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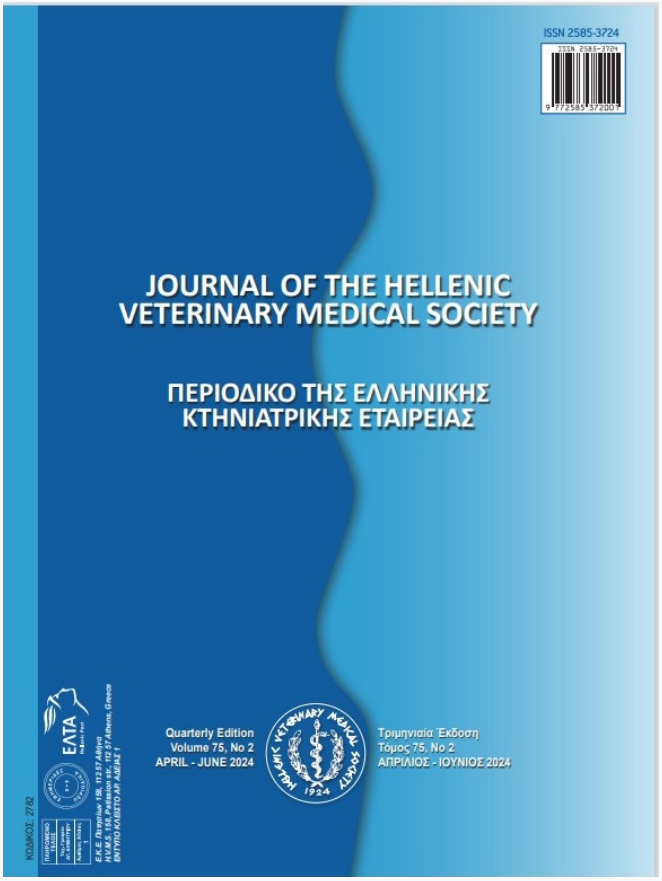

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Research article
Ερευνητικό άρθρο

Isolation, genotyping, and sub-genotyping of Newcastle disease virus from commercial broiler chickens in Northern Egypt

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ABSTRACT: In poultry, viral diseases, including the Newcastle virus (NDV), are considered among the causes of profitable losses in poultry, which directly affect livestock and the general health of birds. NDV is a ssRNA paramyxovirus causing a contagious disease in chickens ranging from subclinical infections to 100% death. Therefore, this research was conducted to follow up on the Newcastle disease virus recently circulating in commercial poultry farms in northern Egypt and compare the master amino acids that determine the velogeneity of the ND virus. In addition, this research compared their relationship to the vaccines currently in use. A specific-pathogen-free chicken (EE-SPF) egg was used to isolate NDV from chicken farms. Then the inoculated allantoic fluids were tested for NDV using the Hemagglutination Test and Hyper-Immune Serum against LaSota NDV. Furthermore, the F gene was molecularly characterized and sequenced. The results showed that 15 allantoic fluids were positive for NDV. From 9 isolates that were sequenced, seven isolates (ON497007, ON497008, ON497010, ON497011, ON497012, and ON497013, and ON532692) clustered into sub-genotype VII.1.1 and the other 2 (ON497006 and ON497009) into sub-genotype XXI.1.1. In addition, all the isolates in this investigation carry the velogenic strain's characteristic velogenic motif 112RKQKR*F117. In conclusion, the circulating ND in vaccinated chicken flocks in Egypt genotype VII.1.1 and XXI.1.1. Moreover, there was a residual substitution between most isolates, and genotype VII vaccine's strain (R/K), and autogenous vaccine strain (E/R) at position 78. These results are expected to help provide the latest characteristic information on NDV in northern Egypt.

Keywords: *Newcastle disease; Chicken; Genotyping; Sub-genotyping; Northern Egypt.*

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INTRODUCTION

Newcastle disease virus (NDV) causes Newcastle disease (ND), a highly contagious disease that is the third most important poultry disease recorded in 109 participating nations of the World Organization for Animal Health (OIE). Due to its global impact on poultry production, the disease has attracted the interest of various researchers in recent years (Abd Elfatah *et al.*, 2021).

