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Molecular Characterization of the Newcastle Disease Virus Currently Circulating Among Broiler Chicken Flocks During 2021 in Sharkia Province, Egypt

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Abstract

Newcastle disease (ND) outbreaks have been occurred in the Egyptian poultry causing high mortalities with severe economic losses. NDV infection of genotype VII has been reported to cause outbreaks in several commercial poultry farms in Egypt. In the present study, NDV was isolated from 6 broiler chicken flocks in Sharkia province suffering from respiratory and/or neurological signs into the allantoic sac of 11-day-old commercial embryonated chicken eggs (ECEs) as well as these isolates were subjected to reverse transcriptase - polymerase chain reaction (RT-PCR). Six isolates (one NDV isolate/each flock) were sequenced to characterize their whole fusion (F) protein gene of NDV. The sequence analysis and phylogenetic study of the six isolates revealed that they were velogenic, belonging to the class II subgenotype NDV VIId with the characteristic amino acid sequences of the F0 protein proteolytic cleavage site motifs (112-RRQKRF-117). As well as the nucleotide blast analysis showed 97.4% - 98.6% nucleotide identity to virulent NDV isolated in Egypt and neighboring countries. On the other hand, the nucleotide blast analysis showed 82.3% - 83% nucleotide identity to reference commercially available vaccine strains. This genetic variation between the commercially available vaccine strains and six studied NDV strains during 2021 may clarify the failure of the currently used vaccines to protect chicken against vNDV subgenotype VIId. Further studies are needed to monitor the protection of the currently used vaccines against recently isolated vNDV strains.

KEYWORDS

Newcastle disease virus, Reverse transcriptase- polymerase chain reaction, Virulence, Subgenotype, Vaccine, Chicken, Egypt.

INTRODUCTION

Newcastle disease (ND) is a globally reported viral disease affecting over 200 species of birds (OIE, 2012). It is an Organization International des epizootics (OIE) notifiable disease (OIE, 2021) however, only few countries report its incidence to OIE, especially in developing countries where the disease is enzootic among vaccinated and unvaccinated poultry (Shittu *et al*., 2016).

The etiology of ND is virulent strain of Avian orthoavulavirus1 also known as Newcastle disease virus (NDV) of the genus Orthoavulavirus belonging to the family Paramyxoviridae and order Mononegavirales (OIE, 2021). It is a negative sense, non-segmented, single stranded enveloped RNA virus (Mayo, 2002). NDV Fusion (F) glycoprotein mediates fusion between the viral and host cellular membranes (Yussof and Tan, 2001). During replication, the fusion protein gene is translated into a precursor protein (F0) that must be cleaved by host cell proteases into F1 and F2 subunits for viral particles to become infectious (Morrison *et al*., 1993). A virulent and virulent strain may also be distinguished on the basis of the cleavage site sequence of their fusion protein. Most virulent strains exhibit the consensus sequence 112(R/K) RQ (R/K)R/F117 at the cleavage site of the F0 precursor, in contrast to 112(G/E) (K/R)Q(G/E)R/L117 in a virulent viruses (Collins *et al*., 1993). The additional basic amino acids in the virulent viruses allow the F0 precursor to be cleaved by ubiquitous proteases. Thus, virulent viruses have the ability to replicate in a range of tissues and organs, causing fatal systemic infections (Nagai *et al*., 1976).

Most of the commercially available ND vaccinations do not entirely prevent infection or virus shedding, and disease can spread in vaccinated birds, according to several investigations on the use of various vaccines to control ND (Xiao *et al*., 2012). However, several investigations also indicate that the majority of commercially available ND vaccinations are not performing optimally, and there is disagreement regarding the degree of protection provided by these vaccines against newly emerging ND viruses (Kilany *et al*., 2015). For example, common NDV genotype II vaccine strains such as LaSota fully protected vaccinated chickens against clinical signs and mortalities but did not fully stop virus shedding post-challenge with velogenic NDV genotype VII.1.1 (Ali *et al*., 2019). For creating genetically matched vaccines that can stop ND vaccine failure and perhaps future outbreaks in the nation, knowledge of the vNDV's constantly changing genetic variety is crucial. The objective of this study was to isolate

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and characterize the full Fusion protein gene of the Newcastle Disease Virus (NDV), which was detected in a dead chicken of a vaccinated flock that was presented to a veterinary clinic for post-mortem examination in Sharkia province, Egypt during January to May 2021. As well as the sequence of fusion protein gene was then compared to previously reported vNDV isolates from Egypt and commercially available NDV vaccine strains.

