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Molecular Characterization of Newcastle Disease Virus Genotype VII.1.1 from Egyptian Mallard Ducks with Nervous Manifestations

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ABSTRACT

In Egypt, Newcastle disease virus (NDV) strains of genotype VII are known to be mild in domestic waterfowl and considered reservoirs. This is the first report for the detection of NDV GVII.1.1 from ducks showing severe clinical signs with high mortalities and nervous manifestations, additionally, isolation of NDV and molecular characterization for full HN and F genes were performed. In the current study, 16 backyard mallard duck flocks showing severe nervous signs with high mortalities were investigated by real-time RT-PCR using primers specific for the Fusion gene of NDV and matrix gene for avian influenza virus (AIV). Fourteen duck flocks tested positive for AIV and only two flocks tested positive for NDV infection. NDV was isolated from the trachea and brain of the same duck from each flock then full HN and F genes were sequenced. The phylogenetic analysis of the F and HN genes indicated that these strains were clustered with NDV genotype VII 1.1. The F gene had a specific mutation that cluster them in a new branch with with A11T in the signal peptide, N30S, T324A, and 480K in the hydrophobic heptad repeat (HRc) compared to the Lasota strain. The duck strains of NDV isolated from the brain had N294K in the hydrophobic heptad repeat-b (HRb) of F protein compared to the strains isolated from the trachea of the same duck, which may have a role in crossing the blood-brain barrier. The HN protein had a specific mutation that clustered them in a new branch with mutations of A4V, R15K in the cytoplasmic region, A28T in the transmembrane domain, and S76L in the HRa. In addition, HN protein had A50T, S54R T232N, P392S, and T443V, and multiple mutations were detected in the neutralizing epitopes specific to strains in the present study (N120G, K284R, S521T) that can alter virus antigenicity. The current study indicated the continuous evolution of NDV strains from genotype VII circulating in Egypt with increasing pathogenicity in ducks. The present findings demonstrated the urgent need for the vaccination of ducks and geese with killed NDV vaccines to reduce economic losses due to virus infection and prevent transmission to chickens helping in ND control in Egypt.

Keywords: F gene, Genotype VII 1.1, Mallard duck, Newcastle disease virus, Protein

INTRODUCTION

Newcastle disease (ND) is a contagious viral disease which detected in a wide range of bird species causing devastating economic impacts worldwide. Control of the disease relies on vaccination combined with the implementation of biosecurity measures to reduce virus shedding and decrease economic losses from infection. However, the continuous genetic evolution of the virus negatively affects the efficacy of the available commercial vaccines (Moustapha et al., 2023).

The disease is caused by virulent avian orthoavulavirus 1 (AOAV-1), belonging to the

Paramyxoviridae family (Rima et al., 2019), the virus was previously referred as avian paramyxovirus-1 (APMV-1). The virus is enveloped with a negative sense, non-segmented RNA coding six genes (3-N-P-M-F-HN-L-5), which translated into eight proteins with two important surface glycoproteins; haemagglutinin-neuraminidase (HN) and fusion (F) protein (Steward et al., 1993). Important components for the virus's entry and exit from host cells are the HN and F proteins. Hemolysis, cell fusion, and virus entrance are all highly influenced by the F protein (Morrison, 2003).

The clinical signs observed in ND virus infection varied from mild infection to severe clinical symptoms (sometimes mortality reaches 100%) according to the virus virulence and bird species (Jindal et al., 2009). Also, coinfections, immune status, age, health, and environmental conditions affect the disease severity. Newcastle disease viruses are categorized regarding their pathogenicity in the host into velogenic (showing severe clinical signs with high mortality), mesogenic (showing respiratory manifestations, rarely nervous signs), lentogenic (ranging from subclinical to mild respiratory signs), and asymptomatic enteric (No clinical signs, Cattoli et al., 2011; Miller and Koch, 2013).

