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Identification and Sequencing of Genotype VII of Newcastle Disease Virus From Chicken Flocks In Six Egyptian Governorates

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NEWCASTLE disease (ND) is a very contagious disease in chickens and turkeys and one of the most important diseases of poultry in the world. The infection causes sudden death with high mortality. ND in recent years showed symptoms more severe than previous symptoms of this disease. NDV infection of genotype VII has been reported to cause this outbreak in several commercial poultry farms. In this study the isolated viruses were molecularly characterized by RT-PCR targeting the partial F-gene of NDV. Partial F gene sequence analysis showed that the isolated NDV strains belong to genotype VII with the characteristic amino acid sequences of the F0 protein proteolytic cleavage site motifs (^{112}RRQKRF^{117}) for the velogenic NDV (vNDV) strains. From the isolated and molecular identified NDV Genotype VII four selected isolates (NDV/Chicken/EG-MN/NRC/2015, NDV/Chicken/EG-QU/NRC/2015, NDV/Chicken/EG-SH/NRC/2015 and NDV/Chicken/EG -SH2/NR/2015) were sequenced for NDV F gene and nucleotide sequences were submitted to GenBank and given accession numbers (MF418017), (MF418018), (MF418019) and (MF418020); respectively.

Though no significant genetic changes observed, the multiple vNDV outbreaks in vaccinated chicken require continuous monitoring of the evolution and the epidemiology characteristics of the vNDV as well as evaluation of the used vaccines.

Keywords: Identification, Genotype VII , Sequencing, Newcastle disease virus, Chicken.

Introduction

Newcastle disease (ND) is a highly contagious viral disease causing high mortality and severe disease in birds especially chickens as the most susceptible host [1]. Newcastle Disease Virus (NDV), is classified in the genus Avulavirus, sub-family Paramyxovirinae, family Paramyxoviridae, order Mononegavirales [2]. The genome contains 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′ [3].

Infection of the disease varies from subclinical to severe or systemic infection with high mortality rate depending on the virulence of virus strain and the host susceptibility. Based on the clinical signs in chicken, the NDV are categorized into five pathotypes including viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic respiratory and asymptomatic enteric [4]. Genetic diversity between NDV strains was proved using DNA sequencing and phylogenetic analysis of either complete or partial nucleotide sequences of the F gene. The amino acid sequence of the F0 precursors in NDV of low virulence (loNDV) are characterized by a monobasic amino acid sequence motif at the C-terminus of the F2 protein and a leucine at the N-terminus of the F1 protein, 112G-R/K-Q-G-R-L117. The F0 precursors of loNDV are cleaved only extracellularly by trypsin-like proteases present in the respiratory and intestinal tract. While, virulent NDV (vNDV) have a multibasic amino acid sequence motif at the C-terminus of the F2 protein, and a phenylalanine at the N-terminus of the F1 protein, 112R/G/K-R-Q/K-K/R-RF117 and that are cleaved intracellularly by ubiquitous furin-like proteases found in

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most host tissues. This difference in protease activation is the major determinant of disease severity of NDV [5].. Based on sequence analysis of the fusion (F) gene, NDV strains are divided into two distinct classes (I and II). Class I viruses are mostly loNDV found in wild birds, and contain nine genotypes (1–9), but class II viruses were divided into multiple genotypes representing loNDV and vNDV. Almost all virulent NDV strains isolated from wild and domestic birds belong to class II, which can be further subdivided into at least 18 genotypes from I to XVIII [6].

Genotype VII (class II genotype VII) was firstly classified into two subgenotypes: VIIa, which represents viruses that emerged 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, genotype VII are classified into other subgenotypes VIIc, d, and e, which represents isolates from China, Kazakhstan and South Africa; and VIIf, g, h, and i, which represent African isolates [5].

In Egypt, the disease had been firstly identified in 1948 [7]. Now days, NDV outbreaks are still frequently occurring in vaccinated poultry flocks, despite the intensive vaccination programs [8-14].

From the above mentioned data our trail is designated in order to characterize the NDV strains responsible for multiple outbreaks in vaccinated Egyptian farms and study its genetic relation with available vaccine and wild viruses in GenBank.