MATERIALS AND METHODS

Sampling

Seventy-five tissue samples from 6 broiler flocks of 21–40 days old suffered from respiratory and/or nervous signs and high mortalities were collected during the period from January to May 2021. These samples were collected from different localities in Sharkia province. Briefly, Postmortem examination was conducted on all specimen, characteristic lesions were noted. Pooled tissue samples (proventricular glands, spleen, lung and cecal tonsils) per each flock were collected from freshly dead chickens with history of vaccination against NDV. All samples were placed into virus transport medium (VTM) with 10% antibiotic solution containing 1000 IU penicillin, 1000 µg streptomycin, 2000 µg gentamycin and the clear supernatant fluid of each tissue homogenate was subjected to cooling centrifugation at 3000 rpm /10 min and kept frozen at -20 °C till being used in virus detection and isolation (Table 1).

Isolation of NDV on commercial embryonated chicken eggs (ECEs)

The supernatant of tissue homogenates (200 µl) was inoculated into the allantoic sac of 9-day-old ECEs (five ECEs for each sample) from commercial non-vaccinated chickens. After 48-72 hours, the allantoic fluids of inoculated eggs were harvested and kept at -70 °C until subsequent use and further analysis. The collected allantoic fluids after three series of blind passages were tested using standard haemagglutination assay (OIE, 2012). All samples showed haemagglutination activity using 1 % chicken RBCs and haemagglutination inhibition using NDV reference antiserum. The further tested were applied against AIV matrix gene by RT-PCR according to Fouchier *et al*. (2000). Inactivated NDV antigen strain (Lasota strain) was used as a positive control.

Total viral RNA extraction, reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from six NDV isolates using RNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Specific primers for amplifying the complete F gene have been used (NDV-F 5′ ATGGGCTCCAAACCTTCTA 3′ and NDV-R5′GGAAACCTTCGTTCCTCAT3′) according to Nagy *et al*. (2020). RT-PCR was done using Qiagen OneStep RT-PCR Kit (Qiagen) according to the manufacturer's instructions. Briefly, 5 µl of extracted RNA was used per reaction with 1 µl of each primer, 5 µl of One-Step RT buffer mix, 1 µl dNTPs (10 mM) mix and 1 µl of RT-enzyme and then up to 25 µl of water nuclease-free. The cycling conditions started with one cycle at 50°C for 40 min, then an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 50°C for 20 s, extension at 72 °C for 30 s and final extension at 72°C for 7 min. PCR products were visualized on 1.5% ethidium bromide stained agarose, at 100 volts, for 20 minutes and compared with a 1Kbp DNA ladder (ThermoFisher), amplified products were visualized under ultraviolet transillumination, and then cut & purified from gel using QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions and kept at -20 °C until sequencing. The purified PCR products were shipped for sequencing at Macrogen, Korea using BigDye terminator v3.1 cycle sequencing kit according to the manufacturer's instructions. Sequences obtained was submitted on the NCBI database and assigned accession numbers OP611416 - OP611421.

Phylogenetic analysis

The amplified full length F gene of six NDV isolates were sequenced in both directions using Bigdye Terminator V3.1 cycle sequencing Kit (Perkinelmer, CA) utilizing the amplification primers in applied Biosystems 3130 genetic analyzer (ABI, USA). The nucleotide sequences of fulllength Fusion protein gene of the six isolates were sub¬mitted to the GenBank database under the accession numbers OP611416- OP611421. The nucleotide and amino acid se¬quences of the six isolates were com¬pared and aligned with the sequences of representative strains of different NDV genotypes existing in GenBank (Table 2). Comparative amino acid alignment was carried out using Clustal W Multiple alignment of Bio Edit Version 7.0 software (Hall, 1999). Amino acid sequence identities and divergences were calculated utilizing Meg Align software (DNA STAR® Laser gene® version 7.2, USA). Phylogenetic tree was created via the maximum-likehood method employing the Kimura 2-parameter model in MEGA6.06 software (Tamura *et al*., 2013) with bootstrapping 1000.

