Molecular Identification of a Velogenic Newcastle Disease Virus Strain Isolated from Egypt

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ABSTRACT

Newcastle Disease Virus (NDV) is still a major concern for the Egyptian poultry industry in spite of the mass vaccination programs implemented from a long years ago. The current study aimed to carry out the molecular identification of surface glycoprotein genes of NDV field strain isolated from the Giza governorate, Egypt. Tracheae were collected from 10 broilers NDV-vaccinated chicken flocks (at least three samples from each flock) suffering from mild to moderate respiratory symptoms; with mortalities varying from 10-40% during October 2019. Only five samples showed HA positive activity after propagation in specific pathogen-free embryonated chicken eggs and only one sample was positive for *Avian avulavirus* 1 by real-time reverse transcription-PCR. Sequencing for the cleavage site of the F protein gene of the positive isolate showed the typical known sequence of velogenic NDV strains ($_{112}$RRQKRF$_{117}$). Phylogenetic analysis of both F and HN genes showed high similarity and close relation to Chinese strains of Genotype VII and more specifically subtype VIId, suggesting the role of migratory wild birds in NDV evolution in Egypt. In conclusion, further epidemiological and surveillance studies are strongly recommended to define the exact role of migratory wild birds in NDV evolution in Egypt.

Keywords: Broilers, Newcastle Disease, Poultry industry, Velogenic

INTRODUCTION

Newcastle Disease (ND) is one of the most important viral diseases affecting poultry which is caused by *Avian avulavirus* 1 (APMV-1) (Abd El Aziz et al., 2016). The natural hosts of ND virus (NDV) include chickens, turkeys, ducks, geese, pigeons, quail, pheasants, guinea fowl, ostriches, and several species of wild birds (Wang et al., 2015).

ND as an acute viral infectious disease affects domestic poultry regardless of gender and age (Saad et al., 2017) and causes great economic losses, especially in developing countries (Westbury, 2001). Production inefficiencies are considered as a greater concern compared to mortality losses in breeders and layers flocks while mortalities usually reported to be more significant in broilers (Shahid Mahboob et al., 2020). Many NDV outbreaks were reported in the past years around the world, as in Japan (Mase et al., 2002), in Brazil (Marks et al., 2014), in China (Kang et al., 2014), in South America (Diel et al., 2012) and in Malaysia (Jaganathan et al.,

2015). NDV was recorded in Egypt since 1942 (Daubeny and Mancy, 1947) and has been reported ever since. (Hussein et al., 2000; Mohamed et al., 2011; Selim et al., 2018). Recently in Egypt, NDV outbreaks have been reported in both vaccinated and non-vaccinated flocks (Abd El Aziz et al., 2016; Ewies et al., 2017). A subclinical infection manifested by respiratory, intestinal, and nervous symptoms, with mortalities up to 100% may be a result of NDV infection according to virus strain pathogenicity in infected birds. Based on the pathogenicity of the virus, NDV strains can be categorized into three main types; lentogenic, mesogenic, and velogenic strains. NDV can be classified into two classes; class I and class II. NDV isolates of class I are grouped into one genotype, whereas NDV isolates of class II are grouped into at least eighteen genotypes, some with subgenotypes. Genotype VII viruses are responsible for the fourth panzootic that has spread from Asia, Africa, Europe, and has even been isolated in South America, which continues today (Dimitrov et al., 2016). The NDV is an enveloped virus that has a linear, single-stranded RNA genome of negative

polarity; with a genome length of about 15.2 kb (Aldous et al., 2003; Ashraf et al., 2016). The genome of NDV consists of 15,186, 15,192 nucleotides or 15,198 nucleotides that contains six genes coding six structural and two non-structural proteins including an RNA-directed RNA polymerase (L), hemagglutinin-neuraminidase protein (HN), fusion protein (F), matrix protein (M), phosphoprotein (P), and nucleoprotein (N). Both F with HN proteins play a collective role in NDV infection prosses. The fusion protein is the most important key in the NDV virulence determining process (Peeters et al., 1999). Mutations affecting NDV viral genome which alter its biological properties and virulence, in addition to altered immunity, and improper vaccination processes can increase the incidence of NDV outbreaks in vaccinated flocks (Kattenbelt et al., 2006). Virulence of ND can be distinguished on the basis of the cleavage site sequence of the F protein (Selim et al., 2018). HN is one of the membrane glycoproteins, through its neuraminidase (NA) activity it mediates attachment to sialic acid-containing receptors (Wang et al., 2015). Recently, the molecular identification and phylogenetic analysis of any new NDV isolates become an important and usual approach to find out which of the applied control measures needs to be improved (Fringe et al., 2012; Hassan et al., 2016). Sequence analysis of mainly F and of HN proteins genes - two surface glycoproteins- is wildly used for molecular identification of NDV isolates. Brevity, the current applied NDV vaccination programs consist of live and/or inactivated genotype I or II NDV or genetically modified vaccines depending on flock age and type.

In the present study, analysis of nucleotides sequences of F and HN genes were done for a recently isolated NDV strain obtained from samples collected from different chicken flocks showing mild to severe respiratory symptoms with variable mortality rates in Giza governorate, Egypt.