NDV belongs to the genus Avian Orthoavulavirus-1 of the Paramyxoviridae virus family (formerly known as Avian Avulavirus-1, identified as Avian Paramyxovirus). Six important structural and non-structural proteins, including hemagglutinin, matrix, fusion, phosphoprotein, neuraminidase, and polymerase proteins, are encoded by the genome. That is the fusion proteins (F) and (HN) form spike-like protrusions on the outer surface of the viral envelope, forming the neutralizing and protective antigens of NDV (Dimitrov *et al.*, 2019; Sultan *et al.*, 2022).

The F protein is 55 KD, which is synthesized and glycated in the endoplasmic reticulum as an inactive form of the F0 precursor. They then become active after cleavage by specific cell proteases that can promote cell division. This cleavage site is located between positions 112 and 117 of the fusion protein precursor responsible for the molecular basis of APMV-1 pathogenicity. Thus, the function of the F protein is to ensure the fusion of the viral envelope with the cell membrane when the virus enters the target cell after activation by attachment of the HN protein to cellular receptors (Dzogbema *et al.*, 2021).

NDV strains are classified into two groups based on the F gene sequence: class I viruses are all members of a single genotype, unlike class II viruses, they are recently classified into 20 genotypes, which are separated by nucleotide lengths greater than 10% from the most common genotypes circulating worldwide are genotypes V, VII, and VIII (Shittu *et al.*, 2016). Recent research has found that Egyptian NDV isolates have genetic changes at various sites in the F gene, such as in the N-glycosylation sites, epitope binding sites, and cysteine residues that could influence and interact with the virus's pathogenic potential, genotype VII includes the safety that conventional vaccines offer (Sultan *et al.*, 2020).

At least three NDV genotypes are currently circulating in Egypt: In addition to the vaccine type genotype 2.II virus, the virulent genotypes 2.VIb and 2.VIIb are present. Genotype 2.VIb was mainly isolated from infected pigeons and is considered Pigeon Paramyxovirus (PPMV) (Rohaim *et al.*, 2016; Sabra *et al.*, 2017). On the other hand, the 2.VIIb genotype is relevant for poultry, with numerous outbreaks of ND in all areas of chicken production. Despite intensive vaccination trials with genotype 2.II vaccines have high mortality, particularly in broiler farms (Orabi *et al.*, 2017). However, due to mixed infections with other avian respiratory viruses (Sultan *et al.*, 2015; Hassan *et al.*, 2016), the contribution of specific pathogens to the disease is difficult to assign.

This research was conducted to identify the spread of NDV genotypes and sub-genotypes suggested as responsible for the current NDV outbreak in commercial poultry farms in northern Egypt during 2021.

MATERIALS AND METHODS

Sample collection

Three hundred broilers representing 30 broiler diseased flocks (10 chicks/flock) of different ages (ranging from 20 days to 35 days) were collected in northern Egyptian governorates including Dakahlia, Behera, Ismailia, Suez, and El-Sharkia in 2021. The flock sizes studied ranged from 1000 to 10000 birds. All chicken flocks were vaccinated against the NDV with different vaccination programs [*HVT-ND vaccine (one day old), Live LaSota strain virus vaccine (10 days old) and clone-30 strain of NDV (18 days old); ** HitchnerB1-ND vaccine (one day old), Dalguban Inactivated ND virus vaccine (10 days old), and*** HitchnerB1-ND vaccine (one day old), Live LaSota strain virus vaccine (10 days old) and clone-30 strain of Newcastle disease virus (NDV) (18 days old)]. All the chickens collected showed nervous, and respiratory symptoms, diarrhea, and increased mortality. In addition, postmortem lesions included petechial hemorrhages at the tips of the proventricular glands, enlarged speckled spleen, tracheitis, and enteritis with ulceration of the cecal tonsils. Each chick (liver, spleen, lung, trachea, and brain) was collected under aseptic conditions. All tissues collected were expressed as 30 pooled samples (10 chickens/flock/1 pool/1 sample). Tissue samples were collected aseptically from suspect chickens on each farm, then shipped on ice and held at -80°C until processing.

Preparation of samples

Tissues were homogenized in sterilized phosphate-buffered saline (PBS) containing 1 mg/mL

streptomycin sulfate, 0.4 mg/mL gentamicin sulfate, and 1000 IU/mL penicillin in 0.9% NaCl (SIGMA) to form a 10% tissue solution. The suspension was thoroughly mixed before three freeze-thaw cycles. Thereafter, the suspension was clarified by chilled centrifugation at 10,000 rpm for 10 min. A 0.45 µm syringe filter was used to filter the supernatant. The filtered supernatant was then kept at -80°C (McNulty, 1998).