RESULTS

Molecular detection of NDV in field samples using RT-PCR

The overall fusion protein gene was detected in the six chicken broiler flocks. A 1662bp fragment was successfully amplified

Table 1. History of the collected field samples; include chicken flock size, farm location, collection date, chicken age, chicken breed, vaccination program and clinical signs.

Chicken farm number	Production type	Farm location	Collection date	Chicken age (days)	Vaccination programme	Postmortem lesions
1	broiler	Abokebeer	Jan-21	30	-H9N2 + NDV (inactivated LaSota)	High mortality rate, ecchymotic haemorrhage on proventriculus,haemorrhagic caecal tonsils, enlarged liver and spleen, Severe congestion in trachea
2	broiler	Awlad saqr	Apr-21	23	-H5N1 + NDV (inactivated LaSota)	
3	broiler	Belbies	Mar-21	21	-LaSota (live)	
4	broiler	Hehya	Jan-21	35		
5	broiler	Faqous	Feb-21	29		
6	broiler	Mashtol soque	May-21	40		

and PCR products of electrophoresis visualized using ultraviolet transillumination (Figure 1). The specific nucleotide sequences were deposited in the GenBank under accession numbers OP611416 - OP611421.

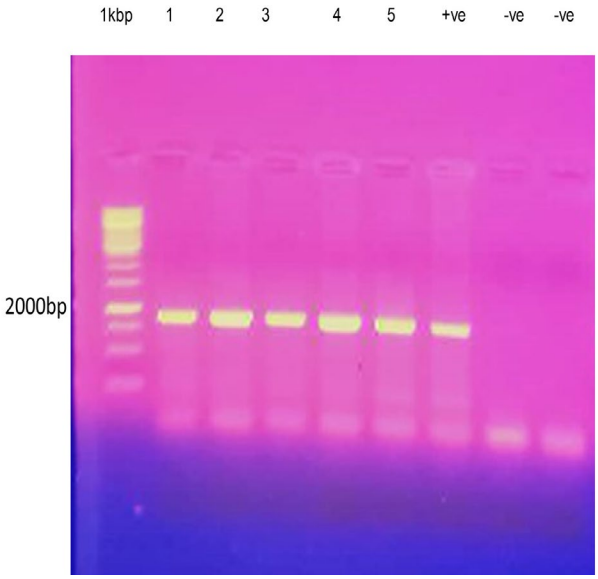

Figure 1. RT-PCR amplification of complete fusion protein gene from NDV isolates in the present study showed a single specific band approximately (1660 bp). Lane 1: 1 kbp DNA ladder (ThermoFisher); Lanes 2–6: positive NDV-infected samples; Lane 7: positive control; Lane 8: negative control; Lane 9: negative sample.

Sequencing and Phylogenetic analysis of fusion protein gene

Sequencing of six confirmed NDV isolates for full fusion protein gene by RT-PCR assay revealed that the expected and corrected size bands. The genetic relatedness of the six studied isolates, reference eight commercially available vaccine strains and NDV genotypes of known F gene sequences obtained from GenBank database was deduced by phylogenetic analysis (Figure 2). All study isolates clustered in the class II subgenotype NDV VIId that widely reported in Egypt. The Nucleotide sequence identities and divergences were calculated utilizing Meg Align software (DNA STAR® Laser gene® version 7.2, USA). Nucleotide blast analysis showed 97.4% - 98.6% nucleotide identity to virulent NDV isolated in Egypt and neighboring countries. On the other hand, The nucleotide blast analysis showed 82.3% - 83% nucleotide identity to reference commercially available vaccine strains (Figure 3).