For decades, waterfowl represented the natural reservoirs of NDVs with unnoticed infection or only mild clinical signs when infected by either lentogenic or velogenic NDVs (Alexander and Senne, 2008; Dimitrov et al., 2016). In Egypt, muscovy ducks were shown to be excellent carriers for NDV-genotype VIId and efficiently transmit NDV to broiler chickens in contact (Elbestawy et al., 2019). However, it has been documented that NDVs can spread from chickens to wild birds, and then wild birds can transmit the virus to other countries (Xiang et al., 2017).

During the last several years, ND outbreaks in domestic waterfowl with obvious clinical manifestations have been reported frequently (Dai et al., 2013). Interestingly, the pathogenicity of one duck and one chicken NDV strain isolated from China was studied (Meng et al., 2018), and the results showed that the mortality reported for NDV chicken isolates in 4-week-old ducks was 70% compared to 20% mortalities caused by NDV duck isolate, both strains were belonging to NDV genotype VII(1.1). This can be explained by Hidaka et al. (2021), who found that consecutive circulation of NDV chicken strain (9a5b strain) in domestic waterfowl can result over time in more virulent strains for chickens and waterfowl. Moreover, outbreaks of ND (NDV/duck/Jiangsu/JSD0812/2008) in laying duck flocks in China were reported with a 70% drop in egg production and mortalities up to 50% (Liu et al., 2015).

In the current study; for the first time in Egypt velogenic NDV GVII.1.1 was isolated from mallard ducks showing severe clinical signs, nervous manifestations, and high mortality. Moreover, molecular characterization of full F and HN genes of the NDV strains isolated from trachea and brain tissues was performed.

MATERIAL AND METHODS

Ethical approval

Ethical approval for this study was given by the Institutional Animal Care and Use Committee under the University of Sadat City No. VUSC-004-1-24.

Sampling

Trachea and brain were collected from 16 backyard duck flocks showing respiratory, enteric, and nervous signs with high mortalities and were examined during the winter of 2023 from Menoufiya governorate. One duck with nervous signs from each flock was humanly euthanized then packed and transported on ice to the birds and rabbit medicine department, faculty of veterinary medicine, university of Sadat City, Menoufiya, Egypt. Post-mortem lesions were recorded like severe congestion in the liver, spleen, pancreas, kidney and intestine then trachea and brain from the same duck collected and processed separately (total 16 trachea and 16 brain samples were collected). The clinical signs and postmortem lesions are shown in (Figure 1). Tissue samples were homogenized, suspended in sterile phosphate-buffered saline (PBS) (with penicillin 2000 units/mL), freezing and thawing three times then clarified by centrifugation at 1500 rpm for 30 minutes at 4^{o}C (WHO, 2002).

Molecular detection of the causative agent by rRT-PCR

The QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) was used to extract RNA from 16 tracheal tissue suspensions in accordance with the manufacturer's recommendations. Real-time reverse transcriptase polymerase chain reaction (rRT-PCR) was performed using Quantitect probe RT-PCR kit (Qiagen, Inc. Valencia CA, USA) with specific primers for matrix (M) gene of avian influenza virus (AIV) (Spackman et al. 2003), and M gene of NDV (Wise et al., 2004), sequences of primers and probes were listed in Table 1.

Virus isolation

The trachea and brain tissue suspensions of two mallard duck flocks that tested positive for NDV by rRT-PCR were used for ND virus isolation. The clarified tissue suspensions were filtered by 0.2 µm bacteriological filter

then 0.2 ml was injected in the allantoic cavity of 10-day-old SPF embryonated chicken eggs (for each sample five eggs were inoculated). Eggs were incubated at 37 ˚C and candling was performed for successive 3 days after allantoic cavity inoculation, then at the end of the third-day eggs were chilled to 4 ◦C, then the allantoic fluids were tested by hemagglutination test (HA). Briefly, two-fold serial dilution for the allantoic fluid in 50 µl PBS was performed then HA activity was tested by the addition of 50 µl of 1% washed RBCs after 20-30 minutes of incubation at room temperature.