Virus Propagation

Allantoic membranes from 9-day-old embryonated eggs of specific-pathogen-free chickens (EE-SPF) were injected with prepared samples (OIE, 2021). Then the eggs were incubated at 37°C for 96 h with daily candling to monitor embryo viability. Thereafter, all injected eggs, and allantoic fluids, whether the embryos were dead or survived, were collected and examined for hemagglutination after overnight cooling.

Hemagglutination test for the samples

Using a slide hemagglutination (HA) test with chicken red blood cells (1%) to determine the hemagglutination-positive samples, the positive allantoic fluids were then tested for NDV using hyper-immune serum HI against LaSota NDV vaccine strain immunized in SPF chickens was prepared at the Department of Avian Diseases, Animal Health Research Institute, ARC, as noted (Horwitz & Scharff, 1969). Allantoic fluids positive for NDV were stored at -70 °C for RT-PCR.

Conventional Reverse Transcriptase (RT-PCR)

EasyPure® viral RNA Kit (China) was used to obtain viral RNA from allantoic fluids according to the manufacturer's guidelines. The following primers were used to detect the partial F gene of vNDV by RT-PCR. F: ATGGGCTCCAGACCTTCTACCA, R: CTGCCACTGCTAGTTGTGATAATCC (Radwan *et al.*, 2013). PCR thermal cycler as follows: RT (reverse transcription) response for 50 °C/15 min, then 95 °C/15 min; initial denaturation at 95°C for 15 minutes; followed by 35 cycles at 95°C/30 s, 54 °C/30 s and 45 s/72 °C. They were followed by a cycle of 62 °C/10 min. The amplicons were examined under UV light.

Amplification of full-length F gene and sequencing

One positive allantoic fluid underwent full-length F gene amplification using specific primers F: GTCAGATCTTGATGGGCTCCAAACCTTC, RATGAATTCTCACGCTCTTGTGGTGGCTC (Orabi *et al*. 2017), The PCR cycling conditions were as follows: RT response for 50 °C/15 min, then 94 °C for 20 s, annealing at 57 °C for 30 s, and elongation at 68 °C for 1 Min 45 sec, final elongation at 68 °C for 7 min. The amplicons were examined under UV light.

Gene Sequencing

Nine bands of specific sizes (8 for the partial F gene and one for the complete F gene) were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. PCR product sequencing was performed by Macrogen® Company using the Sanger sequencer in two directions. The sequencing data were analyzed using NCBI Blast assembled, and edited using BioEdit program version 7.1.5.

The phylogenetic tree was created using the MegAlign module. Neighbor-joining phylogenetic analyses were performed in MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar *et al.*, 2018).

RESULTS

A total of 25 inoculated allantoic fluids of chicken EEs-SPF showed hemagglutination ability by direct HA test. Of 20 HA-positive allantoic fluids, 15 HA-positive allantoic fluids were positive for hyper-immune serum HI test ND antibodies. Using primers specific for the NDV F gene, 15 allantoic fluids were positive for NDV.

Molecular analysis of F gene

From 15 NDV-positive allantoic fluids, 8 isolates were approved for the partial F gene sequence and assigned accession no. ON497006, ON497007, ON497008, ON497009, ON497010, ON497011, ON497012 and ON497013. Additionally, accession number ON532692 was assigned to the complete gene sequence of the F gene.

Sequence and phylogenetic analysis of the F gene

The genotyping classification was based on nucleotide sequences of the F gene (Figure .1) in seven isolates (ON497007, ON497008, ON497010, ON497011, ON497012, and ON497013 and ON532692) out of 9 ND sequenced isolates clustered into genotype VII (VII.1.1), and the other 2 (ON497006 and ON497009) into genotype XXI (XXI.1.1). All of the isolates in this study carry the characteristic 112RKQKR*F117 motif of the velogenic strain (Table 1. supplementary