Mutational analysis of the Functional and Antigenic domains of NDV- fusion protein gene

The complete translated 553 fusion protein amino acid sequences obtained from the study isolates were used to compare their functional and antigenic domains relative to nine vaccine strains using LaSota (AF077761) as reference, six vNDV strains previously reported from Egypt and neighboring countries (Figure 4). Along the Hypervariable region/Signal peptide (residue

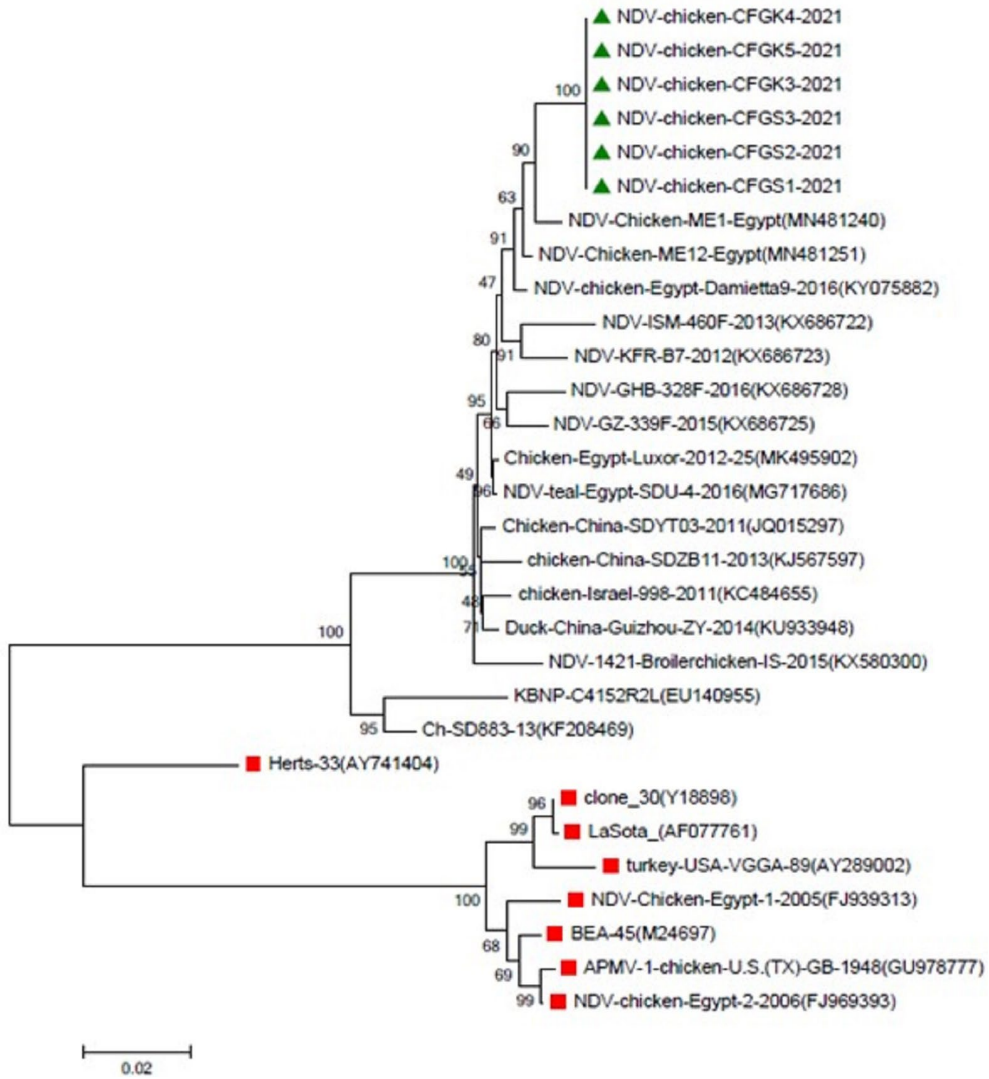

Figure 2. Phylogenetic tree based on nucleotide sequences of fusion protein gene for the six NDV isolates and related reference virulent and vaccine NDV strains using bootstrap consensus of 1000 replicates. The red square denoted vaccine strains and green triangle denoted the studied six NDV isolates.

