Molecular characterization of a recent Newcastle disease virus outbreak in Egypt

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A B S T R A C T

Newcastle disease (ND) is a highly contagious viral disease and is a continuous threat to the poultry industry worldwide. In the early months of 2014, several devastating ND outbreaks affecting broilers had been occurred in Sharqia province, Egypt. The fusion gene of the isolated Newcastle disease virus (NDV) was partially amplified by RT-PCR, then directly sequenced. The NDV isolates were found to have the motif 112RRQKRF117. This motif and the intracerebral pathogenicity index (ICPI) are indicative of the velogenic nature of these NDV isolates. Phylogenetic analysis showed that the new NDV strain belongs to the lineage 2 genotype II and is closely related to the Egyptian strain VRCLU/Giza/2009. Based on the high nucleotide similarity between our isolated NDV isolates and the Egyptian NDV strain, the origin of these recent NDV isolates might be from Egypt.

Keywords: NDV, ICPI, RT-PCR, Velogenic

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1. INTRODUCTION

Newcastle disease (ND) is one of the most serious infections of poultry. Outbreaks of virulent ND require an immediate reporting to the Office of International Epizootes (OIE) (Alexander, 1997). Newcastle disease virus (NDV) has a negative-sense, single-stranded RNA genome, belongs to the Avulavirus genus, within the family Paramyxoviridae, in the order Mononegavirales, and is designated avian paramyxovirus 1 (APMV-1) (de Leeuw and Peeters, 1999). 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(de Leeuw and Peeters, 1999). NDV isolates are classified into two distinct classes (class I and class II) within a single serotype (Czegledi et al., 2006). 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 represents isolates from China, Kazakhstan and South Africa; and VIIf, g, h, and i, which represent African isolates (Miller et al., 2010). 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) (Alexander, 2000). 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 rapid and accurate diagnosis of ND are essential. Therefore, the present study was conducted to isolate and molecular characterize NDV isolated from the recent outbreak in sharqia province, Egypt.

2. MATERIAL AND METHODS

2.1. clinical Specimens

Within two years of this study (January 2013 to December 2014), forty ND-suspected field samples (trachea, liver and brain tissues) were obtained from chickens flocks suspected to have ND in three broiler farms of Sharqia Province. Most sacrificed chickens had diarrhea, also showed nervous symptoms and respiratory difficulties. The specimens were grinded in phosphate buffered saline, pH 7.4, containing antibiotics of (2000 U/ml) penicillin, (2 mg/ml) streptomycin, (50 lg/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.

2.2. Virus isolation in ECE

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 SPFeggs, and the HA titer of the allantoic fluid was again determined. All HA-positive allantoic fluids were further assayed for NDV using RT-PCR.

2.3. 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 60µl elution buffer and stored at -80 °C until further use.

2.4. RT-PCR and sequencing of Fusion gene

The RNAs were reverse transcripted using RevertAidTM first strand cDNA synthesis kit (Fermentas, Germany). The reaction mixture was carried out in a 20µl volume containing 5× buffer (4 µl); 10 mM dNTPs (2 µl); 100 lM 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-ll total volume containing 29 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

Table 1 Sequence and nucleotide positions for primers of fusion gene

Primers name	Sequence (5 –3)	Nucleotide position	Amplicon size	Reference
APMV1-F–F	5-ATGGGCYCCAGACYCTTCTAC-3	47–67	535 bp	Seal et al. (1995)
APMV1-F-R	5-CTGCCACTGCTAGTTGTGATAATCC-3	557–581		

Table 2 Newcastle disease viruses used in the phylogenetic tree construction

Isolate definition	Origin	Genotype (lineage)	GenBank accession
NDV/Chicken/Giza/Egypt/MR0/2012	Egypt	VIId (5d)	JX173098
NDV/Ostrich/Ismailia/2010	Egypt	II (2)	JN193503
NDV/VRCLU/Giza/2009	Egypt	II (2)	HQ455810
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
Mexico 468/01	Mexico	V (3c)	EU518685
BITPI87079	Italy	V (3c)	AY135747
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
Chicken/Sudan/03/2003	Sudan	VIId (5d)	GQ258670
Chicken-2601-Ivory Coast-2008	Ivory Coast	(7b)	FJ772466
Avian-1532-14-Mauritania-2006	Mauritania	(7b)	FJ772455
Chicken-3490-149-Cameroon-2008	Cameroon	(7b)	FJ772478

Basic, Canada). The purified DNA was sequenced in an automated ABI 3730 DNA sequence (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.