Material and Methods

Virus Isolates

Four vNDV selected isolates (NDV / Chicken/ EG-MN / NRC / 2015, NDV / Chicken / EG-QU/ NRC/2015, NDV/Chicken / EG-SH / NRC / 2015 and NDV/Chicken/EG-SH2/NRC/2015) isolated and identified by [15] was further passed into allantoic sac at 9-11 days old specific pathogen free (SPF) embryonated chicken eggs (ECE) then incubated at 37 C for 4 days [1].

Haemagglutination (HA) and Hemagglutination Inhibition (HI) test [1].

Allantoic fluids were tested for haemagglutination (HA) using 1 % chicken RBCs. the HA titer of the allantoic fluid was determined. Field immune sera was used as control positive in HI test.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Extraction of viral RNA

The genomic viral RNA was extracted from harvested HA positive allantoic fluid by using QIAamp viral RNA extraction Kits according to the manufacture's protocol .

Conventional RT-PCR for detection of NDV F-Protein gene [14]

One step RT- PCR was carried using QIAGEN® OneStep RT-PCR kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. RT-PCR was used for the detection of partial F-gene of vNDV using the following primers: forward 5 -ATGGGCYCCAGACYCTTCTAC-3 and Reverse 5/ -CTGCCA CTGCT AGTT GT G ATAATC-3 that flanks a 535 bp of the F gene of NDV as previously described [16]. Thermal cycling RT-PCR conditions included a reverse transcription 50°C for 30 min. then an Initial PCR activation step 95°C for 15 min. followed by 39 cycles at 94°C for 30 sec., 55°C for 30 sec. and 72°C for 45 sec. then the final extension was performed at 72°C for 10 min.

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 (100 bp DNA markers) and visualized by ultraviolet (UV) trans illumination.

Purification of PCR Products from the gel and gene sequencing:

For gene sequencing, the target bands of specific size were excised from the gel and purified with the QIAquick gel extraction kit (Qiagen, Valencia, CA) according to the manufacturer instructions and The purified DNA was sequenced in an automated ABI 3730 DNA sequence (Applied Biosystems, USA).

Phylogenecity and phylogenetic tree

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 1) . A phylogenetic tree of aligned sequences was constructed by Boost-trap method. The deduced amino acid sequences were determined to detect the pathotype of isolated NDV.

TABLE 1. Newcastle disease viruses used in the phylogenetic tree construction.

Reference strain	Accession number	Genotype
APMV1/Chicken/US(TX)GB/1948	GU978777	Virulent
NDV/Chicken/N.Ireland/Ulster/67	AY562991	I
NDV/Mukteswar	EF201805	III
NDV/US/Largo/71	AY562990	V
Pigeon paramyxovirus-1/IT-227/82	AJ880277	VI
NDV/Chicken/Giza/Egypt/MR0/2012	JX173098	VIId
NDV/Ostrich/Ismailia/2010	JN193503	II
NDV/chicken/Egypt/4/2006	FJ969395	II
NDV/chicken/Egypt/2/2006	FJ969393	II
Hitchner B1/47	M24695	II
BeaudetteC/45	M24697	II
Mexico 468/01	EU518685	V
BITPI87079 (Italy)	AY135747	V
Chicken/China/SDYT03/2011	JQ015297	VIId
Turkey/Israel/111/2011	JN979564	VIId
Chicken/Israel/174/2011	JN849578	VIId
Apmv1/Chicken/Jordan/Jo11/2011	JQ176687	VIId
Chicken/Sudan/03/2003	GQ258670	VIId
Chicken-2601-Ivory Coast-2008	FJ772466	VIIb
Avian-1532-14-Mauritania-2006	FJ772455	VIIb
Chicken-3490-149-Cameroon-2008	FJ772478	VIIb
NDV/Chicken/Egypt/1/2005	FJ939313	Virulent
NDV/chicken/Egypt/MR2-1998	JX193769	II
NDV/Chicken-Behira/Egypt/MR6-2012	JX193771	II
NDV/VRCLU/Giza/2009	HQ455810	II
NDV/99/0655/1999/Australia	AY935494	Virulent
NDV/99/PR32/1997/Australia	AY935497	Virulent
NDV/strain NA-1	DQ659677	VII
NDV/chicken/IS/27/2017	KY510688	VII
NDV/chicken/IS/929/2016	KY510684	VII
NDV/chicken/Egypt/Dakahlia28/2016	KY075891	VII
NDV/Chicken/Egypt/3/2006	FJ969394	Virulent
Clone_30	Y18898	II
La_sota	AJ629062	II
VGGA-87	AF419411	II
Komarov_45-LK	AY170137	II
NDV-Chicken-Egypt-MR3-2003	JX193770	II
NDV/chicken/Egypt/Damietta9/2016	KY075882	VIId
NDV/chicken/Egypt/Qualyobia11/2016	KY075884	VIId
APMV1/chicken/EG-KB2/POD.CU/2015	KX353698	VIId
APMV1/chicken/EG-SHR/POD.CU/2015	KX353695	VIId
NDV/chicken/Egypt/Ismailia8/2016	KY075881	VIId
NDV/chicken/Egypt/El-Arish15/2016	KY075887	VIId
NDV/Duck/China/Guizhou/ZY/2014	KU933948	VIId
NDV/Pheasant/H-Israel/2013/746_828	KF792022	VIIb