1-31), 12 substitutions leading to R4K, K8R, N9I, M13L, T16I, I17T, V19I, A20M, V22T, C27R, P28L and A29T mutations was observed (Figure 4). The major epitopes involved in virus neutralization are conserved in all residues except for one amino acid substitution Lys to Arg (K78R) of the A2 neutralizing epitope identified in all the studied isolates (Figure 4). All six isolates share the characteristic virulent motif 112-RRQKRF-117 at the F0 cleavage site indicating that they are velogenic NDV strains (Figure 4). Along the fusion peptide region (117-142), three amino acid substitutions L117F, I121V and G124S substitutions were seen (Figure 4). The three Heptad Repeat regions HRa (143-185), HRb (268-299) and HRc (471-500) in the isolates displayed 1, 3 and 4 amino acid substitutions respectively compared to the LaSota reference strain (AF077761). A176S at HRa; N272Y, Q279H, and T288N at HRb and N479G, K480R, E482A, R486S, D489E, and K494R. Notably, a substitution N479G was seen only in in all studied isolates and but not in any other isolate included in the phylogenetic tree analysis (Figure 4).

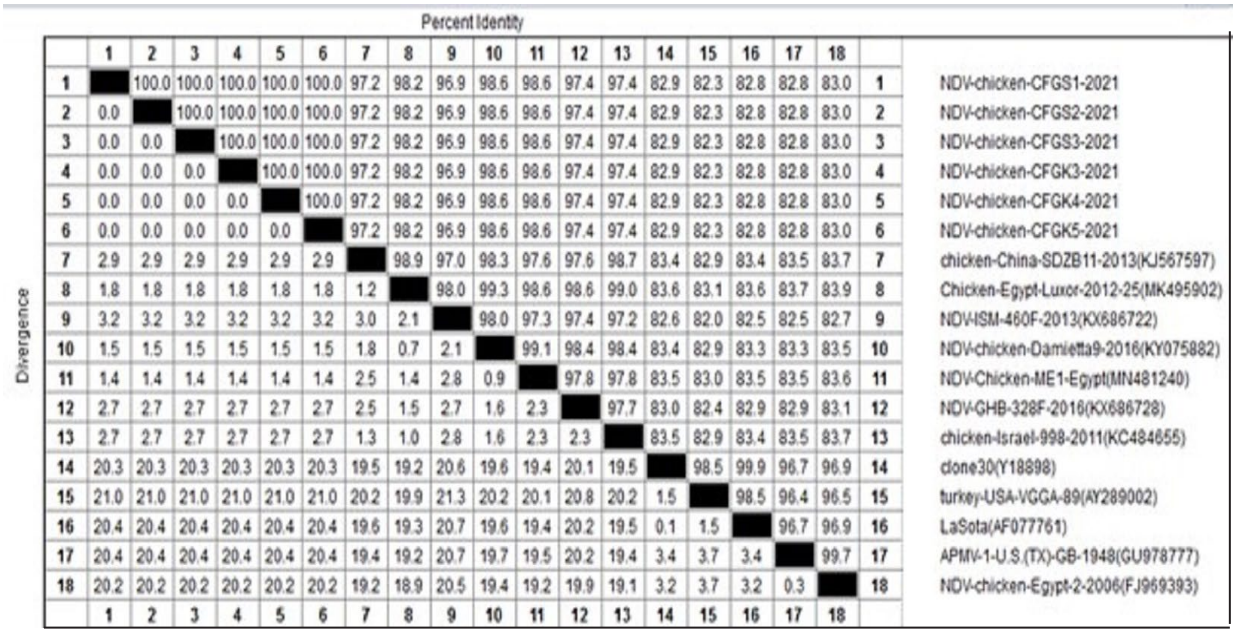

Figure 3. Percentage fusion protein gene nucleotide identity and divergence between the studied isolates, commercial vaccine strains and virulent NDV isolated in Egypt and neighboring countries. Black squares indicate identical sequences.

Table 2. Sequences of reference NDV strains published in GenBank which used in this study for the phylogenetic analysis.

