Research Article

Epidemiological Surveillance for the Newly Classified Newcastle Disease Virus Genotype VII.1.1 in Chicken Flocks in Egypt

Sameh Abdel-Moez Ahmed Amer[1]*, Mohamed Abdel-Aziz Kutkat[1], Mohamed Mahmoud Abdel-Baki[1], Asmaa Mahmoud Maatouq[1], Omnia Mohamed Kutkat[2], Hagar Magdy Ahmed[1], Khaled Mohamed El-Bayoumi[1]

[1]Department of Poultry Diseases, Veterinary Research Institute, National Research Centre, P.O. Code 12622, Dokki, Cairo, Egypt;[2]Centre of scientific excellence for influenza virus, Environmental Research Institute, National Research Centre, P.O. Code 12622, Dokki, Cairo, Egypt.

Abstract | Newcastle disease virus (NDV) has been recently considered as one of the major problematic diseases affecting poultry flocks in Egypt. The conventional vaccines can no longer provide an adequate protection for birds against ND outbreaks, where expected to be due to genotype mis-matched vaccines to the rapidly evoluted NDV field strains. Herein, the present study investigated the prevalence and the phylography of currently epidemic genotypes of NDV. Out of 550 samples were collected during an active surveillance in 2020 and 2021 from different poultry farms in five Egyptian governorates, 100 pooled samples were tested for NDV identification. NDV replication and growth was detected by embryo mortality after inoculation of all samples in one passage in embryonated chicken eggs and further testing by haemagglutination test. Whereas, reverse transcription polymerase chain reaction (RT-PCR) using specific primers to amplify 1681 base pair (bp) of the whole NDV F-gene was carried out to confirm the presence of NDV and then sequenced. Our results demonstrated that, RT-PCR was successfully detecting 62 NDV samples. While, Sequencing and phylogenesis of six selected isolates revealed that, all isolates were virulent NDV with amino acid motive ^{112}RRQKRF^{117} at F protein cleavage site. Furthermore, all isolates were clustered together with NDV genotype VII.1.1 (former GVIId) in a high similarity with previously reported strains from Egypt since 2012 till now, as well as with other neighboring and geographically close countries. In conclusion, the recent NDV outbreaks have been likely caused by the distinctive genotype VII.1.1 which seems to be the predominant strain circulated in Egypt. So as to, it is recommended to be used as a contemporary vaccinal strain against the frequent NDV genotype VII outbreaks.

Keywords | Newcastle disease virus, Genotype VII.1.1, Prevalence, Phylogenesis, NDV outbreaks

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***Correspondence** | Sameh Abdel-Moez Amer, Department of Poultry Diseases, Veterinary Research Institute, National Research Centre, P.O. Code 12622, Dokki, Cairo, Egypt; **Email:** drsamehnrc@hotmail.com
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INTRODUCTION

Newcastle disease, caused by the Newcastle disease virus is a highly infectious disease affecting poultry and wild birds in the whole world. It has been known as the avian paramyxovirus type 1 (APMV-1) and classified in the family Paramyxoviridae, subfamily Avulavirinae, and genus Metaavulavirus (Seal et al., 2000). The NDV is a non-segmented negative-sense RNA virus which encoding six proteins; nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large polymerase (L) of 15,186–15,198 nucleotides (Rima et al., 2019).

Newcastle disease virus virulence is mainly determined by the cleavage site in the F protein, where the cleavage site motif ^{112}R/K-R-Q-R/K-R↓F^{117} that is cleaved by a variety of proteases is indivative for velogenic and mesogenic strains of NDV resulting in systemic infection. While ^{112}G/E-K/R-Q-G/E-R↓L^{117} motif is cleaved by specific respiratory

and gastrointestinal systems trypsin like proteases only is a specific for lentogenic strains (Morrison, 2003).