3. RESULTS

The use of degenerate primers for the fusion protein gene resulted in amplicons with the expected size of 535 bp. The deduced amino acid sequence of the isolate was compared with other strains of NDV.

These revealed that the amino acid sequence surrounding the fusion glycoprotein cleavage site of isolate NDV/chicken/Sharqia/Egypt/2014 carries the motif 112RRQKRF117 that is consistent with viruses of velogenic strains of the 2 lineage of genotype figure (1). Phylogenetic analysis of NDV/chicken /Sharqia /Egypt/2014 with other reference and vaccinal strains of NDV revealed that NDV/chicken /Sharqia/Egypt/2014 was related to the Egyptian strain VRCLU/Giza/2009 figure (2).

4. DISCUSSION

A great genetic diversity has been demonstrated among NDV strains based on phylogenetic analyses of partial or complete nucleotide sequences of the F gene that was reinforced by the wide use of DNA sequencing techniques in the last years (Miller et al., 2010). The amino acid sequence at the F protein cleavage site is a major determinant of NDV virulence, in Lentogenic viruses have a monobasic amino acid motif at the F cleavage site, 112G-R/K-Q-G-R↓L117, and are cleaved extracellularly by trypsin-like proteases found in the respiratory and intestinal tract. Mesogenic and velogenic strains have a multi-basic amino acid motif at the F cleavage site, 112R/G/K-R-Q/K-K/R-R↓F117 and can be cleaved intracellularly by ubiquitous furin-like proteases (Glickman et al., 1988). Analysis of the deduced amino acid sequence and phylogenetic analysis of the NDV F-gene of the strains NDV/chicken /Sharqia /Egypt /2014 proved that it to belong to class II, genotype II and carries the motif 112RRQKRF117 that is consistent with viruses of velogenic strains.

Our results agree with results of Hussein et al., (2013) who reported the importance of studying the genetic diversity of NDV field strains in different geographic regions of Egypt for understanding the genetic relatedness among NDV strains.

Also these results agree with that of Gould et al., (2003) who used genetic analysis as a predictor of the pathogenicity potential of NDV isolates and Mohamed et al., (2009) who reported that NDV genotype II has been reported to circulate in Egypt.

This confirming record of isolation and molecular characterization of this genotype in Egypt is thought to be spreading in Egypt through migratory birds, as previously reported (Mohamed et al., 2011).

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 (Gilchrist, P. 2005).

In conclusion, NDV isolates circulating among chickens belong to class II, genotype II and carries the motif 112RRQKRF117 that is consistent with viruses of velogenic strains and associated with outbreaks in commercial poultry farms in Sharqia province.