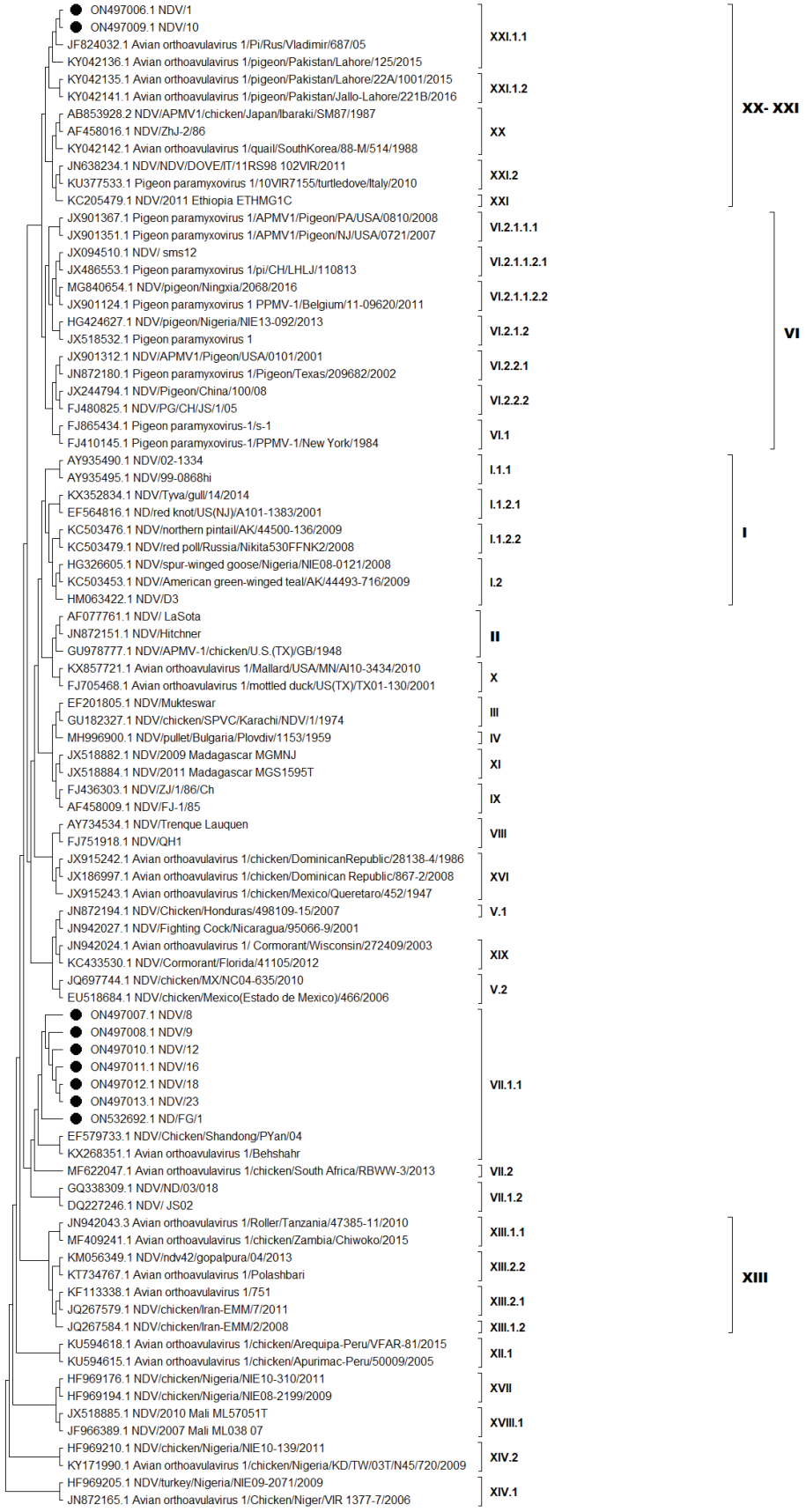

Fig. 1. Genogroup classification Phylogenetic tree based on the F gene nucleotide sequence of nine NDV strains (marked with a black circle) and reference NDV strains obtained from Genebank.