Based on nucleotides sequence, about six distinct lineages of NDV have been identified by (Aldous et al., 2003). While in more traditional classification based on the completed F-gene sequence by Czegledi et al. (2006), NDV was divided into two groups class I and class II within a single serotype. In addition, two major divisions represented as class I and class II was demonstrated by Kim et al. (2007a and b), that Class I strains are a virulent in chickens and recently have been divided further into nine genotypes (1-9) based on a 374 base-pair portion of the F gene. Whereas class II viruses, are further divided into 16 genotypes isolated from wild bird and domestic poultry species (Abolnik et al., 2004). In which genotype VII among class II NDVs is one of the most worldwide prevalent genotypes causing severe outbreaks in recent years in Europe, Africa, the Middle East, South America and Asia (Zhang et al., 2010).

Daubney and Mansy (1948) were considered the first who diagnosed NDV in Egypt as early as 1948 by. Presently and according to Rohaim et al. (2016) and Sabra et al. (2017), NDV genotypes II, VI and VII are the common genotypes circulating in Egypt, in which genotype II viruses are mainly of vaccine type and virulent genotype VI was isolated mainly from infected pigeons and clustered as pigeon paramyxovirus. While, NDV sub-genotype VIId was considered as the predominant strain, that has led to several outbreaks in poultry since it was firstly isolated in Egypt in 2012 (Radwan et al., 2013). Since that time and in spite of intensive vaccination strategies with both live and killed NDV genotype II vaccines, high mortality rates are observed in most of chicken farms in Egypt (Orabi et al., 2017; Saad et al., 2017; Amer et al., 2019).

Dimitrov et al. (2019) have updated a recent nomenclature and classification of NDVs, that class I isolates are clustered in a single genotype with three sub-genotypes (1.1.1, 1.1.2 and 1.2). While class II viruses and due to their high genetic diversity, are clustered into 20 genotypes (I to XXI). In which NDV VII genotype, was sub-divided into sub-genotypes VII.1.1 and VII.1.2. Whereas the former sub-genotypes; VIIb, VIId, VIIe, VIIj, and VIIl, were all classified as sub genotype VII.1.1. While the sub-genotype VII.1.2, only comprises the former sub-genotype VIIf. Finally, formerly sub-genotypes VIIa, VIIh, VIIi, and VIIk were merged all into a separate single sub-genotype, namely VII.2 which considered being responsible for the Asia, Europe, the Middle East and Africa NDV outbreaks (Miller et al., 2015; Abolnik et al., 2018).

Newcastle disease viruses prevalence and genotypic diversity still a challenge for disease control that, ND outbreaks have still occurred annually worldwide with severe economic losses and elevated annual use of ND vaccines in spite of application of an intensive vaccination programs, have raised questions about the antigenic variation of the conventional classic vaccines efficacy against the field NDVs (Cho et al., 2008; Hu et al., 2011; Absalon et al., 2019). So as to, recent studies were investigated the role of either live or killed NDV vaccines designed based on the phylogenetic matching to field virus that can provide better protection in periodical NDV outbreaks (Yang et al., 2016; Kim et al., 2017; Amer et al., 2019). Therefore, the goal of this study was, to identify the most predominant circulated NDV genotypes and sub-genotypes suggested to be responsible for the current NDV outbreak in Egyptian poultry farms.

MATERIALS AND METHODS

Ethics statement

The animal handling and swabs sampling were carried out by specialized veterinarians to ensure birds welfare in accordance with the relevant policies regarding animal care and handling, that was approved with a registration no; 19312 by the Medical Research Ethical Committee (MREC) at the National Research Centre, Dokki, Cairo, Egypt.

Sample collection and preparation

A total collected number of 550 tracheal and cloacal swabs from commercial broilers, commercial layers and native breeds were pooled into 100 samples, which were obtained from five different Egyptian governorates (El-Behera, Alexandria, Menoufia, Kafr El-Sheikh and Giza) during an active surveillance programme in 2020 and 2021. Samples were collected under a septic condition from freshly dead and sick birds. The collected samples were placed in transport media supplied with 1% antibiotic-antimycotic mixture and stored at (-80C) for further isolation and identification of NDV.