Results

Identification of ND virus

The passaged isolates were positive for HA and HI tests. This result of the 4 isolates were confirmed by RT-PCR that indicated the detection F-gene of vNDV result in amplicon 535 bp.

RT-PCR NDV isolates were detected by RT-PCR using degenerate primers for the fusion protein gene result in amplicon 535 bp as shown in Fig. 1.

Phylogeny and genetic analysis of the NDV isolates strains

The phylogenetic analysis of partial sequences of the selected four NDV named (NDV/Chicken/EG-MN/NRC/2015,NDV/Chicken/EG-QU/NRC/2015,NDV/Chicken/EG-SH/NRC/2015 and NDV/Chicken/EG -SH2/NRC/2015) strains for F gene showed that the isolated viruses belong to genotype VII (Fig. 2).

The obtained sequences alignment with a Chinese genotype VII (Chicken-China-SDYT03-2011) reference strain (retrieved from the Genbank) revealed that the obtained 4 isolates A.A sequence are showing (98-99) % identity with the genotype VII. As shown in Fig. 3.

The amino acid sequence of fusion protein cleavage site of the isolates carries motif ^{112}RRQKRF^{117} that is consistent with viruses of velogenic strains. Fig.4.

Sequence Submission to Gene Bank

Sequence of the isolated NDV strains in this study (NDV/Chicken/EG-MN/NRC/2015, NDV/Chicken/EG-QU/NRC/2015, NDV/Chicken/EG-SH/NRC/2015 and NDV/Chicken/EG -SH2/NRC/2015) 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 (MF418017), (MF418018), (MF418019) and (MF418020); respectively.

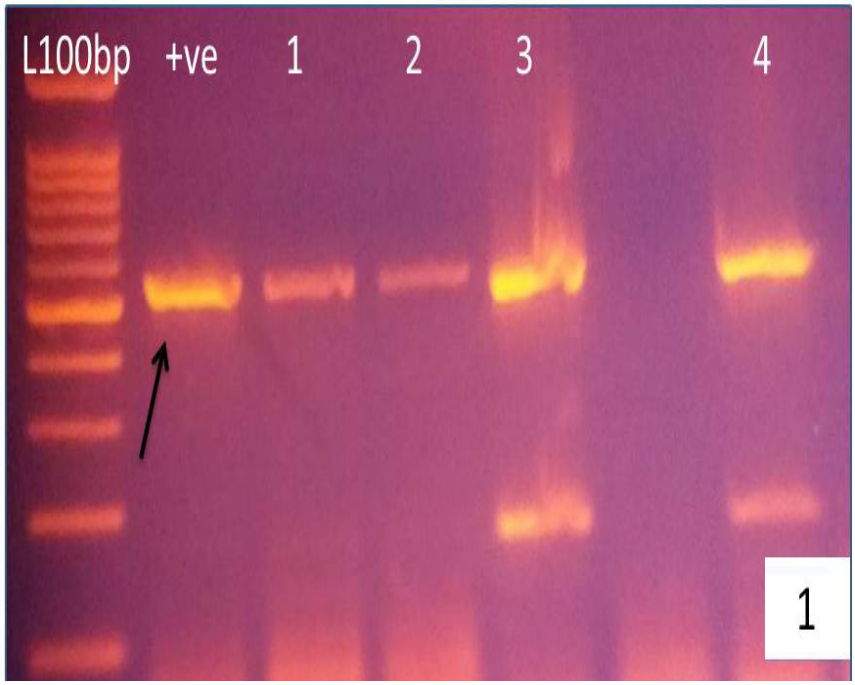

Fig. 1. Agarose Gel Electrophoresis (AGE) pictures showing 535 bp RT-PCR Products of NDV Fusion protein gene.