MATERIALS AND METHODS

Ethical approval

Institutional, national, and international animal care guidelines were followed.

Sampling and samples history

Tracheae (at least three samples from each flock) from 10 freshly dead broilers, NDV vaccinated chicken flocks suffering from mild to moderate respiratory symptoms; with mortalities varying from 10 to 40 % as well as a range of NDV indicative postmortem lesions at Giza governorate during October of 2019. Tracheae from the same flock kept together for isolation. Samples history mentioned in Table 1.

Table 1. History of flock sampled in the present study

Sample	Birds No/Flock	Mortalities (%)	Age of birds (day)	NDV vaccination
S1	10,000	12.5 %.	35	
S2	8,000	14%.	55	
S3	11,000	37.6%.	24	Twice, live LaSota
S4	4,000	35.8%.	26	
S5	15,000	22.4%.	22	
S6	2,000	10.7%.	23	
S7	1,000	34.5%.	35	Once, live LaSota
S8	3,000	21.3%.	40	
S9	4,000	39.1%.	41	Twice, live LaSota
S10	12,000	35.7%.	33	

Isolation

Virus isolation was done from tracheal swabs after immersion in Phosphate-Buffered Saline (PBS) mixed with gentamycin antibiotic (50 μg/ml) and mycostatin (1000 units/mL). Swabs from different birds from the same flock were immersed in the same PBS solutions. Samples were named numerically as sample 1 (S1): sample 10 (S10).

PBS-containing samples were clarified by centrifugation at 5000 rpm for 15 minutes. A 200 μl of supernatant fluid from each sample was inoculated into the allantoic cavity of five 10-day-old Specific Pathogen Free Embryonated Chicken Eggs (SPF-ECE). Allantoic fluid from each egg was harvested 3 to 5 days post-inoculation and was tested for hemagglutination (HA) activity by rapid slide HA test. HA negative samples were submitted for two blind passages of SPF-ECE. Collectively, samples that showed HA positive activity were kept for further molecular identification (OIE manual, 2018).

Viral RNA extraction

Viral RNA from HA positive allantoic fluid was extracted using Pure Link® (Invitrogen, USA) RNA Mini Kit following the manual's instruction.

Real-time reverse transcription-polymerase chain reaction

Real-time **Reverse Transcription** PCR (RT-qPCR) was performed in one step. Using TOPreal™ One-step - SYBR Green with low ROX - RT qPCR Kit (Enzynomics, Korea) according to the manufacturer's instructions and

using the CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories, USA). Primers used were designed according to Wise et al. (2004) which are specific for the matrix protein gene of APMV-1 viruses selected from a conserved region of the M gene (Table 2).

The thermal conditions were as follows; reverse transcription at 50°C for 30 mins followed by 10 mins at 95 °C for reverse transcriptase inactivation and initial denaturation. Then, followed by 40 amplification cycles of 95 °C denaturation for 5 s, 52°C annealing for 10 s, and 60 °C extension for 30 s.

Melting curve analysis was performed to determine the specificity of amplification as follows: 95°C denaturation for 10 s, 65°C annealing for 5 s, and heating to 95 °C with an increment 0.5 °C for 0.05 s.

The melting temperature (Tm) of melting curves and Cp values were calculated using the Bio-Rad CFX manager 3.1 software (Figure 1).

Table 2. Primers used for Newcastle disease virus detection using RT-qPCR.

Primer Name	Sequence
F Primer	5'-AGTGATGTGCTCGGACCTTC-3'
R primer	5'-CCTGAGGAGAGGCATTTGCTA-3'

F and HN genes amplification

Positive NDV RNA samples (by RT-qPCR) samples were subjected to one-step RT-PCR using SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase according to the manufacturer's instructions to amplify full-length F protein gene and HN protein gene using two sets of primes kindly provided by Dr. Mohammed Rohaim, Virology Department, Cairo University, Egypt (Table 3) and using the ProFlex PCR thermal cycler (Applied biosystem, USA).

Thermal amplification conditions were as follows; reverse transcription at 50 °C for 30 min followed by initial denaturation for 2 min at 94 °C. Then followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 65 °C for 30 s for F gene while 51 °C for 30 s for HN gene, and extension at 68 °C for 120 s followed by one cycle of final extension at 68 °C for 5 min.

PCR products were analyzed by agarose gel electrophoresis (1%) and then purified using a QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions.

Table 3. Primers used for F and HN genes amplification and sequencing.

Target gene	Primer Name	Sequence
F protein gene	Fus-F	5'-ATGGGCTCCAAACTTTCT-3'
	Fus-R	5'-CATGCTCTTGTAGTGGCTCTC-3'
HN protein gene	Hae-F	5'-CATGGACCGCGCGGTTAAC -3'
	Hae-R	5'-CTAAACTCTATCATCCTTG-3'

Sequencing

Sequencing of the purified RT-PCR products was done by the Bigdye Terminator V3.1 cycle sequencing kit (Perkin- Elmer, Foster City, CA) and Applied Biosystems 3130 genetic analyzer machine (ABI, USA).

Genetic alignment

The quality of obtained F and HN genes sequences were checked, assembled, edited using Bioedit software version 7.0.4.1 (Hall, 1999), and submitted to GenBank using BankIt tool of the GenBank (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank), with accession numbers MN905162 and MN905163, respectively.