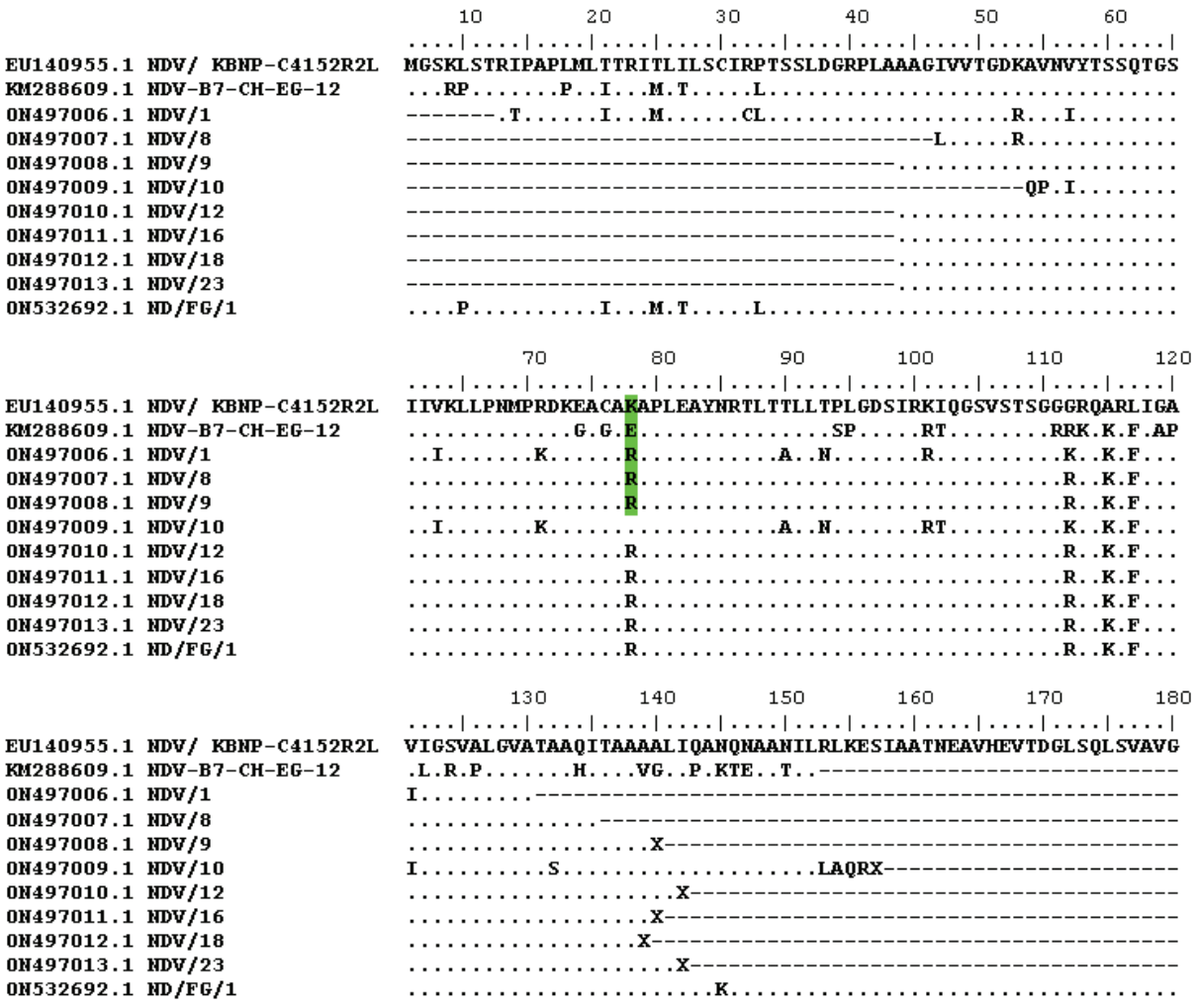

Fig. 2. Alignment of the deduced amino acid sequence of F protein of NDV in nine field NDV strains compared to vaccinal NDV G.VII strains.

data). Regarding the alignment of the isolated strains' F protein amino acid sequence against the autogenic genotype VII.1.1 NDV-inactivated oil emulsion vaccine (NDV-B7-Egy-2012 KM288609) (Sultan et al., 2022) and the genotype. The vaccine strain VII KBNP- C4152R2L showed residual substitutions between most of the isolates and the genotype VII vaccine strain (R/K) and the autogenous vaccine strain (E/R) at position 78 (Figure 2).

An additional residue substitution existed at position 57V (V/I), 71R (R/K), 90T(T/A), 93T(T/N), and 101K(K/R) F protein sequence of ON497006.1 NDV/1 and ON497009.1 NDV/10. In this study, the fusion peptide (FP, 117139 aa) appeared to be largely conserved among all the isolated strains and the vaccine strain of genotype VII (genotype VI), while it was 95% conserved between the other isolates ON497007, ON497008, ON497010, ON497011, ON497012 and ON497012 and ON497013 and ON532692 (genotype VII). Amino acid identity of the autogenous vaccine strain was 86% with ON497006 and ON497009 (genotype VII.1.1), respectively, while it was 90% with isolates ON497007, ON497008, ON497010, ON497011, ON497012, and ON497013 (XXI.1.1).

