Virus Genes (2013) 47:311–316
DOI 10.1007/s11262-013-0950-y

Isolation and molecular characterization of Newcastle disease virus genotypes II and VIId in Egypt between 2011 and 2012

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Received: 18 May 2013 / Accepted: 28 June 2013 / Published online: 11 July 2013

Abstract The current study was conducted to isolate and characterize Newcastle disease virus (NDV) from recent outbreaks affecting poultry farms in Egypt between 2011 and 2012. Trachea, spleen, liver, proventriculus and caecal tonsils were collected from clinically infected NDV ten different vaccinated broiler farms in Fayoum, Behira and Giza Provinces. Inoculation of all the collected samples in 10-day-old embryonated chicken specific-pathogen-free eggs resulted in isolation of haemagglutinating agents in three samples. These haemagglutinating agents were confirmed as NDV by real-time reverse transcription polymerase chain reaction (rt RT-PCR) using matrix (M) gene-specific primers. The deduced amino acid sequences of the fusion protein revealed that one isolate possessed the motif ^{112}RRQKRF^{117} at the cleavage site, indicating that this isolate is velogenic genotype, whereas the other two isolates carries the motif ^{112}GRQGRL^{117} indicating they are lentogenic genotype. The phylogenetic analysis revealed that the velogenic genotype isolate clustered with published class II genotype VII sub genotype d NDVs and closely related to Middle East isolates, whereas the other two isolates clustered with published class II genotype II NDVs. The spread of velogenic genotype strain to Egypt via Middle Eastern countries is likely to be the source of infection.

Keywords Newcastle disease virus · Velogenic and lentogenic genotypes · Egypt

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Introduction

Newcastle disease virus (NDV) and highly pathogenic avian influenza virus (AIV) are regarded throughout the world as the two most important diseases of poultry and other birds (over 200 species of birds), because of the severe nature of the disease and the associated consequences. NDV is included as an Office International des Epizooties (OIE) list A disease [1]. The importance of NDV for poultry is not only due to devastating NDV infections, with flock mortality rates going up to 100 %, but also due to the economic impact that may ensue owing to trading restrictions and embargoes placed on areas and countries where outbreaks have occurred [2].

NDV or avian paramyxovirus type 1 (APMV-1) belongs to the genus *Avulavirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, order *Mononegavirales* [3]. NDV is an enveloped virus with a single stranded, negative RNA genome of approximately 15.2 kb [4]. The genome contains at least six major genes encoding the nucleocapsid protein (NP), phosphoprotein (P), matrix (M) protein, fusion (F) protein, haemagglutinin–neuraminidase (HN)

and RNA-dependent RNA polymerase (L) protein in the order of 3′-N–P-M-F-HN-L-5′[5, 6].

NDV isolates are classified into two distinct classes (class I and class II) within a single serotype [4]. Each class has been further classified into different genotypes; class I (night genotypes from 1 to 9) and class II (ten genotypes from I to X). Genotype VII (class II genotype VII) was firstly categorized into two subgenotypes: 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 spread to South Africa. Later, the two subgenotypes of VII are classified into VIIc, d, and e, which represent isolates from China, Kazakhstan and South Africa; and VIIf, g, h, and i, which represent African isolates [7, 8]. Based on the pathogenicity of NDV isolates in chickens, NDVs are divided into three main pathotypes: velogenic (high virulence), mesogenic (moderate virulence), and lentogenic (low virulence) [9]. Both mesogenic and velogenic strains are classified as virulent strains and can cause high morbidity and mortality rates in commercial poultry farms.

In Egypt, NDV outbreaks are occurring frequently, and the epidemiology of the virulent NDV isolates from these outbreaks is not known. Therefore, the present study was conducted to isolate, molecular characterize and elucidate the epidemiology of NDV isolated from the recent outbreaks in Egypt.