Amplification of fusion and hemagglutinin-neuraminidase genes by RT-PCR

Two primer sets were used for each gene to be amplified into two segments. Primer's sequence and cycling protocols for F gene amplification were carried out according to Munir et al. (2010), and for HN gene amplification was carried out according to Kiani et al. (2021). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using COSMO RT-PCR Master Mix (Willowfort, Birmingham, UK). Size-specific PCR products for each gene were separated by gel electrophoresis and Specific DNA bands were purified for sequencing using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

Fusion and hemagglutinin-neuraminidase gene sequencing

In separate reactions, use forward and reverse primers with each specific DNA fragment. The sequencing procedure was carried out using a cycle sequencing kit, big dye terminator v3.1 (Applied Biosystems, Foster City, California, USA), according to the manufacturer's instructions.

Fusion and hemagglutinin-neuraminidase genes sequence analysis

Using the Clustral W alignment approach of BIOEDIT software (Hall, 1999), the sequences obtained during the study were contrasted with Egyptian field strains and reference strains from various countries that were listed in the GenBank database (NCBI). The maximum likelihood technique in MEGA 6 software was used to generate the phylogenetic trees (Tamura et al., 2013). The percentage of similarity between the strain sequences used in this study and other sequences that have been published and made available in the NCBI database was calculated using Lasergene software (version 7.2; *DNASTAR*, Madison, WI).

The Expasy database, PyMOL software, and SWISS-MODEL were used to simulate the three-dimensional (3D) structure of the F and HN genes (Waterhouse et al., 2018). According to Gupta and Brunak (2016), N-linked glycosylation was detected using NetNGlyc 1.0 Server.

Table 1. Primers used for detection of matrix gene of avian influenza and F gene of Newcastle disease virus

Virus	Gene	Name	Sequence	Reference
AI	M	Sep1	AGATGAGTCTTCTAA CCGAGGTCG	Spackman et al. (2003)
		Sep2	TGCAAAAACATCTTC AAGTCTCTG	
		Sepro	[FAM]TCAGGCCCC CTCAAAGCCGA [TAMRA]	
NDV	M	M-F	5'AGTGATGTGCTCGGACCTTC3'	Wise et al. (2004)
		M-R	5'CCTGAGGAGAGGCATTTGCTA3'	
		M-probe	5'[FAM] TTCTCTAGCAGTGGGACAGCCTGC [TAMRA]3'	

AI: Avian influenza, NDV: Newcastle disease virus.

RESULTS

Virus identification and isolation

By testing the tracheal suspensions of the 16 duck flocks by rRT-PCR; two backyard mallard duck flocks only tested positive for NDV While the remaining 14 flocks tested positive for AIV. The first flock that tested NDV positive was sampled in January 2023 and was 2 weeks old with 70% mortality and the second flock sampled in February 2023 was 4 weeks old with 55% mortality.

Four samples (2 tracheae and 2 brains) of the NDV-positive flocks were inoculated in a total of 20 eggs (5 for each sample) resulting in embryonic death between 36-48 hrs post-inoculation indicating virulent virus. The 4 isolates were tested HA positive with HA titer of 256 HAU. The allantoic fluids were tested by rRT-PCR confirmed that the causative agent in the 4 samples was NDV while the assay gave negative results for the avian influenza virus.

Sequence and phylogenetic analysis of fusion and hemagglutinin-neuraminidase genes

The F and HN gene segments for the four identified and isolated NDV strains (two strains isolated from the

trachea and two strains isolated from the brain) were successfully amplified and sequenced. The obtained sequences were submitted with an accession number to the GeneBank at the National Center for Biotechnology Information (NCBI), and listed in Table 2.

The nucleotide alignment of the complete F and HN gene sequences and the phylogenetic tree of the duck isolates were recognized as a new branch in the genotype VII 1.1 (Class II) as shown in figures 2, 3, 4, and 5.

The amino acid (A.A) identity of the complete F and HN protein sequence compared to other reference strains, vaccines, and Egyptian strains. The two isolates had high similarity and close relatedness 98.5-99.3% and 96.4-96.8% for F and HN genes with Chinese strains related to VIIJ, respectively, and 96.7-99.3% and 97.3-98.2% with other Egyptian strains for F and HN gene, respectively (Figures 6 and 7).

