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Biological and Molecular Characterization of Newcastle Disease Virus Circulating in Chicken Flocks, Egypt, During 2014-2015

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Abstract

Newcastle disease (ND), a highly contagious disease, is responsible for disturbing disease outbreaks in poultry flocks worldwide. This study was carried out to isolate and characterize the circulating Newcastle Disease Virus (NDV) strains in different chicken flocks (broilers, commercial egg layers and breeders) in four Egyptian governorates (Gharbia, El Behera, Dakahlia and Kafer EL Shiekh) during 2014 and 2015. Forty-eight suspected NDV infected flocks were examined clinically and used for virus isolation and characterization. The mortality rates among the examined flocks were 5-85 %. The deduced amino acid sequences of F protein gene revealed that four NDV isolates possessed the motif ^{112}R/K-R-Q-K/RR^{116}at the C-terminus of the F2 protein and F (phenylalanine) at residue 117, indicating that they are velogenic genotype. The present study revealed the circulation of NDV class II genotype VII in Egyptian chicken flocks.

Keywords: Newcastle Disease, Genotype VII, F protein

Introduction

Newcastle disease (ND) is a highly contagious disease causing severe economic losses to the poultry industry due to high morbidities and mortalities. The disease is caused by NDV of *Avian Paramyxovirus* Serotype1 (APMV-1) with in Genus *Avulavirus* in family *Paramyxovidae* [1]. The criteria of NDV virulence are: a) demonstration of multiple basic amino acids at least three arginine or lysine between residues 113 and 116 at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein, b) Intracerebral pathogenicity index (ICPI) in day-old chicks of 0.7 or greater. The in ability to display the characteristic pattern of amino acid residues would require the characterization of the isolated virus by ICPI test [1]. The genome of NDV is composed of six genes that code for the six major structural proteins: hemagglutinin neuramindase protein (HN), nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), and large RNA-dependent RNA polymerase protein (L) [2,3].

ND viruses vary in their virulence or pathogenicity for chickens and based on this difference, NDV strains are classified into three pathotypes: velogenic, mesogenic and lentogenic [4]. The velogenic strains are more virulent than the mesogenic strains, but both are classified as being virulent, while, the lentogenic strains, are considered avirulant [4]. The vaccination strategy should be revised due to the repeated NDV outbreaks which became more severe in the vaccinated chickens. In addition, other factors may have contributed to the vaccination failure, such as the presence of immunosuppressive agents and poor cross immunity between the vaccines and challenge field virus strains [5]. NDV is endemic in many countries including Egypt [6]. The emergence

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and spread of new genotypes across the world is considered a significant threat to poultry suggesting the continuous evolution of velogenic ND strains (VND) leading to more diversity [7]. Viruses from genotype VII that have been spread from Asia, Africa, Europe were responsible for the fourth panzootic and has been isolated in South America [8,9]. The phylogenetic analysis based on the nucleotide sequences of the F gene revealed the classification of the Egyptian NDV isolates from different outbreaks into genotype VIId [10-14]. Therefore, the aims of the current study were the isolation, genotyping and phylogenetic analyses of the circulating NDV from field clinically infected chicken flocks.

Material and Methods

Clinical and post-mortem examination

A total number of 48 chicken flocks of different type of birds (broilers, commercial egg layers and breeders) with different ages suspected to be affected with ND at four governorates (Gharbia, El Behera, Kafer El Shiekh and Dakahlia) during 2014 and 2015 were investigated clinically (Table 1).

Virus isolation and hemagglutination

Tissue samples including trachea, spleen and liver were collected from freshly dead birds for virus isolation. Samples were processed and inoculated in 9-11 day-old embryonated chicken eggs (ECE) according to the protocol of OIE and European standard [1]. The collected allantoic fluids (AF) were screened by rapid hemagglutination test (HA). Quantitative hemagglutination was applied according to Terregino and Capua [15]. At least three blind passages were applied for each sample to be considered negative [16].