Materials and methods

Clinical specimens

Clinical specimens including trachea, spleen, liver, proventriculus and caecal tonsils were collected from suspected cases of NDV in ten different vaccinated (two doses of live attenuated vaccines; one at one-day old in the hatchery, and the other at 14 days old in the farms) commercial broiler farms located in three Egyptian provinces (Fayoum, Behira and Giza) during 2011 and 2012. Chicks at the age of 28–42 days were suffering from moderate-to-severe respiratory sings accompanied with lesions including haemorrhages in the trachea, caecal tonsils, intestine, payer's patches and proventriculus glands. The specimens were grinded in phosphate buffered saline, pH 7.4, containing antibiotics of (2000 U/ml) penicillin, (2 mg/ml) streptomycin, (50 μg/ml) gentamycin and (1000 U/ml) fungizone. After three cycles of freezing and thawing, the samples were clarified by centrifugation at 5000 rpm for 10 min then the supernatants were collected and stored at −80 °C until used.

Virus isolation

The supernatants were inoculated into 10-day-old embryonated chicken specific-pathogen-free (SPF) eggs via the allantoic route using 0.2 ml/egg. The eggs were incubated at 37 °C daily with candles for 5 days. Eggs showing embryonic death within 24 h of inoculation were discarded and considered non-specific, whereas eggs showing embryonic death after 24 h and remaining alive up to 5 days were chilled. Allantoic fluid from each of the inoculated eggs was harvested and tested for haemagglutination (HA) using 1 % (V/V) chicken RBCs. The HA-negative samples were passaged two further times in SPF eggs, and the HA titer of the allantoic fluid was again determined. All HA-positive allantoic fluids were further assayed for NDV using real-time (rt) RT-PCR.

RNA extraction

The total RNA was extracted from 140 μl of the HA-positive allantoic fluid as well as LaSota vaccinal strain as positive control and non-infected allantoic fluid as negative control using QIAamp Viral RNA extraction kit (Qiagen, USA). After lysis of the specimens, the mixture was transferred to a spin column according to manufacturer's protocol. The extracted RNAs were eluted in 60-μl elution buffer and stored at −80 °C until further use.

Real-time RT-PCR testing of allantoic fluid for NDV

The extracted RNA was tested for presence of NDV by rt RT-PCR targeting to the conserved region of the M-gene using the QuantiTect Probe RT-PCR Kit (Qiagen, Valencia, California, USA). The rt RT-PCR was carried out in a 25-μl reaction volume containing 2× RT-PCR master mix (12.5 μl); 10 μM of each forward and reverse primers (1 μl); 3 μM probe (1 μl) RT-PCR enzyme mix (1 μl); nuclease-free water (3.5 μl); and RNA (5 μl). First-strand cDNA synthesis was synthesized by incubating the RT-PCR mixture for 30 min at 50 °C then at 95 °C for 15 min for inactivation of RT enzyme and activation of Hot Start Taq DNA polymerase enzyme. 40 amplification cycles were conducted, with each cycle containing three steps of 94 °C for 10 s (denaturation), 55 °C for 30 s (annealing) and 72 °C for 10 s (extension). The nucleotide sequences and positions of primers and probe are shown in Table 1.

Sequencing

The RNAs of NDV-positive rt PCR were reverse transcripted using RevertAid^{TM} first strand cDNA synthesis kit (Fermentas, Germany). The reaction mixture was

Table 1 Sequence and nucleotide positions of primers and probes

Target gene	Primes/probe name	Sequence (5′–3′)	Nucleotide position	Amplicon size	Reference
M gene	APMV1-M-F	5′-AGTGATGTGCTCGGACCTTC-3′	4100–4119	121 bp	[10]
	APMV1-M-R	5′-CCTGAGGAGAGGCATTTGCTA-3′	4200–4220		
	APMV1-M-probe	5′-[FAM] TTCTCTAGCAGTGGGACAGCCTGC [TAMRA]-3′	4169–4192		
F gene	APMV1-F–F	5′-ATGGGCYCCAGACYCTTCTAC-3′	47–67	535 bp	[11]
	APMV1-F-R	5′-CTGCCACTGCTAGTTGTGATAATCC-3′	557–581		