The duck strain had high A.A. identity 96-96.2% and 94.5% with vaccine strains that cluster with VIId (NDV-KBNP-C4152R2L) Korean vaccine for F and HN gene, respectively, and 87.8-88.5% and 86.2-86.4% with classic vaccine strain (Lasota, clone 30 and VG/GA [Avinew]) genotype II for F and HN, respectively and 90.7-90.9% and 89.1% with D26/76 vaccine genotype I for F and HN gene, respectively (Figures 6 and 7).

Table 2. List of duck Newcastle disease virus strains names, origin, and gene bank accession numbers

Strain Name	Sample origin	F	HN
Avian-orthoavulavirus-1-Duck-Egypt-MN1-2023	Trachea	PP182340	PP182344
Avian-orthoavulavirus-1-Duck-Egypt-MN2-2023	Brain	PP182341	PP182345
Avian-orthoavulavirus-1-Duck-Egypt-MN3-2023	Trachea	PP182342	PP182346
Avian-orthoavulavirus-1-Duck-Egypt-MN4-2023	Brain	PP182343	PP182347

F: Fusion, HN: Hemagglutinin-neuraminidase

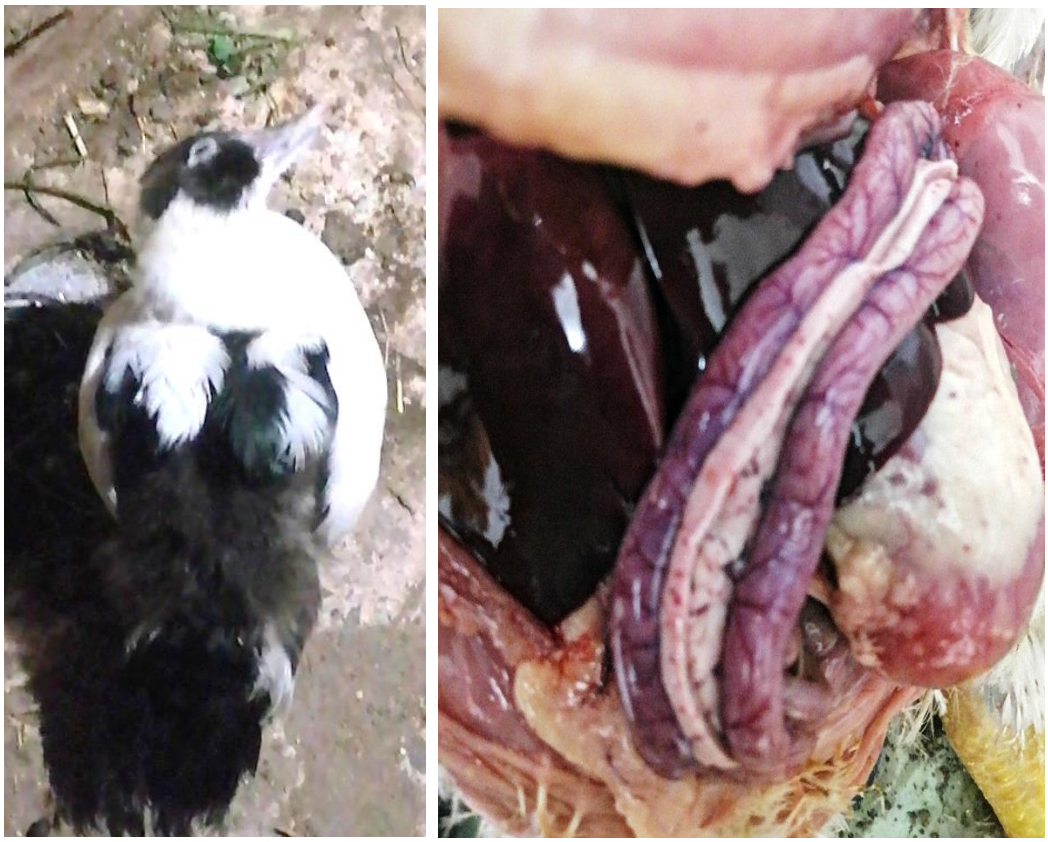

Figure 1. Clinical signs and post-mortem lesions observed in the liver, pancreas, and intestine of a 4-weeks-old mallard duck in February 2023