Virus propagation in embryonated chicken eggs (ECE)

Each sample was processed in 200 µL phosphate-buffered saline containing 1% antibiotic-antimycotic mixture. Only 100 µL of each sample was inoculated through allantoic sac route of inoculation into 9-11 days-old ECE (3 eggs/sample) (Alexander, 2009). The eggs were incubated at 37°C and candled once daily for seven days. Any embryo mortality during the first 24 hours of incubation was discarded and considered as non-specific death. All died or survived embryos till day 7 post-inoculation were chilled over night at 4°C. The allantoic fluid was a septically aspirated and stored at -80°C in sterile screw-capped vials.

Haemagglutination test (HA)

The collected allantoic fluid was tested by slide HA test according to the procedures of the (OIE, 2012). ND LaSota vaccinal strain was used with 4 HAU as a reference control positive HA antigen in slide HA tests of ND (intervet international, B.V. Boxmeer, Holland, supplied by local agency).

Molecular identification by RT-PCR

The genomic RNA was extracted from allantoic fluid using QIAamp viral RNA mini kit (Qiagen™, USA) as recommended by manufacturer. One step RT-PCR method was carried out using specific primer to amplify the full-length F gene as discussed by Wulanjati et al. (2018).

- Forward primer (28 bp): 5'GTCAGATCTTGATG-GGCTCCAA ACCTTC 3'
- Reverse primer (28 bp): 5'ATGAATTCT-CACGCTCTTGTGGT GGCTC 3'

The thermal cycle program of RT-PCR was started by reverse transcription at 50°C for 60 min, initial denaturation at 94°C for 15min, followed by 40 cycles of; denaturation at 94°C for 20 sec, annealing at 57°C for 30 sec and elongation at 68°C for 3 min. Final elongation was done at 68°C for 7 min. The RT-PCR product was then screened by 1.5% agarose gel electrophoresis.

Sequencing and phylogenetic analysis

The targeted DNA Bands for six representative samples were excised from the gel, and were purified with QIAquick® PCR gel purification kits (QIAGEN, Valencia, CA, USA) as per manufacturer's guidelines. The purified DNA of the selected samples was sequenced using the Sanger dideoxy sequencing method and the nucleotide sequences were then registered in Genbank (http://www.ncbi.nlm.nih.gov/Genbank) with accession numbers (Table 1).

All finely adjusted full F- gene sequences were blasted and aligned using DNASTAR® Lasergene software alignment tools and exported as Lasergene DNA files that was used as a base for phylogenetic analysis using Maximum likelihood method with various NDV genotypes that were obtained from Genbank.

RESULTS AND DISCUSSION

Characteristics of NDV outbreaks

Swabs were collected from commercial broilers, commercial layers and native breeds poultry flocks either dead or suspected clinically to be infected with ND. The investigated flocks were showing increased mortalities in the last days before sample collection, layer flocks were suffering from a sudden drop in egg production with the production of abnormal eggs. Whereas, most of broiler and native breeds flocks were showing increased mortalities, decrease in the food consumption, abnormal respiratory sounds and low percent of the flocks were showing nervous signs.

Most of broiler farms were double vaccinated with only live NDV vaccines as LaSota and Hitchner B1. While commercial layer flocks were heavily vaccinated with at least double shots of both live and killed NDV vaccines of either genotype II or VII. Whereas, native breed farms are of the little vaccination attempts with or without vaccination regimes.

Postmortem examination of the investigated flocks showed different lesions of septicemic carcasses, petechial and hemorrhagic spots on the proventricular glands tips, greenish discoloration of the intestinal tract contents and hemorrhagic ulcers in the caecal tonsils, in addition to the non-specific respiratory lesions as nasal discharges, tracheitis and pneumonia.

Virus propagation in ECE and HA test

Samples were inoculated in one blind passage in 9-11 days-old ECE allantoic sac, whereas embryo mortalities were found in 88 samples between 48 to 96 hours post inoculation, as well as revealed pathological lesions of dead embryo include congestion of the entire embryo, edema of the head and petechial hemorrhages on the skin. Furthermore, the slide HA test was carried out for all samples after the blind passage in ECE revealing in which, 75 samples of them were HA positive, however HA negative samples were passaged twice in ECE before recorded as negative.