Micro Haemagglutination Inhibition (HI) test

Identification of NDV isolates was carried out using quantitative HI Test (α technique) with constant-serum diluted- antigen [17]. This method is based on a reaction between the virus and the specific hyper immune serum obtained from rabbits against NDV and contained titer log 2^9 that represents the antibodies (prepared in the laboratory of Poultry Diseases Department, Faculty of Veterinary Medicine, Damanhur University).

Reverse transcription Polymerase Chain Reaction (one step RT-PCR)

The RNA was extracted from HA positive allanoic fluids using the Gene JET Genomic RNA purification kit (Thermo Scientific) according to the manufacturer's instructions. The primers used for the identification of NDV were M2: 5′-TGG AGC CAA ACC CGC ACC TGC GG-3′ and F2: 5′- GGA GGA TGT TGG CAG CAT T-3′ [18]. The extracted RNA was tested for the presence of NDV using GeneJET Viral RNA Purification Kit/ K0821(Thermo Scientific). Reverse transcription was carried out at 50^{o}C for 15 min, followed by an initial denaturation at 95^{o}C for 2 min, cDNA was then amplified with 40 cycles of 95^{o}C for 30 sec, 54^{o}C for 1 min and 72^{o}C for 1 min, followed by a final extension step at 72^{o}C for 10 min. RT-PCR product was visualized by electrophoresis in 1.5% agarose in 1X TAE, ethidium bromide was added to a concentration of 0.5 µg /mL for nucleic acid visualization. For excluding Avian Influenza Viruses, HA positive isolates were screened using H5 and H9 primers [19, 20].

Sequencing

Four NDV isolates were selected for sequencing and phylogenetic characterization to represent the four governorates under investigation. Amplicons of the proper molecular size were purified by QIAquik PCR product purification protocol**,** using QIAquick PCR Product extraction kit (Qiagen Inc. Valencia CA) following the manufacturer guidelines. Sequencing was then carried out using BigDye® Terminator v3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA.USA, cat. no. 4336817) in an Applied Biosystems 3130 genetic analyzer (Hitachi, Japan), with 3130 Genetic Analyzer Capillary Array, 3730 Running Buffer, BigDye® Terminator v3.1 5X Sequencing Buffer,Hi-Di™ Formamide. The nucleotide sequences were submitted to GenBank with accession numbers KU377781, KU377782, KU377783 and KU377784.

Sequence and phylogenetic analysis

The obtained sequences were aligned by the Clustal W method using MegAlign module of DNAStar software (Lasergene version 7.2, DNASTAR, Madison, WI, USA). The nucleotide sequences were compared with NDV sequences available in GenBank and commonly used vaccinal strains. The phylogenetic tree of aligned sequences was constructed by neighbor-joining method [21] in MEGA6 [22] (www.megasoftware.net). The tree topology was evaluated by 1000 boot strap analysis. The deduced amino acid sequences were determined to identify the pathotype of the isolated NDV strains. The evolutionary distances were computed using the Maximum Composite Likelihood method [23] and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA6.

Titration of NDV isolates

The median embryo infection dose 50 (EID50) of each isolate was determined using the method of Reed and Muench [24].

Pathogenicity test

Intracerebral Pathogenicity Index (ICPI)

The ICPI of the four NDVs "NDV/EG/CH/18/2015, NDV/EG/CH/24/2014, NDV/EG/CH/26/2014 and NDV /EG/ CH/ 35/ 2014" were determined according to the OIE [1]. Briefly, freshly harvested infective AF with HA titre > 2^4 was diluted to 1: 10 in sterile isotonic saline (antibiotics free), and then 0.05 mL/chick of the diluted virus was injected intracerebrally into each of ten 1 day-old chicks (24-40 hour after hatching). The chicks were examined at intervals of 24 h for 8 days. At each observation, each bird was scored: 0 = normal, 1 = sick, 2 = dead. The index was calculated as 10 birds observed for 8 days = 80 observations

Index = mean score per bird per observation. The ICPI is the mean score per bird per observation over the 8-day period. The most virulent isolates have an ICPI close to 2.0, lentogenic and asymptomatic enteric viruses have values of 0.0.