DISCUSSION

Poultry production sectors are the principal source of income and food security (having, at all times, both physical and economic access to sufficient food to meet dietary needs for a productive and healthy life, for many people) for many people, while commercial and backyard poultry are still under serious threat from ND. Molecular epidemiology and phylogenetic studies of NDV in countries where this viral disease is endemic are crucial. To identify disease patterns, study relationships between isolates in different geographic locations to assess spread and predict outbreaks, and

inform targeted biosecurity measures such as vaccine development and immunization strategies (da Silva *et al.*, 2020).

A total of 30 broiler farms were examined in the existing study with nervous, respiratory signs, and diarrhea. Moreover, postmortem lesions of tracheitis and hemorrhagic proventriculus, enlarged speckled spleen, tracheitis, and enteritis with ulceration of the cecal tonsils this finding previously mentioned as signs, and lesions of velogenic NDV infection (Alexander & Senne, 2008).

All investigated chickens were from vaccinated farms. This wasn't new, however, as NDV from earlier studies, NDV infestation with high mortality percentages in these flocks was reported from farms with different vaccination programs (Megahed *et al.*, 2018; Otiang *et al.*, 2021).

All the detected isolates were identified as velogenic NDV strains by the F protein phylogeny, which belonged to class II genotype VII.1.1 (7 isolates), and genotype XXI.1 (2 isolates).

Genotype VII.1.1 known to predominate in Egypt contributed to multiple ND epidemics (Shunlin *et al.*, 2009), and causes more significant injuries to lymphoid tissues, especially to the spleen, when compared to other virulent genotypes (Hu *et al.*, 2012).

To perform molecular pathotyping, amino acid sequences of the proteolytic cleavage site motifs of the F protein (residues 112 to 117) of the NDV strains were examined. It is a rapid and authoritative method for pathotyping NDV in comparison to mean time of death, intravenous pathogenicity index, and intracerebral pathogenicity index (De Battisti *et al.*, 2013; Ganar *et al.*, 2014). The findings demonstrated that all of the study's strains had the cleavage site motif 112 RRQKRF117 which describes vNDV strains. Additionally, it has been believed that the presence of the phenylalanine (F) residue at position 117 may be a factor in neurological symptoms (Collins *et al.*, 1993).

Genotype VI viruses, also known as "pigeon-type paramyxovirus" (PPMV-1), first appeared in outbreaks in the Middle East and Asia in the 1960s (Wehmann *et al.*, 2003). The NDV nomenclature proposed by Dimitrov et al. assigns to genotypes XX and XXI the old strains VIai and VIaii, which circulate in poultry but not in pigeons (Chong *et al.*, 2013), while PPMV-1 remains in genotype VI with several sub-genotypes.

In addition, these isolates are also antigenically dissimilar to the vaccine strains as there was a residual substitution between most isolates and genotype VII vaccine strain (R/K), and autogenous vaccine strain (E/R) at position 78. It is known that mutations at positions (72, 74, 75, 78, 79) affect the virus-neutralizing epitopes (Bari *et al.*, 2021; Omony *et al.*, 2021). It was noticed that the ND outbreaks detected in the vaccinated poultry farms showed that the vaccination strategies used to control the ND viruses were not effective, so there is a need to improve the current NDV control strategy. Also, the comparison of the sequence of the F gene of the detected isolates with recent Egyptian different NDV reference strains (Table 2. Supplementary data) revealed that all isolates have nucleotide similarity reaching up to 98% with each other. In addition, when the F gene sequences of the identified isolates were compared to those of the recently detected Egyptian NDV reference strains, they were found to share a high nucleotide identity (Abd Elfatah *et al.*, 2021; Amer *et al.*, 2022).

CONCLUSION

Based on the isolation, genotyping and sub-genotyping results of the NDV field isolates from northern Egypt in 2021, the field isolates are included in the NDV sub-genotype VII.1.1 and XXI.1.1. with 88%-100% similarity to NDV isolates from Egypt. A polybasic F cleavage site motif of 112R-R-Q-K- R-F 117 was present in all isolates. Moreover, there was a residual substitution between most isolates, and genotype VII vaccine strain (R/K) and autogenous vaccine strain (E/R) at position 78.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Fig. 1. Genogroup classification Phylogenetic tree based on the F gene nucleotide sequence of nine NDV strains (marked with a black circle) and reference NDV strains obtained from Genebank.