GenBank accession number	Reference IBV strains	Country of isolation	Year of isolation
KJ567597	chicken-China-SDZB11-2013	China	2013
MK495902	Chicken-Egypt-Luxor-2012-25	Egypt	2012
MG717686	NDV-teal-Egypt-SDU-4-2016	Egypt	2016
JQ015297	Chicken-China-SDYT03-2011	Egypt	2011
KX686722	NDV-ISM-460F-2013	Egypt	2013
KX686728	NDV-GHB-328F-2016	Egypt	2016
KX686725	NDV-GZ-339F-2015	Egypt	2015
KC48465	chicken-Israel-998-2011	Israel	2011
Y18898	clone 30	Germany	1999
AY289002	turkey-USA-VGGA-89	USA	2004
AF077761	LaSota	Netherlands	1999
M24697	M24697	Japan	1989
GU978777	APMV-1/chicken/U.S.(TX)/GB/1948	USA	1948
EU140955	KBNP-C4152R2L	South Korea	2008
HAY741404	Herts-33	Netherlands	2005
KY075882	NDV-chicken-Egypt-Damietta9-2016	Egypt	2016
KU933948	Duck-China-Guizhou-ZY-2014	China	2014
KX686723	NDV-KFR-B7-2012	Egypt	2012
KX580300	NDV/1421/Broiler chicken/IS/2015	Israel	2015
KF208469	Ch-SD883-13	China	2013
FJ939313	NDV-Chicken-Egypt-1-2005	Egypt	2005
MN481240	NDV-Chicken-ME1-Egypt	Egypt	2016
MN481251	NDV-Chicken-ME12-Egypt	Egypt	2006

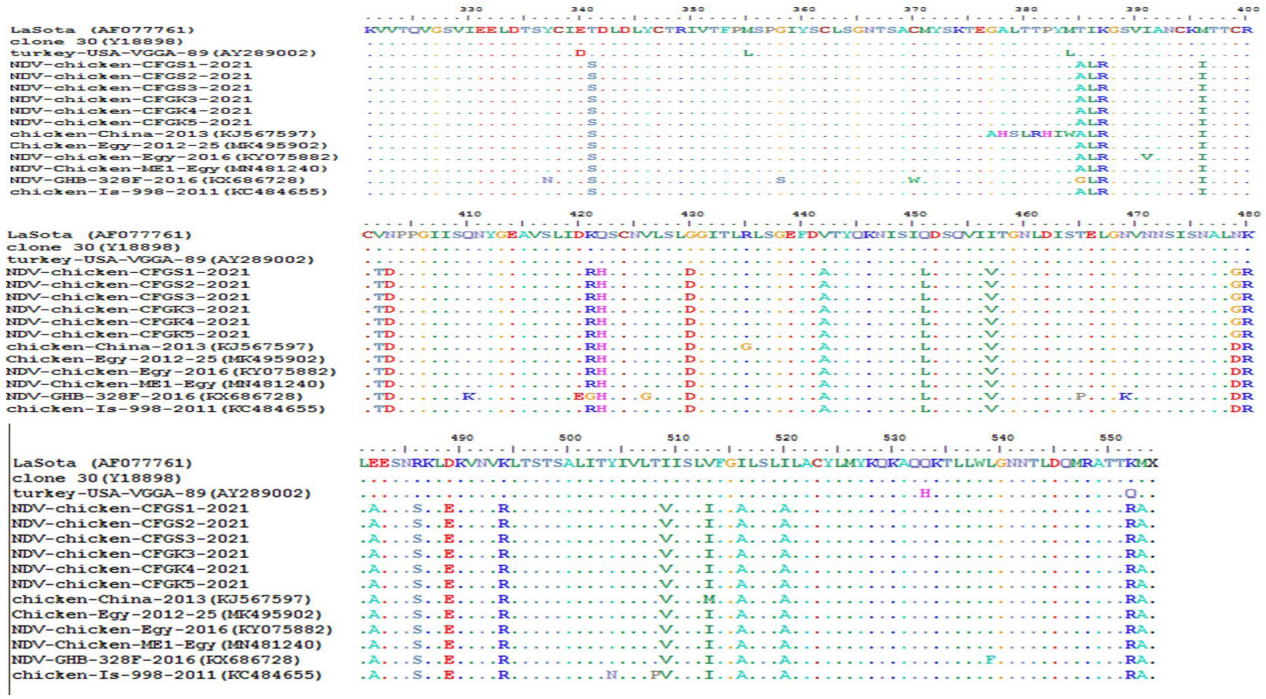

Figure 4. Mutation profile along fusion gene of Newcastle disease virus (NDV). Figures show mutational substitution of study isolates in comparison with vaccine strains LaSota (AF077761), Clone30 (Y18898), and VGGA-89 (AY289002) and the other isolates included in the phylogenetic tree analysis. Mutations along the Hypervariable region/Signal peptide (residue 1-31); the A2 neutralization epitope AGR (residue 78); F0 cleavage site region (residue 112-117); fusion peptide region (residue 117-142); and the three heptad repeat regions HRa (143-185), HRb (268-299) and HRc (471-500).