Mean Death Time (MDT)

Tenfold (10^{-6} to 10^{-9}) dilutions of fresh infective AF in sterile phosphate-buffered saline (PBS) were prepared [25]. From each dilution, 0.1 mL was inoculated into the allantoic cavities of five 9 days old embryonated Specific Pathogen Free (SPF) chicken eggs. The inoculated eggs were incubated at 37°C, examined two times daily for 7 days and the times of the embryo deaths were recorded. The MDT has been used to characterize the NDV pathotypes as follows: velogenic, less than 60 h; mesogenic, 60 to 90 h; and lentogenic, more than 90 h.

Results

Clinical findings

The clinical signs of the examined flocks included respiratory signs such as cough, sneezing, nasal discharge and gasping, peri-orbital edema, nervous signs (prostration, complete reluctance to move, torticollis, opisthotonus and abnormal gait and paralysis of wings and legs). In addition to characteristic greenish diarrhoea. The mortality rates were 5-85% in the examined flocks (Table 1). The most commonly observed post-mortem lesions in this study were haemorrhages on the tip of the glands of proventriculus, button-like ulcers in the wall of intestine, hemorrhagic caecal tonsils, congested larynx and trachea with excess mucus in the lumen and congestion of lungs (Figure1).

Table (1): Clinical history of chicken flocks suspected to be NDV infected

Total Number of flocks	Total Mortality rate	Type of birds	No. of Flocks	Age range by days	Governorate
		Broiler	4	26-35	Gharbia
10		Balady	2	46-50	Gharbia
	5-15%	Breeder pullets	1	38	Dakahlia
		Broiler saso	2	39	Kafer El Shiekh
		Layer pullets	1	40	Kafer El Shiekh
		BroilerSaso	1	29	Gharbia
24	16-30%	Balady	2	33-40	Gharbia
		Broiler	21	26-33	Gharbia
		Broiler	3	28-30	El Behera
8	31-60%	Broiler	5	26-32	Gharbia
1	61-85%	Saso broiler	1	44	Dakahlia
5	Not recorded	Broiler	5	27-32	Gharbia