F protein conventional RT-PCR

Out of 75 HA positive samples were amplified with F protein specific primers, 62 samples were NDV positive showing 1681 bp single specific band (Figure 1).

Sequencing and phylogenetic analysis

Altogether, out of sixty the two positive NDV isolates by RT-PCR which resulted in 1681 bp F-gene amplicons, six isolates were subjected to nucleotide sequencing by Sanger method as representative sequences to five different Egyptian governorates (El-Behera, Alexandria, Menoufia, Kafr El-sheikh and Giza) and the resulted sequences were submitted and registered to Genbank (Table 1).

The prediction of the NDV pathotypes based on the deduced amino acid sequence of the F-gene cleavage site (112 to 117) showed that, the six selected isolates have sequence motif RRQKRF indicative for virulent NDV strains (Table 1 and Figure 2).

Table 1: Characteristics of the obtained Egyptian NDV isolates sub genotype VII.1.1 of the current study.

Isolate name	Accession number	Locus	Cleavage site motive of F- protein	Genotype	Host	Isolation year	Province
NDV-CH-EGY-BH-NRC-2021	MW590307	F- gene	^{112}RRQKRF^{117}	VII.1.1	Broilers	2021	El-Behera
NDV-CH-EGY-BEH- NRC-2021	MW603769	F- gene	^{112}RRQKRF^{117}	VII.1.1	Broilers	2021	El-Behera
NDV-CH-EGY-ALEX-NRC-2021	MW591503	F- gene	^{112}RRQKRF^{117}	VII.1.1	Broilers	2021	Alexandria
NDV-CH-EGY-MENOUFIA-VVT NRC-2021	MW603770	F- gene	^{112}RRQKRF^{117}	VII.1.1	Layers	2021	El-Menoufia
NDV-CH-EGY-KSH-VVTNRC-2021	MW603771	F- gene	^{112}RRQKRF^{117}	VII.1.1	Native breeds	2021	Kafr El-sheikh
NDV-CH-EGY-GIZA-VVTNRC-2021	MW603772	F- gene	^{112}RRQKRF^{117}	VII.1.1	Layers	2021	Giza

RRQKRF: R (Arginin), Q (Glutamine), K (Lysine) and F (Phenyl-alanine) amino acids.

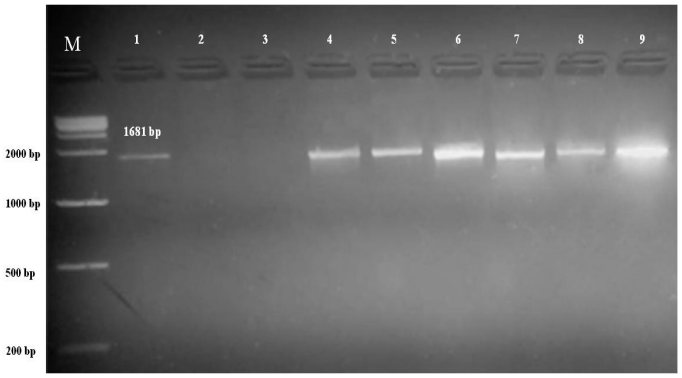


Figure 1: Visualization of 1.5 % agarose gel electrophoresis NDV full F-gene amplification product.
M: 1 kb DNA ladder; Lane 1: Control positive for NDV; Lane 2and3: Negative NDV samples; Lane 4 and 5 and 6 and 7 and 8 and 9: Positive NDV specific bands of 1681 bp amplicon.

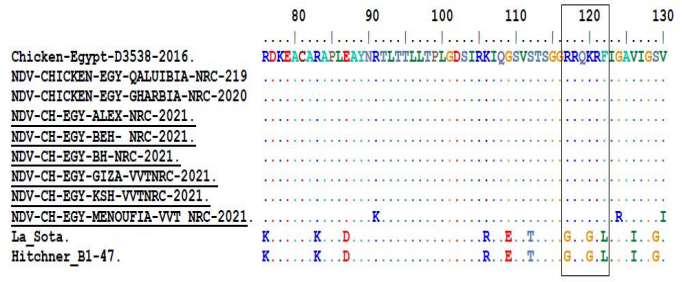


Figure 2: Partial amino acid alignment of the of F- gene cleavage site (112 to 117) of the identified NDV isolates of our study and other NDV strains circulating in Egypt related to velogenic NDV genotype VII 1.1, as well as other vaccinal strains related to NDV genotype II using Bio-edit program. The dot (.) represents identity while single alphabet represents the difference in the amino acid sequence. Underlined isolates are isolates of our study.