Phylogenetic analysis

The tree was constructed using the neighbor-joining method; bootstrapping at 500 repeats using Mega 6 software version 7.0.26 (Tamura et al., 2013).

RESULTS

Hemagglutination activity

After three blind passages only S3, S4, S7, S9, and S10 samples were positive for hemagglutination activity. S7 and S10 were positive for HA after the 1st egg passage, S3, and S4 were positive for HA after the 2nd egg passage, and S9 was positive for HA after the 3rd egg passage. RNA from 5 positive HA samples were sent for one-step RT-qPCR.

NDV detection by RT-qPCR

Only S4 was positive for *Avian avulavirus* 1 by RT-qPCR with a threshold cycle (CT) of 29.34 with a starting quantity of 3.033 log10 in comparison with a standard curve (Figure 2) with melting peak at 79 °C (Figure 3 and 4).

Amplification of full F and full HN proteins genes by RT-PCR

RT PCR products gel electrophoresis revealed the expected and correct size bands for full-length F and HN proteins genes.

Genetic and phylogenetic analysis
F protein gene

Blasting of sequence results obtained for the full F protein gene showed similarities with Chinese genotype VII strains with similarities varying from 95.5 % to 97.28% and with many Egyptian isolates varying from 94.5% to 95.5 %. The phylogenetic tree of the full F protein gene showed that S4 isolate is closely related to genotype VII subtype D (Figure 5).