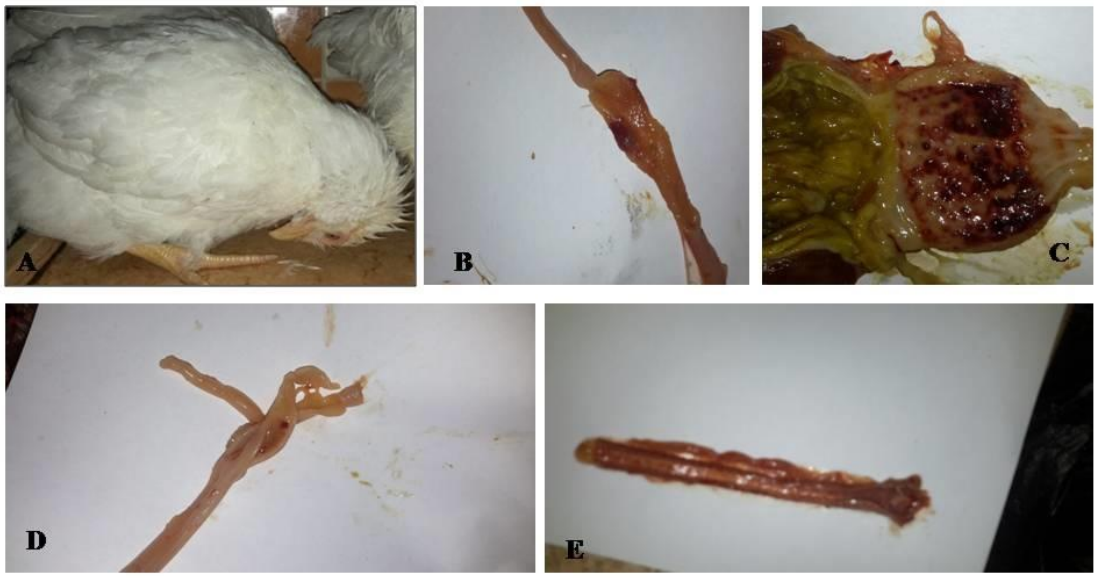


Figure (1): Clinical signs and gross lesions in chickens suspected to be naturally infected with NDV A) Nervous signs (torticollis, head shaking), B) Ulceration in intestine, C) Hemorrhages on proventriculus gland tips, E) Hemorrhages on cecal tonsils, D): Tracheal congestion.

Virus isolation and hemagglutination

Out of 48 samples, 21 (43.8%) were positive for isolation of haemagglutinating viruses in embryonated eggs of 9-11 days. The positivity was distributed among the examined localities as 14, 4, 2 and 1 in Gharbia, El Behera, Dakahlia and Kafer EL Shiekh with the percentages of 36.8%,100%, 66.7% and 33.3%, respectively. The HA unit of allantoic fluid ranged from 1/8 to 1/128 (Table 2).

Micro Haemagglutination Inhibition (HI) test

The positive HA allantoic fluids (n=21) were submitted to α technique constant-serum diluted-antigen HI Test for the detection of NDV. The NDV was detected in 15 samples with a percentage of 71.4% using specific ND antiserum.

Molecular identification by one step conventional RT-PCR

All 21 HA positive allantoic fluids were screened by qualitative detection and confirmation of NDVs and AIVs using specific one step RT-PCR. Only 6 allantoic fluids were confirmed as NDV by RT-PCR giving specific amplicon size at 766 bp of matrix and fusion gene including the F_0 cleavage site. Three HA positive isolates were identified as AIV H5 and one isolate was AIV H9 (Table 2), while the remaining samples were negative for NDV and AIV PCR.

Table (2): Results of HA, HI and RT-PCR of infected allantoic fluids for detection of NDV and AIV (H5 and H9 subtypes)

Sample No	HA* titer log_2	HI titer log_2	RT-PCR		
			NDV	AIV H5	AIV H9
18	5	5	+ve	-ve	-ve
24	4	6	+ve	-ve	-ve
25	3	-	-ve	+ve	-ve
26	4	6	+ve	-ve	-ve
28	7	-	-ve	+ve	-ve
31	4	6	+ve	-ve	-ve
34	4	-	-ve	-ve	+ve
35	7	9	+ve	-ve	-ve
45	5	-	-ve	+ve	-ve
48	4	2	+ve	-ve	-ve

***The Micro plate HA and HI tests were carried out on the HA positive allantoic fluids after 1st passage**

Sequence and phylogenetic analysis

Four representative NDV isolates were chosen for further investigation by sequencing. The obtained results indicated that the four isolates possessed the F protein cleavage site amino acid motif ^{112} R/K-R-Q-K/RR^{116} and phenylalanine at residue 117 indicated they are velogenic (Figure 2). The phylogenic analysis of the four NDV isolates indicated that they were clustered with genotype VII and resembling the genotype VIId strain (NDV strain Chicken/China/SDWF07/2011GenBank accession number JQ015295) and the genotype VIId recently isolated Egyptian strains (Figure 3). The results also indicated that these four isolates are far from vaccine strains which are commonly used in the poultry field to protect the chicken from infection with NDV.