The phylogenetic relationship analysis using full F-gene sequences was carried out by maximum likehood method revealing that, all study sequences were clustered together within class II NDV genotype VII, sub-genotype VII.1.1 strains as recently mentioned by (Dimitrov et al., 2019).

Moreover, our isolates were found closely related to other NDV egyptian sub-genotype VII.1.1 (formerly GVIId), as well as genotype VII isolates from neighboring and regional countries like Turkey, Israel, Sudan and Mauritania in parallel with other worldwide genotype VII isolates from China, Taiwan, Ivory coast and Cameroon revealing the panzootic NDV genotype VII circulation all over the world (Figure 3).

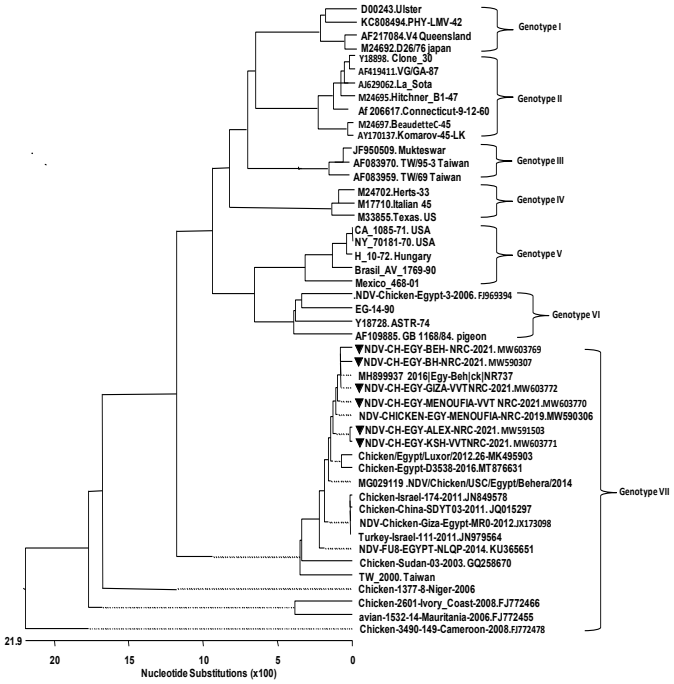
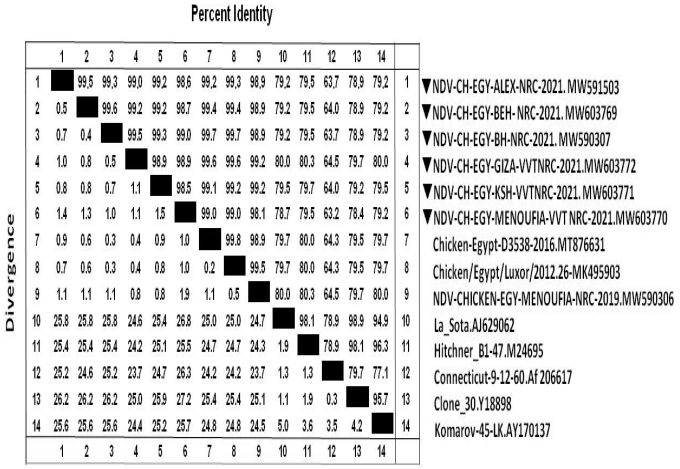


Figure 3: Phylogenetic relationship between the NDV isolates obtained in this study and other previously isolated in Egypt with some reference and vaccinal strains retrieved from the Genbank by Maximum likelihood method using Lasergene® software. ▼ Isolates of the study.