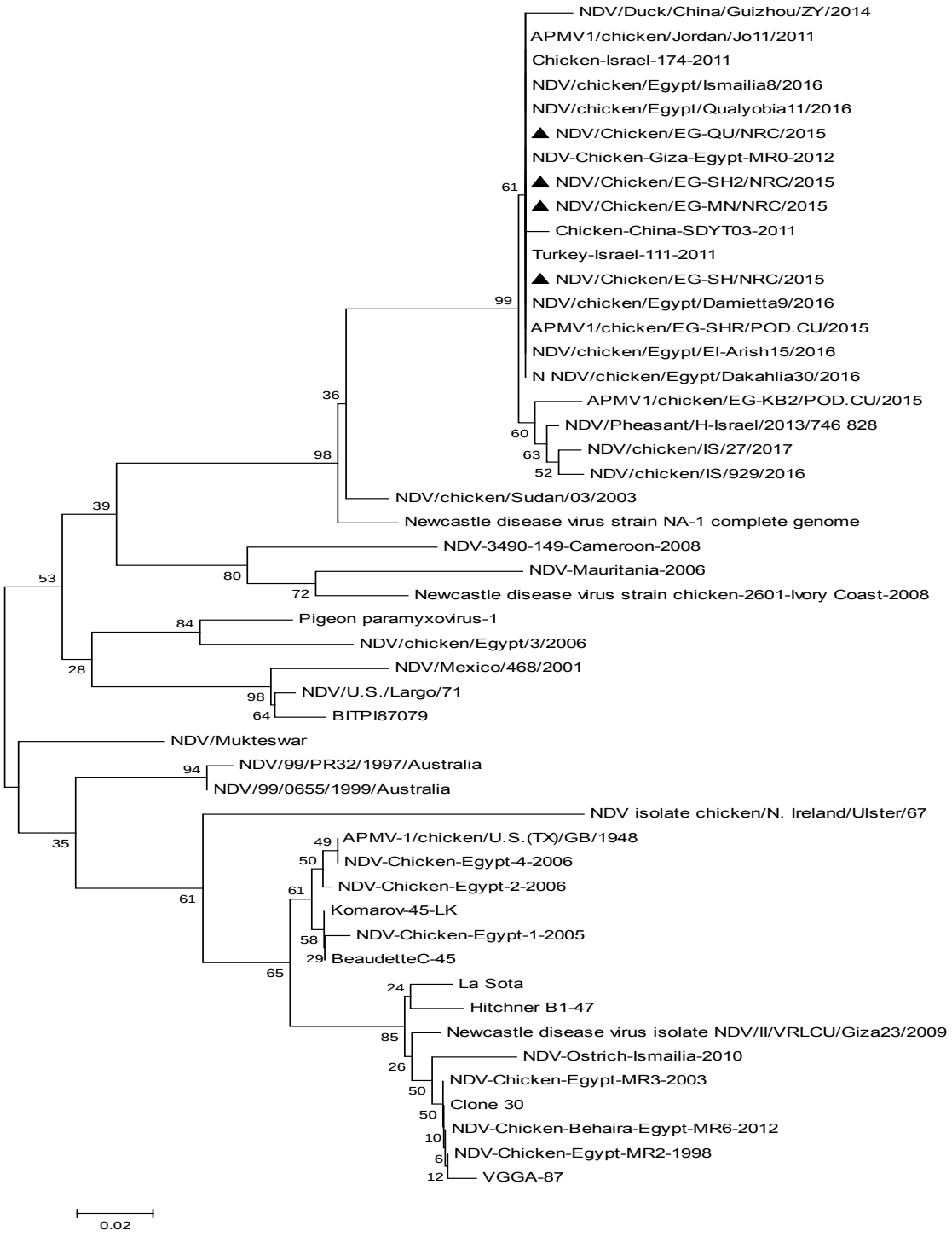

Fig. 2. Phylogenetic tree based on a partial sequence of NDV F gene. showing the relationship between the selected Egyptian NDV isolates in the present study with vaccinal strain and reference NDV strains from gene bank. Black dots refer to viruses isolated in current study.