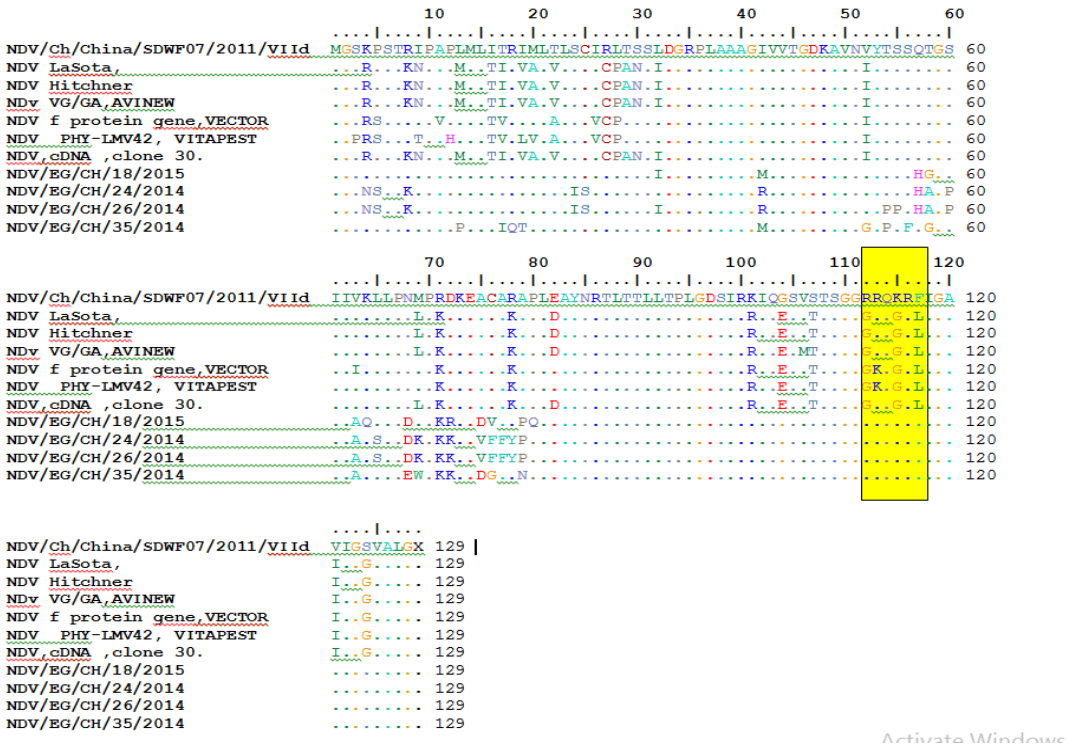


Figure (2): Deduced amino acid sequences of the fusion protein of NDV strains isolated in the current study in comparison to commonly used vaccines strains and reference isolates obtained from GenBank. Dots denote identical amino acids. Boxed segment indicate sequences of F protein cleavage site motif.

The pathogenicity of NDV field isolates

Intracerebral Pathogenicity Index

The ICPI for the four representative NDV isolates were classified as velogenic in sample No. 18 (ICPI value1.89), whereas the other two samples (No. 26, 35) had lower ICPI values (0.9 and1.1), respectively, and were classified as mesogenic and velogenic strains. Finally, sample No. 24 had ICPI value of 0.38 and it was considered lentogenic.

Mean Death Time

The MDT for the NDV isolates No. 18 ,35 were 48 h and were considered velogenic. While, isolate No. 26 was less than 60 h and isolate No. 24 was lentogenic because MDT was 96 h.

Discussion

Multiple NDV lineages circulating worldwide are genetically highly diverse [26-28]. Since 2011, NDV outbreaks occur in several vaccinated and non-vaccinated poultry farms in different governorates of Egypt [12]. The NDV is still one of the most important avian diseases of the poultry industry in Egypt, despite the vaccination against the disease for more than 60 years. Such situation could be attributed to many factors including: poor induced immunity by NDV vaccines or failure of vaccination due to inappropriate dose and regimens, existence of immunosuppressive diseases such as AIV, IBD or Marek's disease, nutritional deficiencies, as well as mycotoxins in feed [29]. Molecular epidemiology and phylogenetic analysis of NDV in Egypt is an important tool to determine and trace the source of infection and the possible routes of transmission that may help in the control of NDV [11]. This study was carried out to isolate, genotype and assess the genetic relatedness of the isolated NDVs circulating in four Egyptian governorates.