Furthermore, Amino acid identity percent of our isolates with each other found ranged from 98.5%-99.6%. In addition, the study isolates were highly related to previously isolated and registered Egyptian NDV GVII.1.1 as Chicken-Egypt-D3538-2016, Chicken-

Egypt-Luxor-2012 and NDV-Chicken-Egy-Menoufia-NRC-2019 with amino acid identity percent from 98.1% to 99.7%. While, identity percent of our isolates with different NDV vaccinal strains like LaSota, Hitchner- B1, clone -30, Connecticut and Komarov were found of high divergence and low similarity ranged from 63.2% to 80.3% (Table 2).

Table 2: Amino acid (A.A) sequence identity of obtained NDV isolates with NDV strains circulating in Egypt and NDV vaccines showing identity and divergence percent based on A.A sequence comparison using Lasergene® software, black squares indicate identical sequence.



▼ Isolates of the study.

Newcastle Disease virus is considered one of the most infectious diseases and a global threat to poultry and other bird all over the world. Velogenic strains of ND especially genotype VII viruses had been associated with the most current worldwide outbreaks in Africa (Abolnik et al., 2018; Twabela et al., 2021), the Middle East (El-Naggar et al., 2018; Abd El-Fatah et al., 2021; Naguib et al., 2021), Asia (Rui et al., 2010; Zhang et al., 2011), Europe (Dimitrov et al., 2016; Fuller et al., 2018) and South America (Perozo et al., 2012; Absalon et al., 2019). NDV genotype VII was firstly isolated and identified in Egypt through an active surveillance from recent outbreaks affecting poultry farms between 2011 and 2012 by Radwan et al. (2013) and reported as velogenic NDV clustered in class II genotype VII which found closely related to other middle east isolates. Since that time, trials for isolation of VNDV genotype VII was successfully carried out by (Hussein et al., 2014; Abdel Aziz et al., 2016; Hagar et al., 2017; Naguib et al., 2021).

This study was dedicated mainly to investigate the epidemiology of NDV and characterization of NDV strains isolated from potential outbreaks in five Egyptian governorates in a period from 2020 to 2021. The epidemiological investigation of ND in this study was closely similar and related to those reported for NDV infection, 100 pooled samples were collected from broiler, commercial layer and native breed flocks in five different Egyptian governorates (El-Behera, Alexandria, Menoufia, Kafr El-Sheikh and Giza). The investigated flocks were showing increased mortalities in the last days before sample collection layer flocks were suffering from a sudden significant drop in egg production with the production abnormal eggs. Most broiler flocks were showing increased mortalities, decrease in the food consumption and abnormal respiratory sounds with the presence of greenish diarrhea and low percent of the flocks were showing nervous signs. While, postmortem examination of the investigated flocks were showing septicemic carcasses, petechial hemorrhagic spots on the tips of the proventricular glands, greenish coloration of the intestinal tract contents and hemorrhages with ulcerations in the caecal tonsils, in addition to the non-specific respiratory lesions as; nasal discharges, tracheitis and pneumonia as mentioned in previous studies by (Alexander and Senne, 2008; Alexander, 2009; Terregino and Capua, 2009; Han et al., 2017).

The propagation in ECE 9-11 days old remains the technique of choice as a confirmatory method for virulent NDV isolation than other techniques (OIE, 2012). In a trial for isolation of NDV, one blind passage was performed to 100 samples in 9-11 days old ECE allantoic sac, whereas embryo mortalities were found in 88 samples between 48 to 96 hours post inoculation, as well as revealed pathological lesions of dead embryo include; congestion of the entire embryo, edema of the head and petechial hemorrhages on the skin as previously detected by Pedersen (2011). The replication and existence of NDV in the harvested allantoic fluid was tested by haemagglutination via rapid slide HA test as NDV induces HA activity in consistent with the results reported by Terregino and Capua (2009); OIE (2012). Whereas, our results for slide HA test were showing 75 HA positive samples.

Polymerase chain reaction-based assays for NDV always have many benefits for identification of the virus and also play an important role in discrimination between the virus genotypes, as well as can be used in assessment of the NDV pathogenicty and virulence (Aldous et al., 2003). In the present work, conventional RT-PCR amplified 1681bp F-gene in 62 samples from out of 100 samples of tracheal and cloacal swabs pool. In this context, NDV was easily screened via RT-PCR in 3.6% of tested tracheal and cloacal samples in Egypt by (El-Naggar et al., 2018), while in contrary Schelling et al. (1999) failed to amplify the NDV RNA extracted from cloacal swabs of 115 different wild bird species using RT-PCR.