DISCUSSION

Egypt is endemic for Newcastle disease virus (NDV) with continuous evolution outbreaks causing great economic losses in chicken industry due to high mortality which may reach 100% in velogenic strains of NDV, despite the intensive vaccination programs. NDV outbreaks occurred in several vaccinated and non-vaccinated poultry farms in different gover¬norates since 2011 causing severe respiratory and nervous signs and high mortalities that resulted in severe economic losses in 3-4-week-old broiler chickens in Egypt (Radwan *et al*., 2013). In Egypt, a vigorous vaccination program is applied, including several live and killed boosts, during a breeder's life to provide high maternal antibodies. In the present study, the six samples were positive for NDV using RT-PCR. These samples were collected from different localities in Sharkia governorate and during different time (Table 1). All samples were collected from vaccinated broiler chicken farms with 2 combination regimes of live attenuated genotype II vaccine with inactivated conventional genotype II ND vaccine. These samples were propagated in commercial ECEs and then tested against the HA activity using HA assay for confirmation. In this study, comparison of the fusion protein gene sequence of the studied isolates with different NDV reference strains and available vaccines in Egyptian market showed that 97.4% - 98.6% nucleotide identity to virulent NDV isolated in Egypt and neighboring countries (Figure 3). On the other hand, the nucleotide blast analysis showed 82.3% - 83% nucleotide identity to reference commercially available vaccine strains indicating considerable diversity (Goldhaft, 1980). While all isolates have 100% nucleotide similarity with each other and most Egyptian NDV strains which belong to genotype VII. As shown in (Figure 4) more than 29 amino acid mutations were detected when comparing the selected Egyptian isolates with LaSota (AF077761), and this will reveal on antibody production which will be a heterologous antibody, and this is at the level of F protein. Sequence analysis of fusion protein gene confirmed that all the studied isolates shared the cleavage site motif 112-RRQKRF-117 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 (Liu *et al*., 2003) and Middle East (Khan *et al*., 2010). Sequence analysis showed K78R mutation in the A2 antigenic epitope in all isolates, and more along the fusion protein gene which vary in some instances within the isolates. Mutation in this A2 antigenic epitope has been reported to induce escape mutation to monoclonal antibodies generated using NDV LaSota strain (Nagy *et al*., 2020). The F protein is capable of provoking host immune response, and it is necessary for producing neutralizing antibodies against NDV induced by vaccines (Omony *et al*., 2021). Mutations along this gene will impact antibody production which will be heterologous even at the level of F-gene (Cornax *et al*., 2012). In the present study, several nucleotide substitutions occur at specific and conserved antigenic sites which in some instances resulted in amino acid substitution (mutation) (Figure 4). The neutralizing epitopes are important in forming antigenic epitopes, aa substitution in this region induce the formation of neutralizing escape variants (Hu *et al*., 2010). The single point K78E mutation of the A2 antigenic epitope seen in all study isolates is due to AAA-AGA nt substitution at codon 232-234 has been previously reported to induce escape mutation (Yussof and Tan, 2001). Some recent findings suggested that the use of homologous NDV vaccines under commercial field conditions showed improvements in clinical protection in suboptimally vaccinated birds, pointing an advantage for the use of close antigenically matched vaccine seeds from the circulating viruses (Dimitrov *et al*., 2017).

CONCLUSION

The study detects virulent NDV genotype VIId among vaccinated poultry in Sharkia province, Egypt during 2021. Based on nucleotide and amino acid analysis, several mutations present along the fusion protein gene compared to commonly used LaSota and others commercially available vaccines. In order to generate genetically matched vaccines, ND surveillance and molecular analysis of circulating strains should be encouraged and reported.

CONFLICT OF INTEREST

The authrs declare that they have no conflict of interest.

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