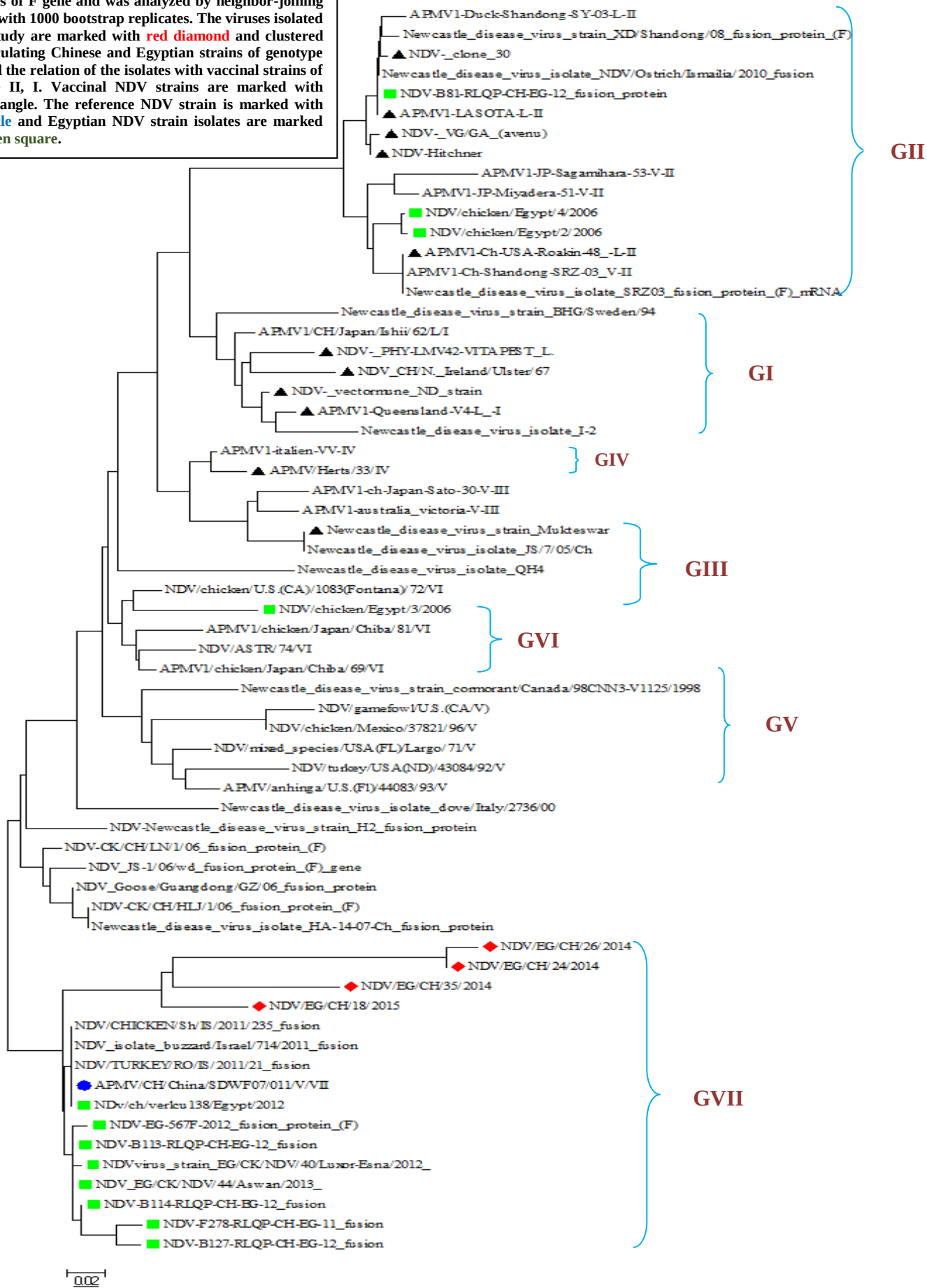
The clinical manifestations reported in this study confirmed the suspicious of NDV infection as previously mentioned by many authors [2,15,30-32]. Necrosis, lymphoid depletion and ulceration in intestine, payer's patches, cecal necrosis, enlarged spleen, and hemorrhages on proventriculus gland tips are common with virulent viscerotrropic NDV [30].