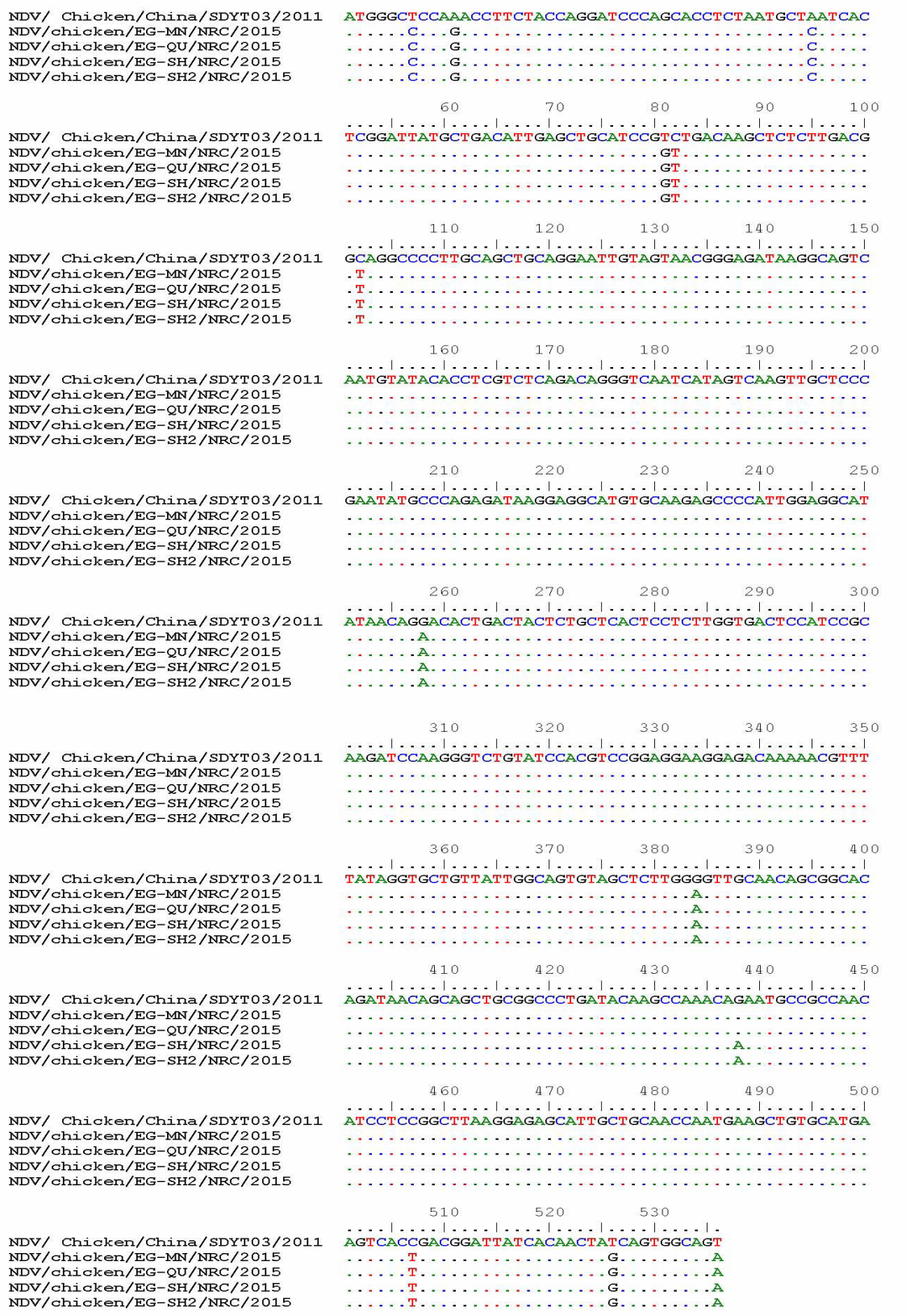

Fig.3. Alignment of the obtained isolates nucleotides sequences with reference isolate