carried out in a 20-µl volume containing 5× buffer (4 µl); 10 mM dNTPs (2 µl); 100 µM random hexamer primers (1 µl); 20 units RNase inhibitor (1 µl); 200 units reverse transcription enzyme (1 µl); nuclease-free water (8 µl); and RNA (3 µl). The RT reaction was composed of one cycle at 25 °C for 5 min, 42 °C for 60 min and 70 °C for 10 min. The hyper-variable region of F gene containing the cleavage site was amplified using degenerate primers as shown in Table 1. The PCR reaction was carried out in a 50-µl total volume containing 2× DreamTaq green PCR master mix (Fermentas, Germany) (25 µl); 10 µM of each forward and reverse primers (1 µl); nuclease free water (18 µl); and cDNA (5 µl). The PCR cycling profile was initial denaturation 1 min at 94 °C followed by 35 cycles of 30 s for denaturation at 94 °C, 30 s for annealing at 55 °C, and extension at 72 °C for 45 s and final extension at 72 °C for 10 min. The amplified PCR products were electrophoresed in 1.5 % agarose gel stained with ethidium bromide and documented using ultraviolet transilluminator. The specific bands were excised from gel and purified using EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic, Canada). The purified DNA was sequenced in an automated ABI 3730 DNA sequencer (Applied Biosystems, USA). 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.

GenBank accession number

The obtained sequences of the isolated NDV were submitted to the GenBank database with the following accession numbers: NDV/Chicken/Fayoum/Egypt/MR7/2011 (JX193772); NDV/Chicken/Behaira/Egypt/MR6/2012 (JX193771); and NDV/Chicken/Giza/Egypt/MR0/2012 (JX173098).

Results and discussion

Detection of NDV

Using primers and probe specific for the conserved region near the 5\ end of the M gene of NDV, validated by the USDA for screen detection of NDV having a sensitivity for class II NDV reach 100 % [7, 10, 12], the three HA-positive allantoic fluids were identified as NDV. The cycle threshold (Ct) values of the isolated NDV were 25.46, 32.03 and 36.66 for NDV/Chicken/Giza/Egypt/MR0/2012, NDV/Chicken/Behaira/Egypt/MR6/2012 and NDV/Chicken/Fayoum/Egypt/MR7/2011, respectively, in comparison with LaSota strain positive control that gives Ct value as 17.30 (Fig. 1).

Cleavage site of the fusion protein

The use of degenerate primers for the fusion protein gene resulted in amplicons with the expected size of 535 bp. The deduced amino acid sequences of the three isolates were compared with other strains of NDV. These revealed that the amino acid sequence surrounding the fusion glycoprotein cleavage site of isolate NDV/Chicken/Giza/Egypt/MR0/2012 was different from the other two isolates NDV/Chicken/Behaira/Egypt/MR6/2012 and NDV/Chicken/Fayoum/Egypt/MR7/2011. The sequence of isolate NDV/Chicken/Giza/Egypt/MR0/2012 carries the motif ^{112}RRQKRF^{117} that is consistent with viruses of velogenic strains of the 5d lineage [13], whereas isolates NDV/Chicken/Behaira/Egypt/MR6/2012 and NDV/Chicken/Fayoum/Egypt/MR7/2011 carry the motif ^{112}GRQGRL^{117}, which is consistent with viruses of lentogenic/low virulence strains [14].