Twenty-one (43.8%) of the samples contained hemagglutinating viruses from different localities indicated the endemic spreading of virus infection in the governorates under study. Allantoic fluids from the inoculated ECE with pooled samples give positive reaction in hemagglutination and the variation may be due to the nature and virus titre in the used samples or the variable amount of the virus present in most tissue specimens [2]. Hemagglutination inhibition using specific hyperimmune serum containing anti-NDV in HI test is considered confirmatory to NDV. Fifteen samples were inhibited and the other 6 samples were negative for NDV, therefore, they could be other hemagglutination viruses such as AIV subtypes H5 or H9.

Reverse-transcriptase polymerase chain reaction (RT-PCR) is one of the most widely used methods to detect NDV, due to the advantages of being extremely specific and fast [33-37]. Out of 15 HI positive isolates, 6 were positive for NDVs. Although the specificity of RT-PCR, the negative results might be false negative and there are more sensitive molecular techniques such as real time quantitative PCR (rt-PCR) which is 100 folds sensitive and rapid when the amount of the virus is fewer than the limits of detection [38]. Also, as a result of the loss of the virus during sample processing especially samples with very low virus loading, inefficient amplification might give weak bands in gel electrophoresis.

ICPI results indicated that sample number 18 is virulent with ICPI value 1.89 and 48h MDT associated with periventricular hemorrhages, enteritis and pneumonic lungs, congestion of brain and liver and splenomegaly which agree with what was mentioned previously by Susta *et al.* [32].

Figure 3: Phylogenetic analysis of F gene amino acid sequences of NDV isolated from chickens in Egypt and other sequences available on GenBank. The tree was constructed via multiple alignments of amino acid sequences of F gene and was analyzed by neighbor-joining analysis with 1000 bootstrap replicates. The viruses isolated in this study are marked with red diamond and clustered with circulating Chinese and Egyptian strains of genotype VIId and the relation of the isolates with vaccinal strains of genotype II, I. Vaccinal NDV strains are marked with black triangle. The reference NDV strain is marked with blue circle and Egyptian NDV strain isolates are marked with green square.



Sequencing and phylogenetic studies of the fusion protein gene of NDV are more sensitive and rapid methods for pathotyping of NDV compared to the conventional pathotyping method [39]. Moreover, they provide valuable data to trace the origins and spread of NDV, and to distinguish low virulent viruses that could have a great potential for mutation to virulence by few point mutations which represents a great risk. Sequencing is also used to fulfill the conditions adopted by the OIE for determination of virulence of NDV strains [1,40,41]. All the NDV isolates in the present study had the amino acid sequence ^{112}RRQKRF^{117} at the C-terminus of the F2 protein and at the N-terminus of the F1 protein, residue 117. These characteristics classify them as virulent strains according to the definition of OIE [1]. The results of sequence analysis of the four NDV isolates revealed that the F protein cleavage site sequence is typical to virulent strains and the isolates were related to virulent /CH/China/SDWF07/011/V/VIId in F protein cleavage site sequence. In strain (NDV/ EG/CH/ 18/2015 and NDV/ EG/CH/ 35/2014) the amino acid identity was 89.5% and the divergence was 11.4% between amino acids. Regarding strains NDV/ EG/CH/ 24/2014 and NDV/ EG /CH/26/2014 the amino acid identity was 98.6% and the divergence between amino acids was 1.4%. Isolate NDV/EG/CH/24/2014 although was considered lentogenic by ICPI (0.38) and MDT 96 h it possesses the motif sequence of virulent NDV. This was in agreement with a study that demonstrated that some strains of Pigeon Paramyxovirus 1 (PPMV-1) had F protein cleavage site of velogenic but with low ICPI and was then considered lentogenic for chicken [42]. Genotype VII NDV strains of class II have maintained a constant threat to domestic poultry since the 2000s and the most recent Chinese isolates belong to sublineage d of genotype VII (VIId) [43-45]. The phylogenetic analysis of our four isolates based on the nucleotide sequences of the F gene revealed that they were classified into genotype VII. This was consistent with other studies [10-14].