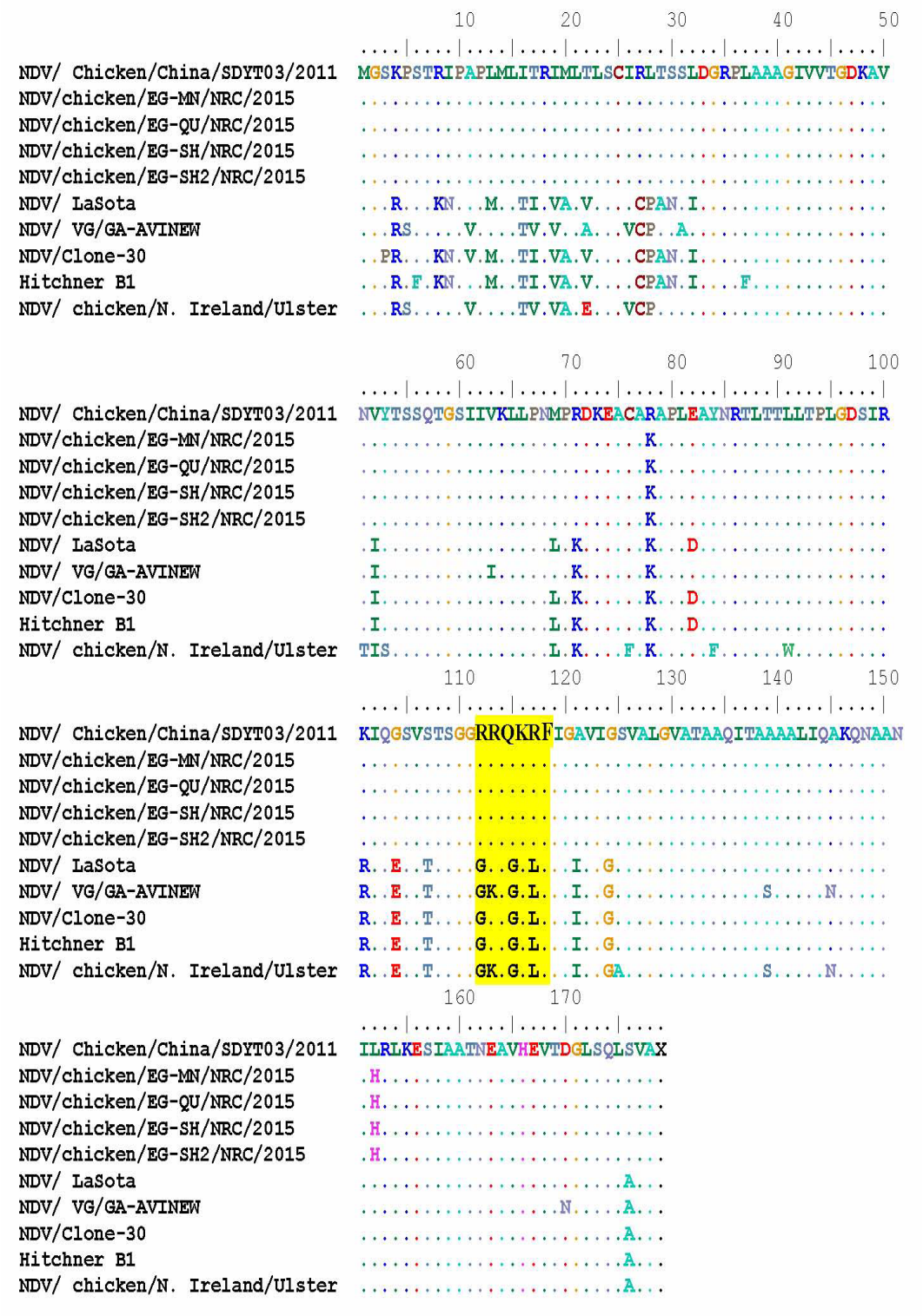

Fig.4. Deduced amino acid sequences of the isolated NDV strains in comparison to commonly used vaccine strains and reference isolate.