Mostly, amino acid sequences in the cleavage site of F-gene can predict the NDVs pathotypes and can be used instead of traditional methods such as intracerebral pathogenicty

index test (ICPI) and mean death time (MDT) (Panda et al., 2004). Fusion protein has a prominent role in the virulence of NDV and could provide a specific protective immunity in birds against NDV (Kim et al., 2013), that can be used in the development of genotype-matched vaccines to the currently epidemic viruses to overcome the repeated ND outbreaks, as previously mentioned by (Amer et al., 2019). In this regard, Panda et al. (2004) and Dharmayanti et al. (2014) reported that, velogenic NDV strains have a motif ^{112}RRQKRF^{117} with phenylalanine (F) at residue 117 of the F1 protein. Meanwhile, the low virulent strains comprise a motif ^{112}GRQGRL^{117} in the same region with leucine (L) at the same position. In this regard and in agreement with our results, the six isolates of present study have multibasic amino acids sequence on cleavage site ^{112}RRQKRF^{117} descriptive for velogenic NDV strains when sequenced with Sanger method.

In retrospect Miller et al. (2015) discussed that, genotype VII viruses were mostly generated from the Far East in the 1980s and then spread to other regions in Asia, Europe, Africa and even South America in the 1990s. Currently, genotype VII viruses are the most prevalent in ND outbreaks in Middle East (Naguib et al., 2021). In the present study, the phylogenetic relationship in accordance with the new classification system proposed by Dimitrov et al. (2019) revealed that, the six isolates were in a closely similarity to each other and related to sub-genotype VII.1.1 within genotype VII in class II NDVs which already registered in Egypt, neighboring countries and even geographically close countries.

In accordance with deduced amino acid identity, our study validates that the six isolates were highly related to the previously isolated Egyptian velogenic NDVs genotype VII.1.1 as Chicken-Egypt-D3538-2016, Chicken-Egypt-Luxor-2012 and NDV-Chicken-Egy-Menoufia-NRC-2019 with amino acid identity percent ranged from 98.1% to 99.7% as in agreement with previously referenced public genotype VII.1.1 in Egypt (Abd El-Fatah et al., 2021; Naguib et al., 2021). Otherwise, F-gene amino acid sequence identity of our isolates against LaSota and B1 vaccine strains revealed higher values of divergence in compared with the values between samples and other genotype VII isolates (Table 2) as in agreement with a previous study of Xiao et al. (2012) when conducted a symmetric analysis based on sequence identity of F protein for 8 virulent ND viruses isolated from Indonesia during 2009 and 2010 outbreaks that was also found higher than the sequence identity value of 8 NDVs against B1 and LaSota vaccine strains.

CONCLUSIONS AND RECOMMENDATIONS

This study provides precious informations on the epidemiological situation and the prevalence of NDV strains in Egypt, so as to continuous monitoring of NDV in Egyptian poultry field is essential for the evaluation of these viruses' genetic traits. In addition, NDV genotype VII.1.1 may probably be the predominant circulated sub-genotype in Egypt. Therefore, it is recommended to be applied as a candidate vaccine strain homologous to field virus to control the future outbreaks of genotype VII virus in Egypt.

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This work is mainly dedicated to the soul of Professor. Doctor Mohamed Abdel-Aziz Kutkat may Allah almighty rest and bless him.

NOVELTY STATEMENT

Our study is considered one of the first trials in middle-east to isolate and characterize the newly classified NDV genotype VII 1.1 as the most prevalent strain currently circulated. Also, provides valuable and recent techniques in isolation and identification of the circulated viruses in poultry field.

AUTHOR'S CONTRIBUTION

All authors equally participated in design, experimental procedure, writing, revised, and reviewing the manuscript.

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Conflict of interest

The authors have declared no conflict of interest.

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