HN protein gene

Blasting of sequence results obtained for the full HN protein gene showed similarities with Chinese genotype VII strains with similarities varying from 95.69 % to 98.72% and with some Egyptian isolates varying from 94.9% to 95.45 %The phylogenetic tree of the full HN protein gene showed that S4 isolate is closely related to the Chinese genotype VII (Figure 6). Three-dimensional structure of F and HN monomer for S4 isolate was created by SWISS-Model modeling online server and visualized by PyMOL program version 2.3.4 (Figure 7 and 8).
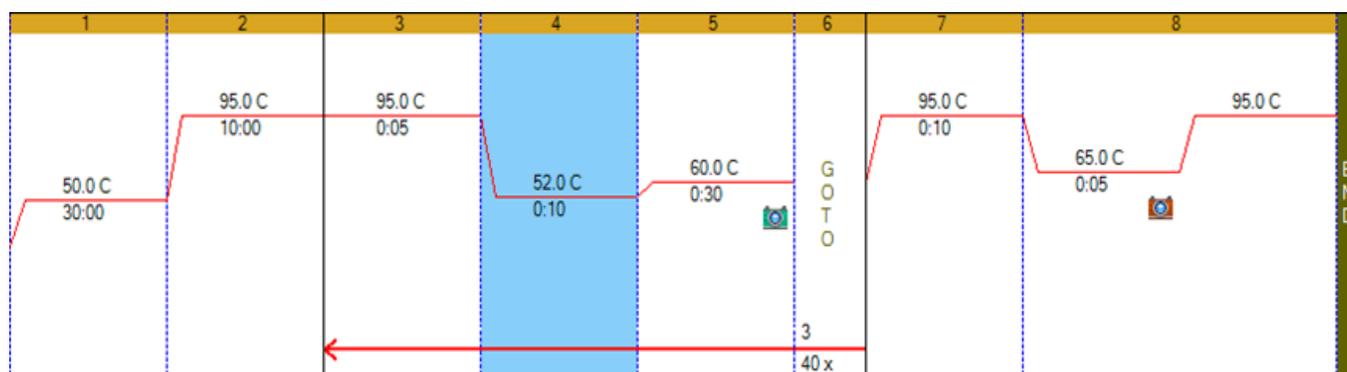
Figure 1. Thermal conditions applied at RT-qPCR and for the melting curve.
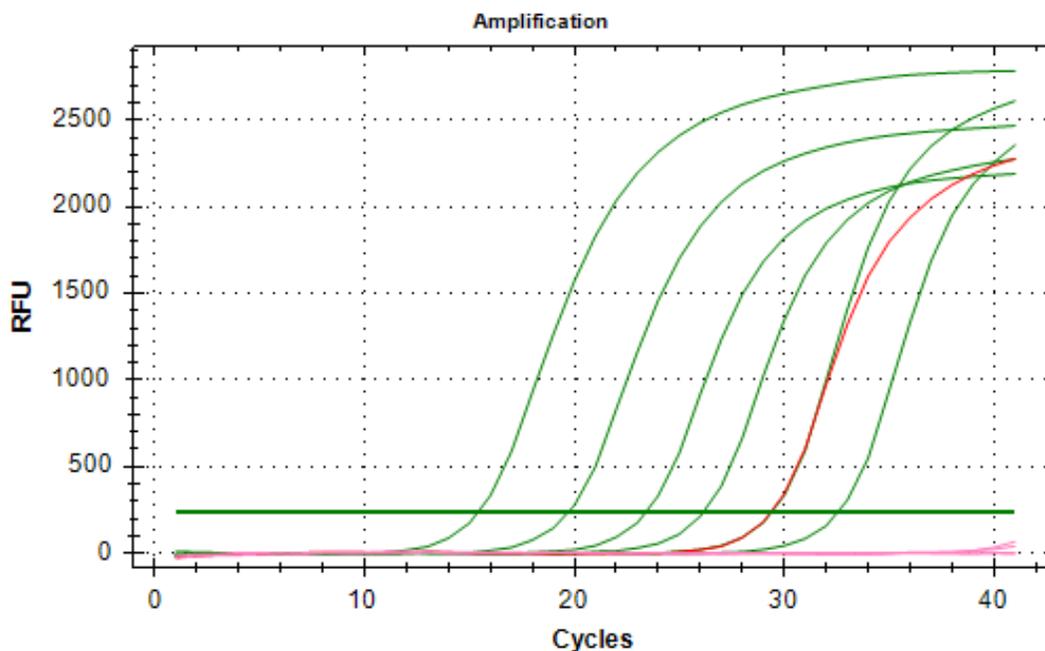
Figure 2. Threshold cycles of tested samples, green lines represent positive control samples (standard curve samples), the red line represents positive for *Avian avulavirus* sample (S4) appeared after 29.34 CT, and pink lines represent the negative for *Avian avulavirus* samples.
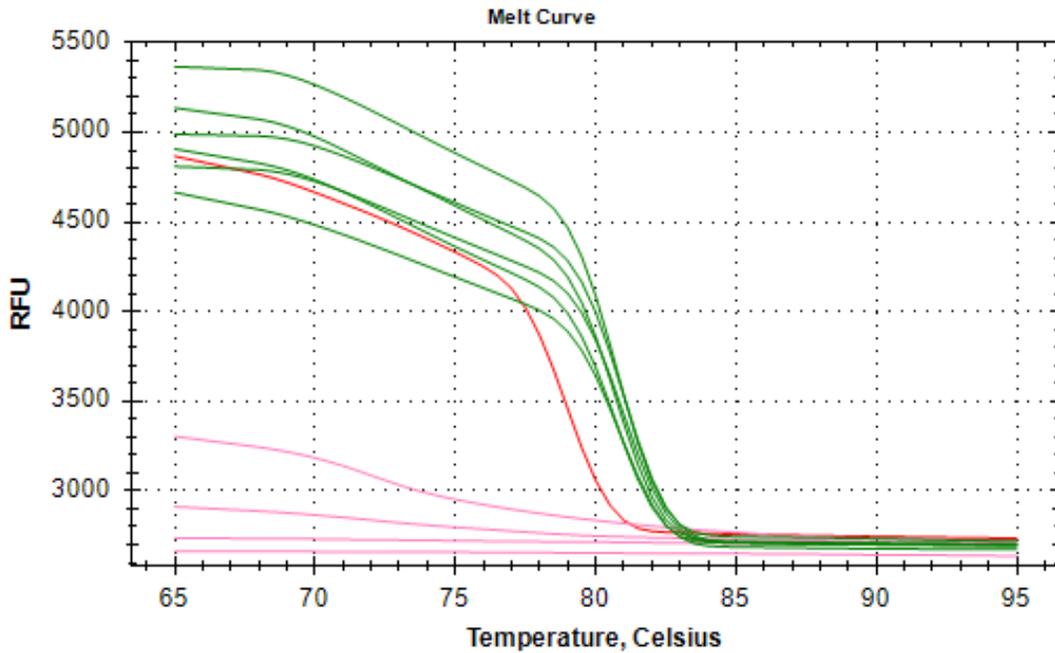
Figure 3. Melting curve of tested samples**,** green lines represent positive control samples (standard curve samples), the red line represents positive for *Avian avulavirus* sample (S4), and pink lines represent the negative for *Avian avulavirus* samples.
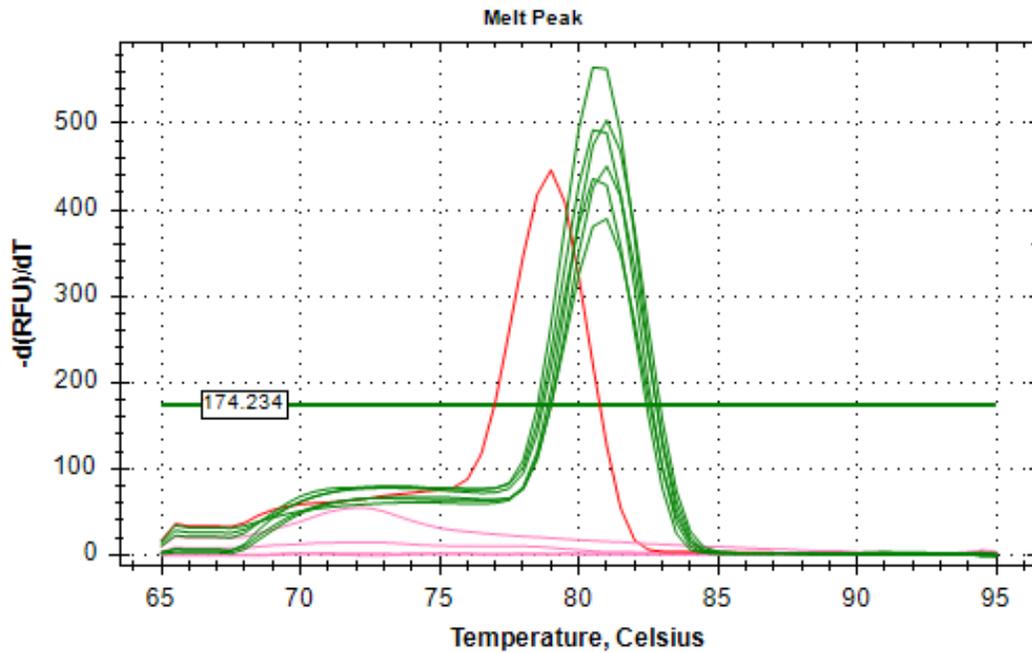
Figure 4. Melting peak of tested samples, green lines represent positive control samples (standard curve samples), the red line represents positive for *Avian avulavirus* sample (S4) showed a different melting peak, and pink lines represent the negative for *Avian avulavirus* samples.