Phylogenetic tree analysis

We determined the nucleotide sequences of a fusion protein gene segment of three Egyptian NDV isolates during 2011 and 2012, which allow us to conduct a phylogenetic study to determine the origin of these Egyptian isolates. One of the

Table 2 Newcastle disease viruses used in the phylogenetic tree construction

Isolate definition	Origin	Genotype (lineage)	GenBank accession
NDV/Ostrich/Ismailia/2010	Egypt	II (2)	JN193503
NDV/chicken/Egypt/4/2006	Egypt	II (2)	FJ969395
NDV/chicken/Egypt/3/2006	Egypt	II (2)	FJ969394
NDV/chicken/Egypt/2/2006	Egypt	II (2)	FJ969393
NDV/Chicken/Egypt/1/2005	Egypt	II (2)	FJ939313
LaSota/46	USA	II (2)	M24696
Hitchner B1/47	USA	II (2)	M24695
BeaudetteC/45	USA	II (2)	M24697
Clone 30	USA	II (2)	Y18898
VGGA/87	USA	II (2)	AY289002
Connecticut/9-12-60	Canada	II (2)	AF206617
Komarov/45/LK	Sri Lanka	II (2)	AY170137
Mexico 468/01	Mexico	V (3c)	EU518685
Brasil AV 1769/90	Brasil	V (3c)	AY175649
CA 1085/71	USA	V (3c)	AF001106
H 10/72	Hungary	V(3c)	AF001107
NY 70181/70	USA	V (3c)	AF001105
AALCK90163	Albania	V (3c)	AY175647
BITPI87079	Italy	V (3c)	AY135747
1SACK00184	Saudi Arabia	VIId (5d)	AY135754
Chicken/China/SDYT03/2011	China	VIId (5d)	JQ015297
Turkey/Israel/111/2011	Israel	VIId (5d)	JN979564
Chicken/Israel/174/2011	Israel	VIId (5d)	JN849578
Apmv1/Chicken/Jordan/Jo11/2011	Jordan	VIId (5d)	JQ176687
TW/2000	Taiwan	VIId (5d)	AF358786
Chicken/Sudan/03/2003	Sudan	VIId (5d)	GQ258670
Chicken-2601-Ivory Coast-2008	Ivory Coast	(7a)	FJ772466
Avian-1532-14-Mauritania-2006	Mauritania	(7a)	FJ772455
Chicken-3490-149-Cameroon-2008	Cameroon	(7b)	FJ772478

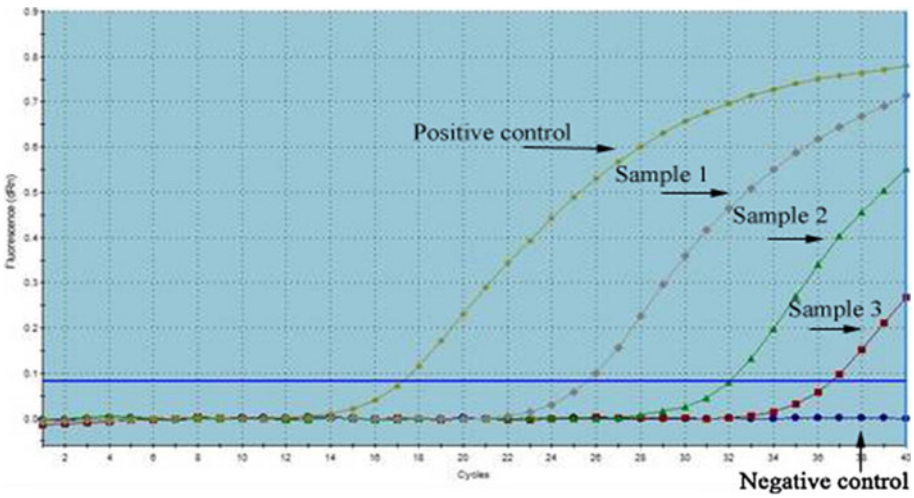

Fig. 1 Amplification curves obtained during rt RT-PCR: *positive control* (LaSota strain), *sample 1* (NDV/Chicken/Giza/Egypt/MR0/2012), *sample 2* (NDV/Chicken/Behaira/Egypt/MR6/2012), *sample 3* (NDV/Chicken/Fayoum/Egypt/MR7/2011), and *negative control* (non-infected alantoic fluid)