The four isolates were velogenic resembling the genotype VII strain (NDV strain Chicken/ China/SDWF07/2011 GenBank accession number JQ015295). High amino acid sequence similarity of strains NDV/EG/CK/18/2015, NDV/EG /CK /35/2014, isolated with in the period of 2014 and 2015 but from different geographical regions, indicated the spread of the virus and the uncontrolled extensive movement of live poultry within the area of study. High phylogenetic distance between vaccines and current isolated strains in this study may facilitate the evolution of virulent NDV [46]. Evolution of NDV may be related to the accumulation of point mutations that induce amino acid substitution in the neutralizing epitopes, the cysteine residues, and N-linked glycosylation sites of the F protein [47].

Regularly, avian flocks are vaccinated by live avirulent and attenuated vaccines such as Hitchiner B1, La Sota and Clone 30 which belong to genotype II NDV but ND outbreaks still frequently occur. The phylogenetic analysis indicated that the four selected Egyptian isolates are far from La Sota vaccine, Hitchiner, AvinewVG/GA, Vectore Immune ND strain ND26/76, Clone30, Vitapest, Ulster. The amino acids sequence alignment of partial F0 gene of the selected four NDV field isolates of the current study with the reference strains and vaccinal strains from GenBank revealed that strain NDV/EG/CK/18/2015 was divergence in amino acid sequences by 40.1% with La Sota strain, 40.1% with Hitchiner, 36.2% with Avinew VG/GA, 35.4% with Vector Immune ND, 40.1% with Clone30, 38.5% with Vitapest indicating that protection from field infection by these vaccines will be low. Newer NDV vaccines should not only protect birds against the disease but, preferably, they also reduce the amount of virus shed by vaccinated birds to a level that will prevent transmission of the virus from bird to bird. In addition, a vaccine that lacks adverse reactions is also very much needed by the poultry industry.

Conclusion

Newcastle disease is still a major problem for poultry industry in Egypt. Deduced amino acid sequences and phylogenetic analysis of F gene of NDV revealed the circulation of NDV class II genotype VIId in Egypt during 2014 and 2015 outbreaks. Therefore, effective vaccination protocols to help in controlling the disease and economic losses in the Egyptian poultry industry are recommended. In addition, education of poultry farmers on biosecurity protocols is essential.

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربى

التوصيف البيولوجي والجزيئي لفيروس مرض النيوكاسل الذي يدور في قطعان الدجاج بمصر خلال ٢٠١٤-٢٠١٥

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مرض النيوكاسل الشديد العدوي والواسع الإنتشار بين مختلف أنواع الطيور يسبب خسائر فادحة بين قطعان الدواجن في مصر. لذلك تم فحص عدد ثمانية وأربعون من قطعان دواجن يشتبه أن بها عدوى NDV إكلينيكيا وعزل وتوصيف الفيروسات المعزولة. أشارت النتائج أن معدل النافق يتراوح مابين ٥% الى ٨٥% في هذه القطعان. تم إختيار4 من المعزولات وذلك لتحليل الحمض النووي وتحليل العلاقة لشجرية المتفرعة. جميع العترات لديها تتابع الأحماض الأمينية فى موقع الانشطار(112R-R-Q-K-R-F117) في نهاية الكربوكسيل من البروتين F2 والفينيل ألانين في الموقع ١١٧ من البروتينF1 ، وتم تصنيف العترات على انها من النوع الضارى.أشار تحليل مقارنة تسلسل النوكليوتيدات مع الأخرى المودعة في بنك الجينات إلى أن العترات الحالية تنتمى إلى النمط الجيني السابع (VII).