TABLE 2. Amino acid sequence identity showing identity percent based on A.A sequence comparison of 535 bp of fusion gene , black squares indicate identical sequences

sequence		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Chicken/China/SDYT03/2011	1		98.8	98.3	98.3	98.3	98.3	78.2	100	87.1	78.2	84.9	100	67.5	99.4	55.3	81.5	84.3	83.2	84.3	84.9	56.4
chicken/Israel/174/2011	2	98.8		97.2	97.2	97.2	97.2	79	98.8	88.1	79	83.7	98.8	66.4	98.3	54.1	80.4	83.2	82.1	83.2	83.7	57
NDV/Chicken/EG-MN/NRC/2015	3	98.3	97.2		100	100	100	78.7	98.3	87.1	78.2	86.5	98.3	67.5	97.7	56.4	82.1	84.9	84.9	84.9	85.4	56.4
NDV/Chicken/EG-QU/NRC/2015	4	98.3	97.2	100		100	100	78.7	98.3	87.1	78.2	86.5	98.3	67.5	97.7	56.4	82.1	84.9	84.9	84.9	85.4	56.4
NDV/Chicken/EG-SH/NRC/2015	5	98.3	97.2	100	100		100	78.7	98.3	87.1	78.2	86.5	98.3	67.5	97.7	56.4	82.1	84.9	84.9	84.9	85.4	56.4
NDV/Chicken/EG-SH2/NRC/2015	6	98.3	97.2	100	100	100		78.7	98.3	87.1	78.2	86.5	98.3	67.5	97.7	56.4	82.1	84.9	84.9	84.9	85.4	56.4
NDV/chicken/Behaira/Egypt/MR6/2012	7	78.2	79	78.7	78.7	78.7	78.7		78.2	72.1	96.4	89.9	78.2	74.8	77.6	60.8	82.6	92.1	92.1	83.2	92.1	50.8
NDV/chicken/Egypt/Dakahlia28/2016	8	100	98.	98.3	98.3	98.3	98.3	78.2		87.1	78.2	84.9	100	67.5	99.4	55.3	81.5	84.3	83.2	84.3	84.9	56.40
APMV1/chicken/EKB2/POD.CU/2015	9	87.1	88.1	87.1	87.1	87.1	87.1	72.1	87.1		72.1	74.8	87.1	72.3	86.	59.5	70.3	73.7	73.1	73.7	74.3	62.5
NDV/chicken/Egypt/MR2/1998	10	78.2	79	78.2	78.2	78.2	78.2	96.4	78.2	72.1		89.3	78.2	74.8	77.6	60.8	82.1	91.6	91.6	82.6	91.6	51.4
NDV/Chicken/Egypt/1/2005	11	84.9	83.7	86.5	86.5	86.5	86.5	89.9	84.9	74.8	89.3		84.9	79.3	84.3	69.8	86.5	96.6	97.2	87.1	96	50.2
Pheasant/H-Israel/2013/746 828	12	100	98.8	98.3	98.3	98.3	98.3	78.2	100	87.1	78.2	84.9		67.5	99.4	55.3	81.5	84.3	83.2	84.3	84.9	56.4
NDV/Ostrich/Ismailia/2010	13	67.5	66.4	67.5	67.5	67.5	67.5	74.8	67.5	72.3	74.8	79.3	67.5		67	79.3	70.3	81.5	80.4	71.5	81	58
Duck/China/Guizhou/ZY/2014	14	99.4	98.3	97.7	97.7	97.7	97.7	77.6	99.4	86.5	77.6	84.3	99.4	67		54.7	81	83.7	82.6	83.7	84.3	55.8
Komarov/45/LK	15	55.3	54.1	56.4	56.4	56.4	56.4	60.8	55.3	59.5	60.8	69.8	55.3	79.3	54.7		58.1	66.4	67	58.6	65.9	72.2
chicken/N. Ireland/Ulster/67	16	81.5	80.4	82.1	82.1	82.1	82.1	82.6	81.5	70.3	82.1	86.5	81.5	70.3	81%	58.1		88.2	88.2	93.2	88.8	46.9
LaSota	17	84.3	83.2	84.9	84.9	84.9	84.9	92.1	84.3	73.7	91.6	96.6	84.3	81.5	83.7	66.4	88.2		98.8	88.8	99.4	48.6
clone 30	18	83.2	82.1	84.9	84.9	84.9	84.9	92.1	83.2	73.1	91.6	97.2	83.2	80.4	82.6	67	88.2	98.8		88.8	98.3	48.6
VG/GA-AVINEW, complete genome	19	84.3	83.2	84.9	84.9	84.9	84.9	83.2	84.3	73.7	82.6	87.1	84.3	71.5	83.7	58.6	93.2	88.8	88.8		89.3	49.1
Hitchner B1/47	20	84.9	83.7	85.4	85.4	85.4	85.4	92.1	84.9	74.3	91.6	96	84.9	81	84.3	65.9	88.8	99.4	98.3	89.3		48.6
chicken/Sudan/03/2003	21	56.4	57	56.4	56.4	56.4	56.4	50.8	56.4	62.5	51.4	50.2	56.4	58	55.8	72.2	46.9	48.6	48.6	49.1	48.6	