three iolates, NDV/Chicken/Giza/Egypt/MR0/2012, was classified as genotype 5d, class II. The nucleotide similarity in the sequenced fragment of the fusion gene of NDV/Chicken/Giza/Egypt/MR0/2012 to the Jordanian strain apmv1-chicken-Jordan-jo11-2011 is 98.9 %. The Jordanian strain was isolated from outbreak affecting broilers, layers and

breeders in 2011 with a mean death time (MDT) of 46 h, which confirms the velogenic nature of this isolate [15]. However, the nucleotide similarity in the sequenced fragment of the fusion gene of NDV/Chicken/Behaira/Egypt/MR6/2012 is 99.7 % to the clone 30 vaccinal strain and is more closely related to the vaccinal strains that are present in class II, genotype II than those of field strains that were previously isolated from Egypt belonging to the same genotype, indicating that this strain is probably a vaccine strain. The same condition is found for the strain Chicken/Fayoum/Egypt/MR7/2011, which bears the highest identical match with both vaccinal strains clone 30 and VGGA/87 (83.7 %) among the field strains isolated from Egypt (Fig. 2).

The isolated velogenic genotype Egyptian strain, NDV/Chicken/Giza/Egypt/MR0/2012 that fell within genotype VII sub genotype d is closely related to strains predominantly in Jordan (ampv1/Chicken/Jordan/Jo11/2011), Israel (chicken/Israel/174/2011 and Turkey/Israel/111/2011), and China (Chicken/China/SDYT03/2011). The pairwise distance revealed that the NDV/Chicken/Giza/Egypt/MR0/2012 is identical with ampv1/Chicken/Jordan/Jo11/2011(pairwise distance 0.012), which is identical with the both isolates chicken/Israel/174/2011 and Turkey/Israel/111/2011 (pairwise distance 0.008) and identical with the Chinese isolate Chicken/China/SDYT03/2011(pairwise distance 0.012). On the other hand, NDV/Chicken/Giza/