(38) gi 705927774 gb KM288610.1 NDV strain NDV-B23-RLQP-CH-EG-12
(45) gi 705927819 gb KM288620.1 NDV strain NDV-B161-RLQP-CH-EG-12
(40) gi 705927791 gb KM288613.1 NDV strain NDV-B102-RLQP-CH-EG-12
(44) gi 705927815 gb KM288619.1 NDV strain NDV-B127-RLQP-CH-EG-12
(43) gi 705927811 gb KM288618.1 NDV strain NDV-B114-RLQP-CH-EG-12
(37) gi 705927770 gb KM288609.1 NDV strain NDV-B7-RLQP-CH-EG-12
(41) gi 705927802 gb KM288616.1 NDV strain NDV-B110-RLQP-CH-EG-12
(49) gi 823075337 gb KP316015.1 NDV isolate NDV-F460-RLQP-CH-EG-13
(39) gi 705927787 gb KM288612.1 NDV strain NDV-B83-RLQP-CH-EG-12
(42) gi 705927807 gb KM288617.1 NDV strain NDV-B113-RLQP-CH-EG-12
(50) gi 823075339 gb KP316016.1 NDV isolate NDV-F388-RLQP-CH-EG-14
(56) gi 906846883 gb AB871428.1 NDV F gene for fusion protein strain: NDV EG CK 67 11
(53) gi 906846873 gb AB871423.1 NDV F gene for fusion protein strain: NDV EG CK 129 12
(47) gi 814944470 gb KR010946.1 NDV strain VNDV VII chicken Egypt BSU-BS-KN2 2013
(54) gi 906846879 gb AB871426.1 NDV F gene for fusion protein strain: NDV EG CK 13 11
(48) gi 814944476 gb KR010949.1 NDV strain VNDV VII chicken Egypt BSU-BS-KB63 2014
(55) gi 906846881 gb AB871427.1 NDV F gene for fusion protein strain: NDV EG CK 101 12
(36) gi 411169868 gb JX647839.1 NDV strain NDV-EG-567F-2012
(51) gi 891481119 gb KP119141.1 NDV strain EG CK NDV 44 Aswan 2013
(46) gi 728054734 gb KM659400.1 NDV isolate chicken USC Egypt 2014
(81) KC542912.1 NDV isolate Chicken China Shandong 01 2012
(24) FJ882014.1 NDV SG Liaoning 2009 (VIId)
(52) gi 891481134 gb KP119146.1 NDV strain EG CK NDV 7B Qena 2012
(76) KC542905.1 NDV isolate Chicken China Liaoning 01 2009
(79) KC542909.1 NDV isolate Chicken China Shandong 02 2011
(77) KC542906.1 NDV isolate Chicken China Hebei 01 2010
(80) KC542911.1 NDV isolate Chicken China Beijing 01 2012
(82) KC542914.1 NDV isolate Chicken China Hebei 01 2012
(78) KC542908.1 NDV isolate Chicken China Shandong 01 2011
(85) KP742770.1 NDV isolate Sheldrake duck China Guizhou SS1 2014
(71) KC542899.1 NDV isolate Chicken China Jilin 01 2008
(75) KC542903.1 NDV isolate Chicken China Beijing 02 2009
(57) gi 930306421 gb KR082481.1 NDV strain NDV House sparrow Desouk Egypt MS12 2014
(58) gi 930306508 gb KR082482.1 NDV strain NDV House sparrow Gallin Egypt MS14 2014
(60) gi 930306822 gb KR082485.1 NDV strain NDV Cattle Egret Desouk Egypt MS3II 2014
(16) DQ659677.1 NDV strain NA-1 (VIId)
(69) KC542897.1 NDV isolate Chicken China Tianjin 01 2007
(61) GQ849007.1 NDV isolate JSD0812
(74) KC542902.1 NDV isolate Chicken China Beijing 01 2009
(67) JN986838.1 NDV strain APMV-1 chicken ZA AL495 04
(72) KC542900.1 NDV isolate Chicken China Liaoning 01 2008
(73) KC542901.1 NDV isolate Chicken China Liaoning 03 2008
(70) KC542898.1 NDV isolate Chicken China Tianjin 02 2007
(22) EU583503.1 NDV isolate Hebei China 2004 (VIId)
(68) KC542895.1 NDV isolate Chicken China Hebei 01 2006
(87) S4
(90) MK124761.1 Avian avulavirus 1 isolate goose/China/ZJ-1/2000 complete genome
(19) EF589134.1 NDV strain H2 (VIId)
(5) AF458010.1 NDV isolate JS-3 00 (VIIc)
(15) DQ439910.1 NDV strain NDV05-055 (VIIc)
(13) DQ227246.1 NDV isolate JS02 (VIIc)
(14) DQ227254.1 NDV isolate SWS03 (VIIc)
(3) AF109886.1 NDV strain Q-GB 445 97 (VIIa)
(2) AF083961.1 NDV strain TW 94P (VIIa)
(88) U62620.1 NDU62620 NDV Taiwan 95 (VIIa)
(1) AF001134.1 NDV strain RI-1 88 (VIIe)
(86) KR732614.1 NDV strain NDV peacock Peru 2011
(11) AY928933.1 NDV isolate MK13 75 (VIIb)
(63) HQ589257.1 NDV strain Bareilly (VIIb)
(62) GU182323.1 NDV strain chicken SPVC Karachi NDV 43 2008 (VIIf)
(65) JN682185.1 NDV isolate chicken CP Rawalpindi1 2010 (VIIf)
(66) JN682189.1 NDV isolate chicken CP Rawalpindi2 2010 (VIIf)
(7) AY562986.1 NDV isolate anhinga U.S.(Fl) 44083 93
(17) EF065682.1 NDV isolate rAnhinga
(8) AY562988.1 NDV isolate chicken U.S.(CA) 1083(Fontana) 72
(27) gi 265020449 gb FJ969394.1 NDV strain NDV chicken Egypt 3 2006
(59) gi 930306702 gb KR082484.1 NDV strain NDV pigeon Desouk Egypt MS3I 2014
(6) AJ880277.1 Pigeon paramyxovirus-1
(25) gi 1014242823 gb KU522142.1 Pigeon paramyxovirus 1 isolate pigeon Egypt VRLCU 2014
(18) EF201805.1 NDV strain Mukteswar
(21) EU293914.1 NDV strain Italien
(10) AY741404.1 NDV strain Herts 33
(23) FJ436303.1 NDV strain ZJ 1 86 Ch
(83) KC920893.1 NDV isolate duck China Guangxi19 2011
(9) AY562991.1 NDV isolate chicken N. Ireland Ulster 67
(12) AY935499.2 NDV isolate I-2
(84) KM056356.1 NDV isolate Avinew
(26) gi 265020447 NDV strain NDV chicken Egypt2006
(28) gi 265020451 gb FJ969395.1 NDV strain NDV chicken Egypt 4 2006
(20) EU289028.1 NDV strain VG GA
(4) AF309418.1 NDV B1
(33) gi 397870247 gb HQ455809.2 NDV isolate NDV II VRLCU Gharbia19 2009
(30) gi 397870241 gb HQ455806.2 NDV isolate NDV II VRLCU Behaira9 2009
(34) gi 397870249 gb HQ455810.2 NDV isolate NDV II VRLCU Giza23 2009
(29) gi 397870239 gb HQ455805.2 NDV isolate NDV II VRLCU Giza27 2009
(32) gi 397870245 gb HQ455808.2 NDV isolate NDV II VRLCU Gharbia18 2009
(35) gi 397870251 gb HQ455811.2 NDV isolate NDV II VRLCU Giza24 2009
(31) gi 397870243 gb HQ455807.2 NDV isolate NDV II VRLCU Gharbia17 2009
(64) JF950510.1 NDV strain LaSota
(89) Y18898.1 clone 30