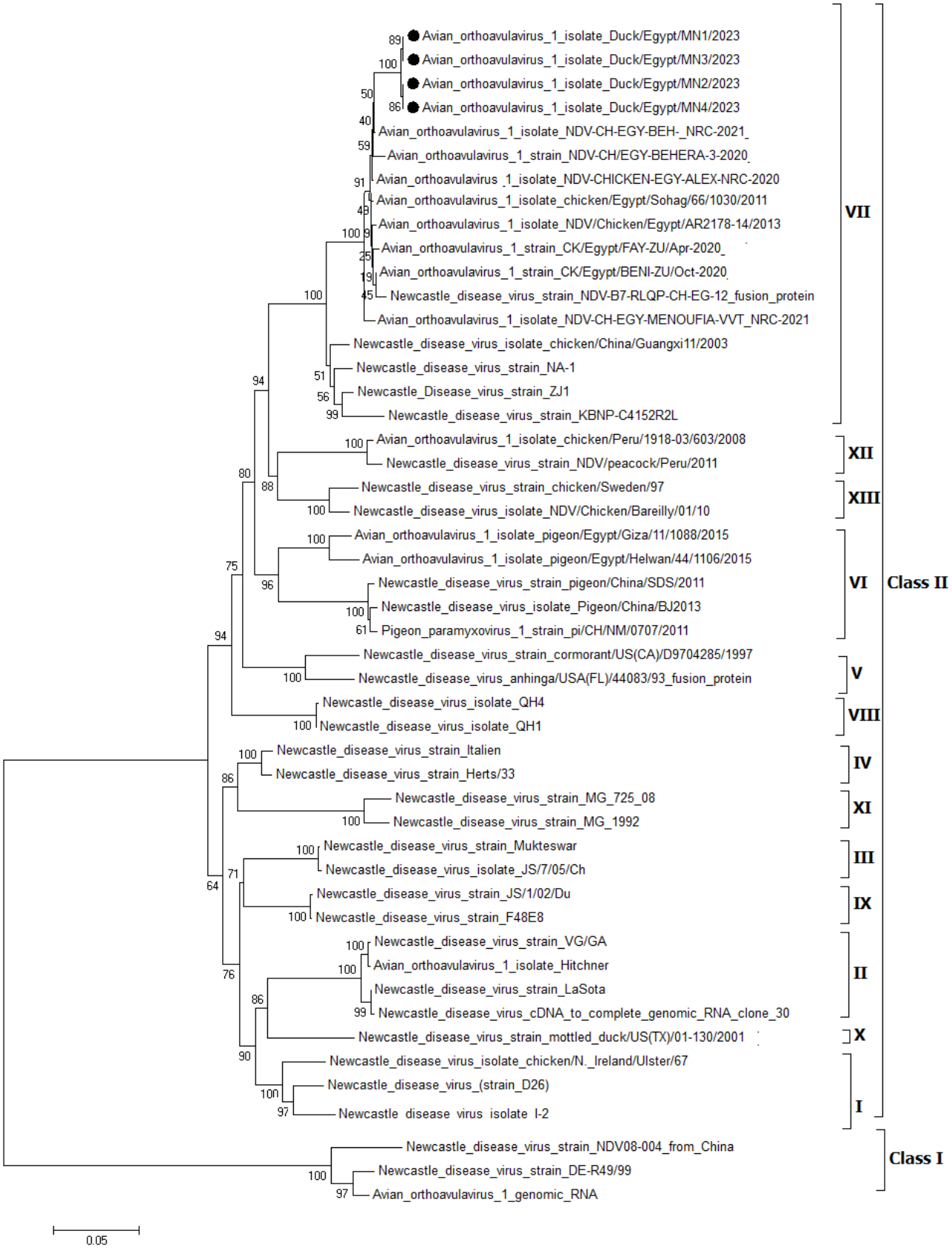

Figure 2. Phylogenetic tree based on nucleotide sequences of the fusion gene using reference sequences representing different genotypes. The black bullets indicated the mallard duck Newcastle disease virus strains isolated in this study