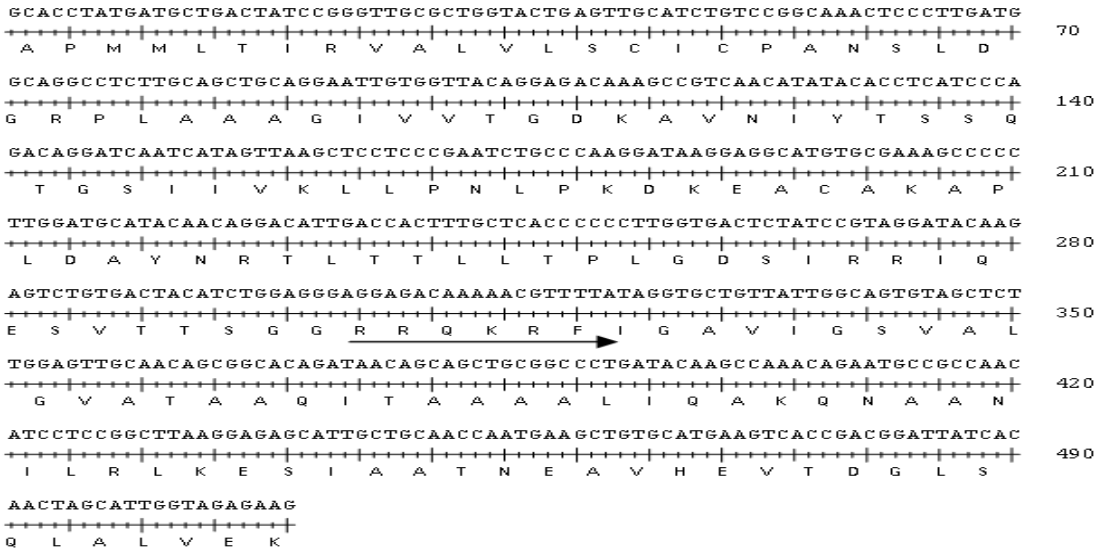

Fig. 1. Nucleotide and amino acid sequence of of the partial fusion gene fragment of Egyptian isolate (NDV/chicken/Sharqia/Egypt/2014), the arrow underline characteristic amino acid motif RRQKRF indicate velogenic strain.

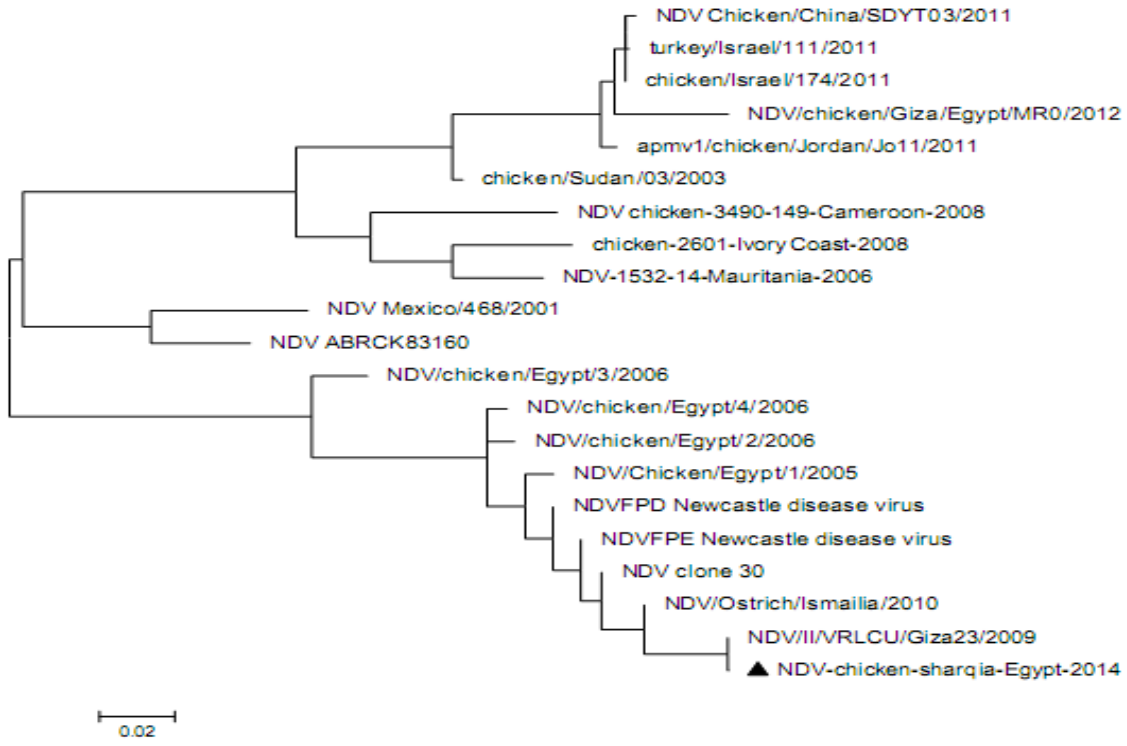

Fig. 2; Phylogenetic tree of the nucleotide sequences of the partial fusion gene fragment of Egyptian isolates (marked with solid triangle) and the references strains from GenBank using maximum likelihood method with bootstrap values for n = 100 replicates.

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