0.02

Figure 5. Neighbor-joining phylogenetic tree of the full-length F gene of Egyptian isolate of Newcastle disease virus (NDV) (S4) in comparison to other NDV strains from GenBank. Bootstrap values are shown above the branches. S4 isolate is indicated by a solid green circle.

MG717684.1:6418-8133 Avian avulavirus 1 isolate NDV/teal/Egypt/SDU-3/2016 complete genome
MG717686.1:6418-8133 Avian avulavirus 1 isolate NDV/teal/Egypt/SDU-4/2016 complete genome
MK673139.1:6418-8133 Avian avulavirus 1 isolate chicken/Egypt/Sohag/66/1030/2011 complete genome
MG717683.1 Avian avulavirus 1 isolate NDV/quail/Egypt/SDU-2/2016 complete genome
KC542911.1:6418-8133 Newcastle disease virus isolate Chicken/China/Beijing/01/2012 complete genome
JN400897.1:6418-8133 Newcastle disease virus strain Chicken/China/SDLY01/2010 complete genome
KC542903.1:6418-8133 Newcastle disease virus isolate Chicken/China/Beijing/02/2009 complete genome
JQ244774.1 Newcastle disease virus isolate Pigeon/China/SD/55/06 hemagglutinin-neuraminidase protein (HN) gene complete cds
MG717685.1 Avian avulavirus 1 isolate NDV/cattle egret/SDU-1/2016 complete genome
HQ917081.1 Newcastle disease virus strain A/goose/Guangxi/1/2008 hemagglutinin-neuraminidase (HN) gene complete cds
MK124761.1:6404-8119 Avian avulavirus 1 isolate goose/China/ZJ-1/2000 complete genome
S4
JN400896.1:6418-8133 Newcastle disease virus strain Chichen/China/SDSG01/2011 complete genome
KC020308.1 Newcastle disease virus NDV/Chicken/TC/4/2011 hemagglutinin-neuraminidase gene complete cds
KF011207.1 Newcastle disease virus isolate Malaysia/1041-632/2008 hemagglutinin-neuraminidase mRNA complete cds
KY212126.1:6222-7937 Avian avulavirus 1 isolate chicken/Jordan/17/2004 partial genome
EU526308.1 Newcastle disease virus isolate TW03-333 hemagglutinin-neuraminidase gene complete cds
KY042134.1 Avian avulavirus 1 isolate pigeon/Egypt/El Fayom/84/1113/2015 complete genome
M24707.1 Newcastle disease virus ULS/67 hemagglutinin-neuraminidase gene complete cds
AY845400.2:6412-8145 Newcastle disease virus LaSota complete genome
FJ939313.1 Newcastle disease virus NDV/Chicken/Egypt/1/2005 complete genome