Discussion

Newcastle disease virus considered the most predominant avian viral diseases affecting poultry industry in Egypt causing high economical losses , although intensive vaccination programmes carried out in field [17]. This may be due to genetic diversity among NDV strains in last years [5] resulting in field outbreaks or emerging of new pathotype resulting in severs infection [10]. So, the isolation and pathotyping of NDV from outbreaks among chickens is a critical for the control of NDV and vaccination evaluation [13].

Molecular identification by RT-PCR revealed that; our isolate is Newcastle field virus this result obtained from positive sample from clinical cases after primary isolation in specific pathogen free embryonated chicken egg (SPF-ECE). Many researcher used RT-PCR for isolation of Newcastle disease as reliable method for detection of positive cases [18,19] which considered accurate method for detection of NDV.

For relatedness detection of isolated NDV field isolates sequencing is carried out,This method is a rapid and reliable method for NDV pathotyping as compared to the mean death time, the intravenous pathogenicity index or intracerebra pathogenicity index tests [20,21]. Results confirmed that all strains of this study shared the cleavage site motif112 RRQKRF117 which is characteristic for vNDV strains and these results also revealed that our isolated strain belonged to class II genotype VIId which considered velogenic strain causing severe outbreaks in china [22] and middle east [23].

The recent vNDV isolates in this study (genebank acc. no.MF418017 , MF418018 , MF418019 and MF418020) showed complete identity to each other with 100% identity and 96%-99% identity with other vNDV strains present on genbank.

It is concluded that, NDV isolates circulating among chickens are virulent (Genotype VIId) and associated with outbreaks in poultry farms and is responsible for severe economic losses.

For prevention control of such virus it is recommended for further protective trial using different vaccination regimes for choosing the best protocol in order to control such pathotype under our Egyptian field conditions which needs further investigations.

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تحديد وتسلسل النمط الوراثي السابع من فيروس مرض النيوكاسل من قطعان الدجاج في ست محافظات مصرية

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مرض النيوكاسل هو مرض معدى جدا في الدجاج والديوك الرومي وأحد أهم أمراض الدواجن في العالم. تسبب العدوى بهالنفوق المفاجئ مع ارتفاع معدل النافق بين قطعان الدجاج . أظهر مرض النيوكاسل في السنوات الأخيرة أعراض أكثر حدة من الأعراض السابقة له. وقد أثبت أن العدوى بالسلالة السابعة لمرض النيوكاسل هى السبب الرئيسى فى انتشار هذا المرض فى العديد من مزارع الدواجن التجارية. في هذه الدراسة تم تصنيف الفيروسات المعزولة جزيئيا عن طريق تفاعل انزيم البلمرة المتسلسل استهدف جزء من الجين F الخاص بالنيوكاسل. وقد أظهر تحليل تسلسل الجينات لجزء من الجينF أن سلالات النيوكاسل المعزولة تنتمي إلى النمط الوراثي السابع مع تواجد تسلسل الأحماض الأمينية المميزة لمنطقة الانقسام البروتيني للبروتين112RRQKRF117) F0) لسلالات النيوكاسل شديدة الضراوة. تم اختيار 4 معزولات من معزولات النيوكاسل شديدة الضراوة NDV/Chicken/EG-MN/NRC/2015, NDV/Chicken/EG-QU/NRC/2015, NDV/) (Chicken/EG-SH/NRC/2015 and NDV/Chicken/EG-SH2/NR/2015 وتسجيلها على بنك الجينات تحت الارقام التالية ((MF418019), (MF418018), (MF418017 *MF418020))* بالترتيب.

وعلى الرغم من عدم وجود تغييرات جينية ملحوظة فى تلك المعزولات ما زال مرض النيكاسل منتشر بشدة بين قطعان الدجاج المحصنة ويتطلب التطور الدورى للتحصينات المستخدمة ضد هذا المرض وتقييمها ايضا.

الكلمات الدالة: التعرف على ، التسلسل الجينى ، فيروس مرض النيوكاسل ، الدواجن.