleavage site motif characteristic to velogenic NDV strains and the NDV local isolates from Qualubiya 2014 showed a pattern of ^{112}G/E-K/R-Q-G/E-R↓L^{117} F protein cleavage site motif characteristic to lentogenic NDV strains.

Phylogenetic analysis of these isolates showed that NDV Giza 2014 was in a separate branch independent from other Egyptian isolates of NDV and it is more related to NDV Lasota strain (genotype II NDV) and NDV Clone 30 vaccinal strain, while NDV Qualubiya 2014 was grouped more related to genotype I Ulster NDV strain and Australian isolates originating from the same ancestral node but it is distantly related to other Egyptian NDV strains 2005 and 2006 grouped together with a common ancestral node but on a separate branch (Figure 2).

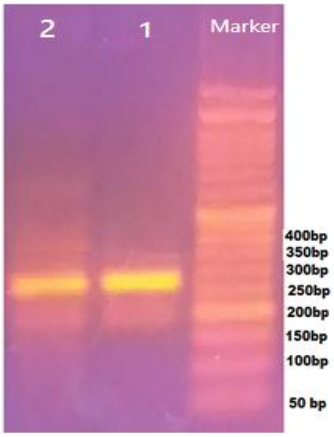

Fig. 1: Electrophoretic pattern of the amplified products of the F protein gene at 275 bp for NDV isolates. Marker: 50 bp nucleic acid marker. Lane 1: NDV isolate from Giza governorate sample #1G. Lane 2: NDV isolate from Qualubiya governorate sample #6K.

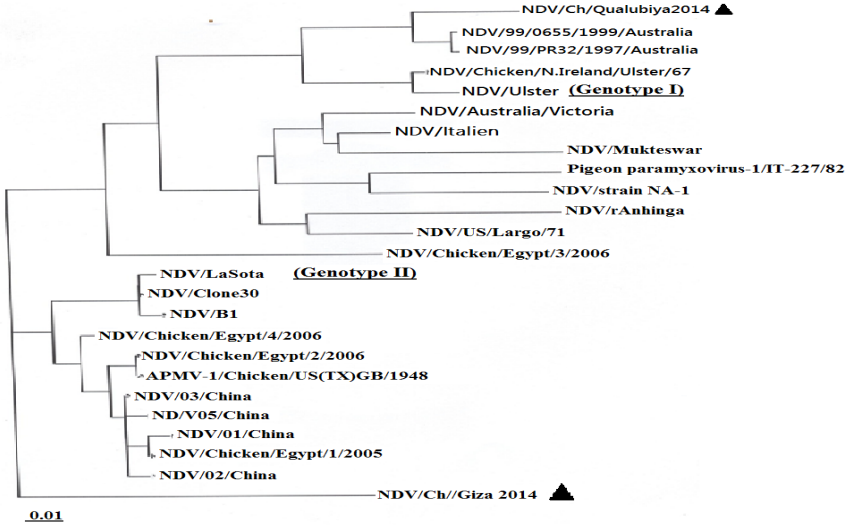

Fig. 2: Phylogenetic tree based on nucleotide sequence of fusion protein gene of local NDV isolates from Giza and Qualubiya governorates and other reference and vaccinal NDV strains published on gene bank.

DISCUSSION

NDV episodes occurred repeatedly in broiler chicken flocks from different governorates in Egypt in spite of vaccination which bringing about severe economic losses. This work aims to study the genetic changes in the F- gene cleavage site Newcastle disease viral isolates from vaccinated flocks affected by the disease problem, which can have an impact on the antigenic properties of the viral isolates from these herds fortified by isolating and classifying these isolates, pathotyping, genetic characterization, studying homology with other Egyptian strains and other vaccinal strains. Trials for isolation of NDV from field samples of suspected broiler chicken flocks in SPF-ECE followed by detection of the isolates in the collected allantoic fluid using HA test showed positive results in 5 out of 7 samples (3 out of 5 samples from Giza and 2 out of 2 samples from Qualubiya) with titers ranged between 4 and 10 $\log_2$ HA units/ml for positive samples after the 3rd passage (**OIE, 2012).** Identification of suspected NDV isolates using HI test showed positive results with the suspected isolates (#1G, #3G, #5G, #6K and #7K) with the highest serum titers 6 $\log_2$ for samples #1G and #6K **(Sidahmed and Elhag, 2014; El-Bagoury et al., 2015).**

Biological characterization of NDV isolates through pathotyping showed that NDV isolate from Giza governorate (#1G) was velogenic with 48 hours and 1.66 for MDT and ICPI, respectively while NDV isolate from Qualubiya governorate (#6K) was lentogenic with 96 hours and 0.44 for MDT and ICPI, respectively (**Sidahmed and Elhag, 2014**).

Genetic characterization is started using detection of NDV isolates from Giza and Qualubiya with RT-PCR for amplification of the F protein encoding gene using specific primers presenting specific PCR product with the specific size at 275 bp for both isolates (**Jestin and Jestin, 1991 and Muhammad et al., 2012)**, followed by nucleotide and deduced amino acid sequences detection of the F protein for local NDV isolates with phylogenetic analysis compared with other reference and vaccinal strains of NDV published on the gene bank. Local NDV isolate Giza 2014 showed a pattern of ^{112}R/K-R-Q-R/K-R↓F^{117} F protein cleavage site motif characteristic to velogenic NDV strains and the local NDV isolate Qualubiya 2014 showed a pattern of ^{112}G/E-K/R-Q-G/E-R↓L^{117} F protein cleavage site motif characteristic to lentogenic NDV strains. These results agreed with that of **Glickman et al., (1988)** and confirmed the results of pathotyping using MDT and ICPI.

Phylogenetic analysis showed that NDV Giza 2014 was in a separate branch independent from other Egyptian isolates and it is more related to Lasota strain (genotype II) and Clone 30 vaccinal strain, while NDV Qualubiya 2014 was grouped more related to Ulster strain (genotype I) and Australian isolates and originating from the same ancestral node but it is distantly related to other Egyptian strains 2005 and 2006 grouped together with a common ancestral node but on a separate branch. These results agree with that of **Gould et al., (2003)** who predicted pathotype of NDV using genetic analysis of F protein cleavage site and **Mohamed et al., (2009)** who reported genotype II NDV in Egypt. The genetic diversity of NDV is shown using partial or complete nucleotide sequence of F gene confirmed by phylogenetic analysis **(Miller et al., 2010)** and plays an important role in studying this genetic diversity or relatedness between NDV field isolates and other vaccinal strains **(Hussein et al., 2014).**

Finally, clear diversity between the isolated velogenic NDV Giza 2014 strain and other vaccinal and circulating NDV strains was proved, however further studies on the antigenic characters of that variant isolate is required to study antigenic variation between NDV strains circulating in Egypt.

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