0.02

Figure 6. Neighbor-joining phylogenetic tree of the full-length HN gene of Egyptian isolate of Newcastle disease virus (NDV) (S4) in comparison to other NDV strains from GenBank. Bootstrap values are shown above the branches. S4 isolate is indicated by a solid green circle.

Figure 7. 3D structure for F protein of Newcastle disease virus (S4 isolate) created by SWISS-Model modeling online server and visualized by PyMOL program version 2.3.4, red color represents the cleavage site.
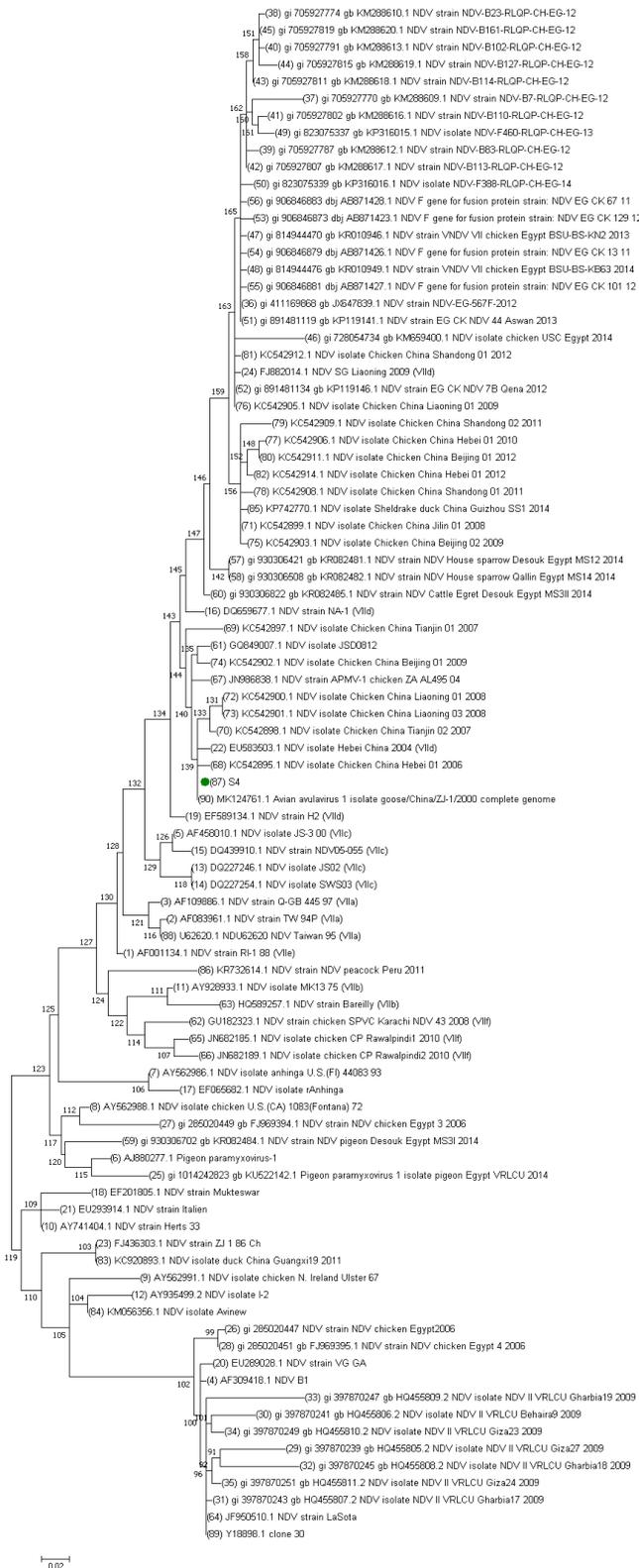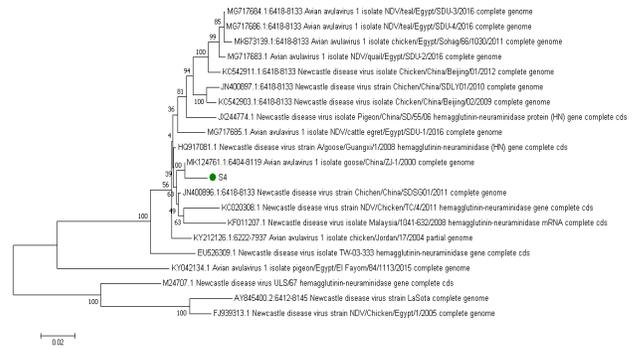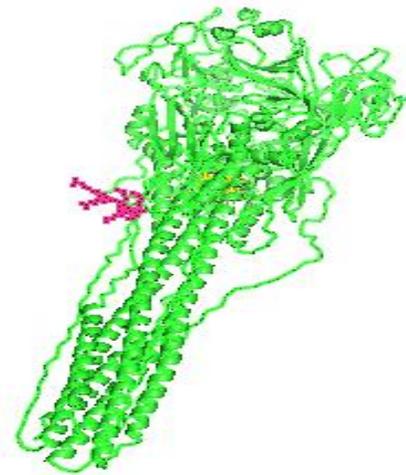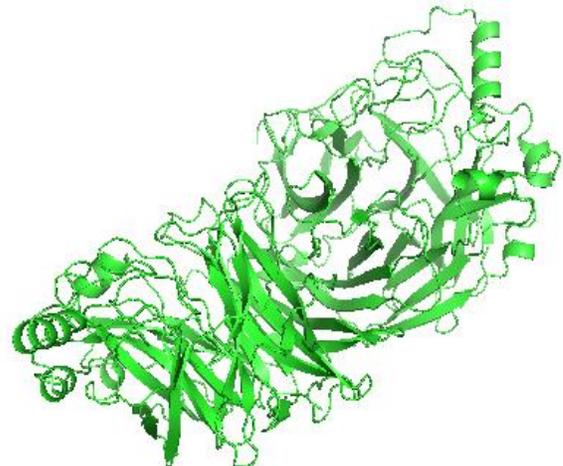