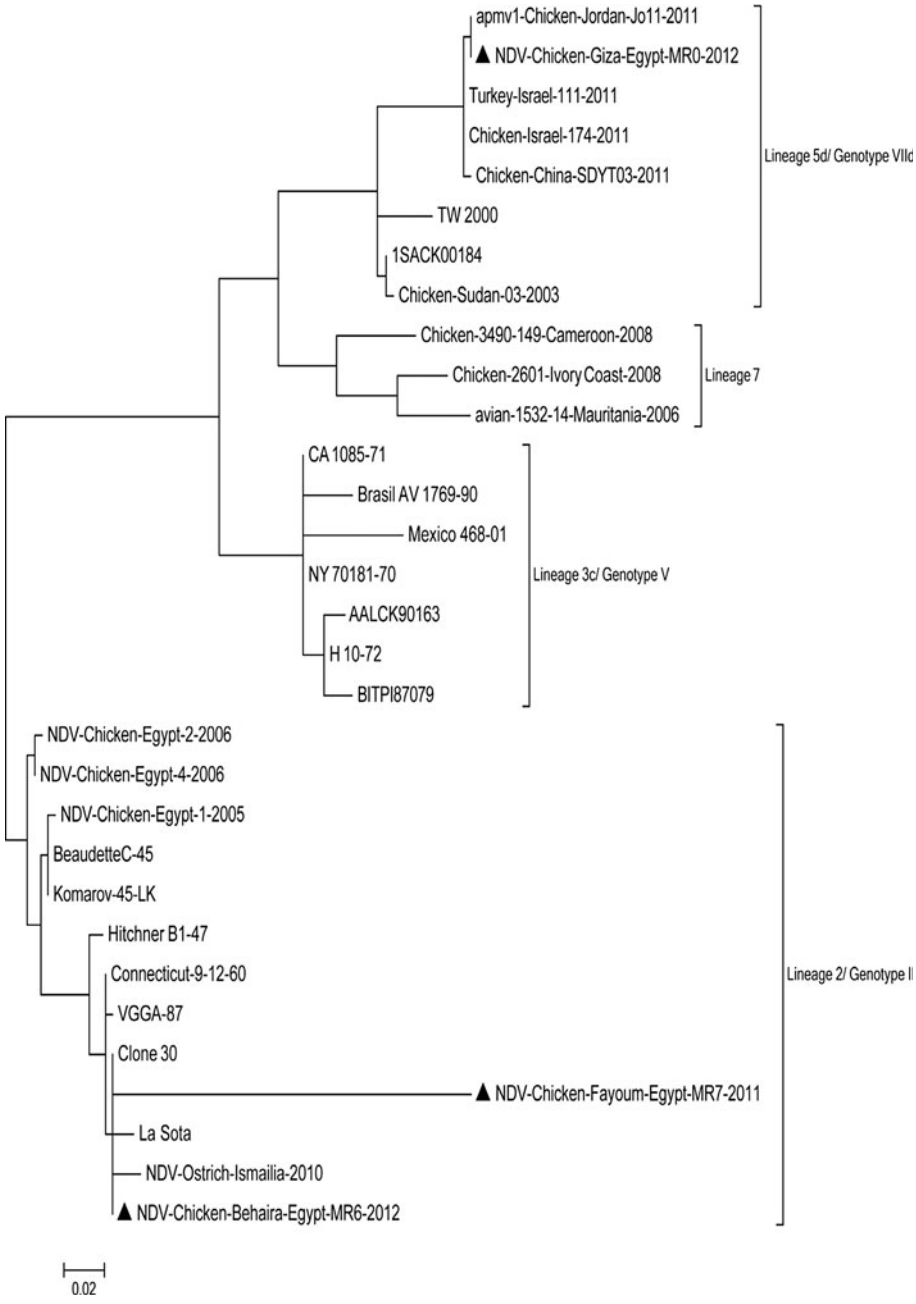

Fig. 2 Phylogenetic tree of the nucleotide sequences of the partial fusion gene fragment of Egyptian isolates (marked with *solid triangle*) and the references strains from GenBank using maximum likelihood method with bootstrap values for $n = 100$ replicates

Egypt/MR0/2012 is distinct from West African viruses isolated from Mauritania, Ivory Coast and Cameroon (pairwise distances 0.158, 0.163 and 0.170, respectively). Also, it is distinct from the Sudanese genotype 5d (pairwise distance, 0.054) as well as distinct from the Saudi Arabian strain (pairwise distance, 0.084) (data not shown).This identity proves that the virus circulating in Egypt is probably extending from Middle Eastern region.

Conclusion

Analysis of the deduced amino acid sequence and phylogenetic analysis of the NDV F-gene of the strains NDV/Chicken/Giza/Egypt/MR0/2012 proved that it to belong to class II, genotype VII, sub genotype d (lineage 5 sub lineage d) which were not present in Egypt before, as the previous dominant strains were identified to be class II, genotype II [16, 17] and genotype VI [18]. This is the first record of isolation and molecular characterization of this genotype in Egypt, which is thought to be spreading in Egypt through trading of poultry and poultry products with Middle Eastern countries and China. Another probable scenario, namely, of virus transfer to Egypt is through migratory birds, as previously reported [19, 20]. There are some reports of wild birds becoming infected during outbreaks in poultry farms and a few reports of mortalities in wild birds, and in countries with widespread native chicken populations. The reservoir for NDV may well be such chickens, which could be possibly infected by wild birds and become a medium for intermediary spread of infection [21]. Thus, molecular epidemiology and phylogenetic analysis of NDV in Egypt, Africa, and Middle East countries is an important tool to determine the current situation, trace the source of infection and to determine the possible root by which the virus spreads, assisting these countries in developing their disease control measures, which needs to be improved.

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