Figure 8. 3D structure for HN protein of Newcastle disease virus (S4 isolate) created by SWISS-Model modeling online server and visualized by PyMOL program version 2.3.4.

DISCUSSION

In the current study, only five samples (50% samples) showed HA positive activity indicating infection with a hemagglutinating virus. To confirm NDV infection, RT-qPCR was performed using the HA positive samples.

Only S4 isolate was positive for NDV using universal primers for APMV-1. Negative RT-qPCR results for S3, S7, S9, and S10 may indicate an infection with another hemagglutinating virus-like avian influenza H9 or H5; however, history of mortalities and symptoms severity indicated H9 infection mixed with other respiratory pathogens other than H5 (Hussein et al., 2014; Sedeik et al., 2018). The most important pathogenicity indicator for NDV is the F protein gene sequence analysis mainly for cleavage site in which velogenic strains have polybasic amino acid sequences; therefore, molecular identification and phylogenetic analysis of the F gene is a major determinant

of NDV virulence instead of conventional methods (Mohamed et al., 2011; Damena et al., 2016). Also, it can be considered as a reliable way for NDV virulence evaluation when compared to traditional ways of evaluation (Ganar et al., 2014).

Results of F protein gene sequencing revealed that the cleavage site motif of S4 isolate has the sequence of velogenic NDV strains $_{112}$RRQKRF$_{117}$ in agreement with (Sedeik et al., 2019). Also, the neurological effects of NDV infections by is thought to be due to the presence of the phenylalanine (F) residue at position 117 (Collins et al., 1993). The full sequence of both F and HN protein genes were submitted to the GenBank database with accession number MN905162 for the full F protein gene sequence and MN905163 for the full HN protein gene sequence.

F and HN proteins genes genetic and phylogenetic analysis in the present study revealed high similarity of S4 isolate with Chinese isolates and relatively fewer similarities with the Egyptian isolates which may strongly refer to the role of migratory wild birds in NDV evolution in Egypt.

CONCLUSION

Newcastle disease still occurs in sporadic cases despite massive vaccination programs implemented in the Egyptian poultry field. Migratory wild birds are supposed to have a big role in the continuous evolution of NDV in Egypt. Further epidemiological and surveillance work is strongly recommended to define the exact role of migratory wild birds in NDV evolution in Egypt with defining the main causes of the inability of currently used vaccines to protect chickens against infection with Newcastle disease virus.

DECLARATION

Authors' contributions

All authors reviewed the final manuscript. This work is a part of Mira Maher, and Abdulrahman S. Metwally thesis under the supervision of Shakal M, and Gehan Safwat. Shakal M. designed, supervised the experiments, and co-wrote the paper. Gehan Safwat co-designed the experiment and co-wrote the paper. Mohammed A. Abdel Sabour conducted samples pooling, virus isolation, and co-wrote the paper. Mira Maher and Abdulrahman S. Metwally conducted RNA extraction, genes amplification by PCR, and conducted genetic alignment. Yahia M. Madbouly conducted RNA extraction, real-time reverse transcription PCR, GenBank submission, phylogenetic analysis, and co-wrote the paper.

Competing interests

The authors declare that they have no competing interests.

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