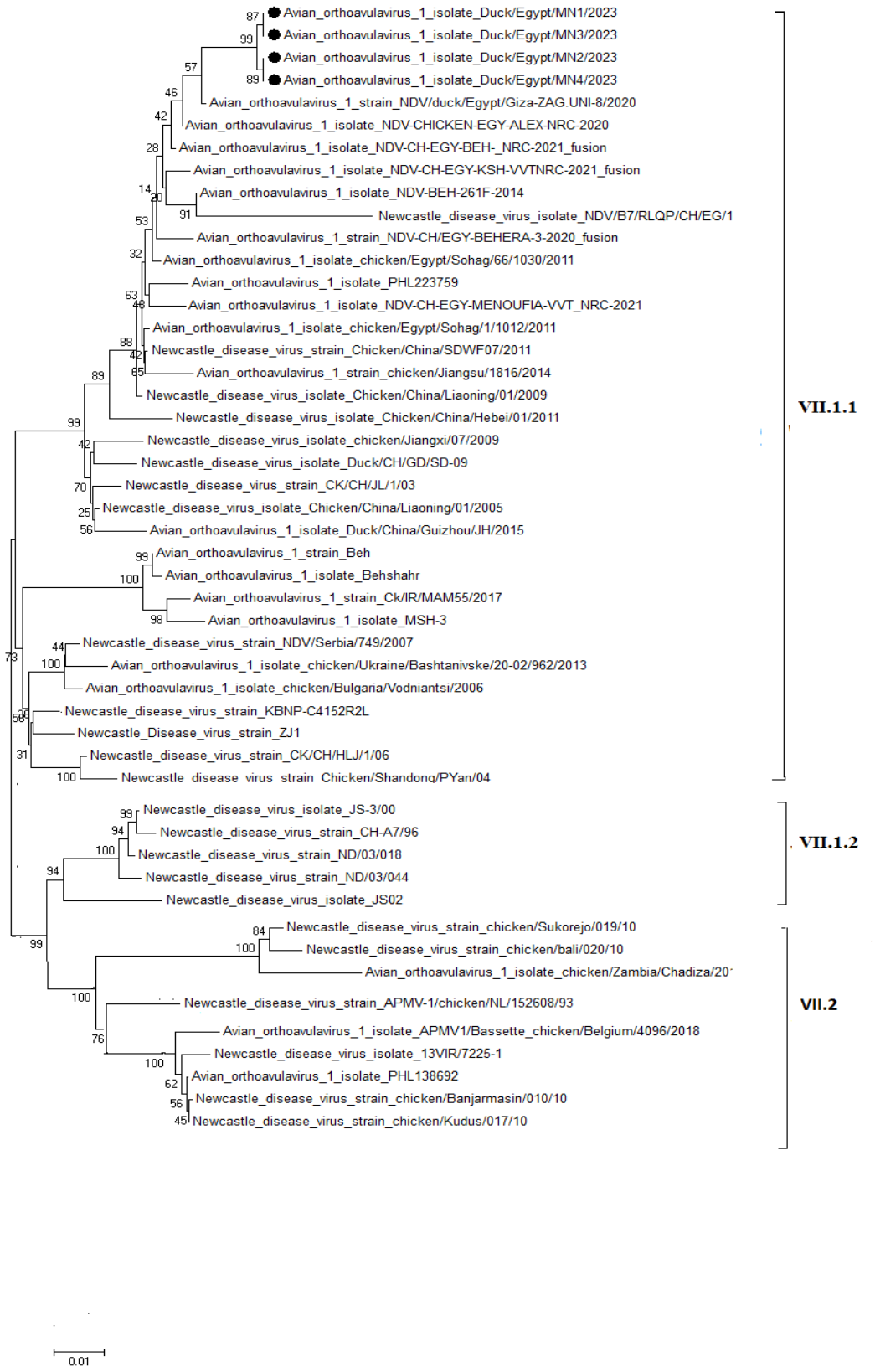

Figure 3. Phylogenetic tree based on nucleotide sequences of the fusion gene using reference strains for Newcastle disease virus GVII. The black bullets indicated the mallard duck Newcastle disease virus strains isolated in this study

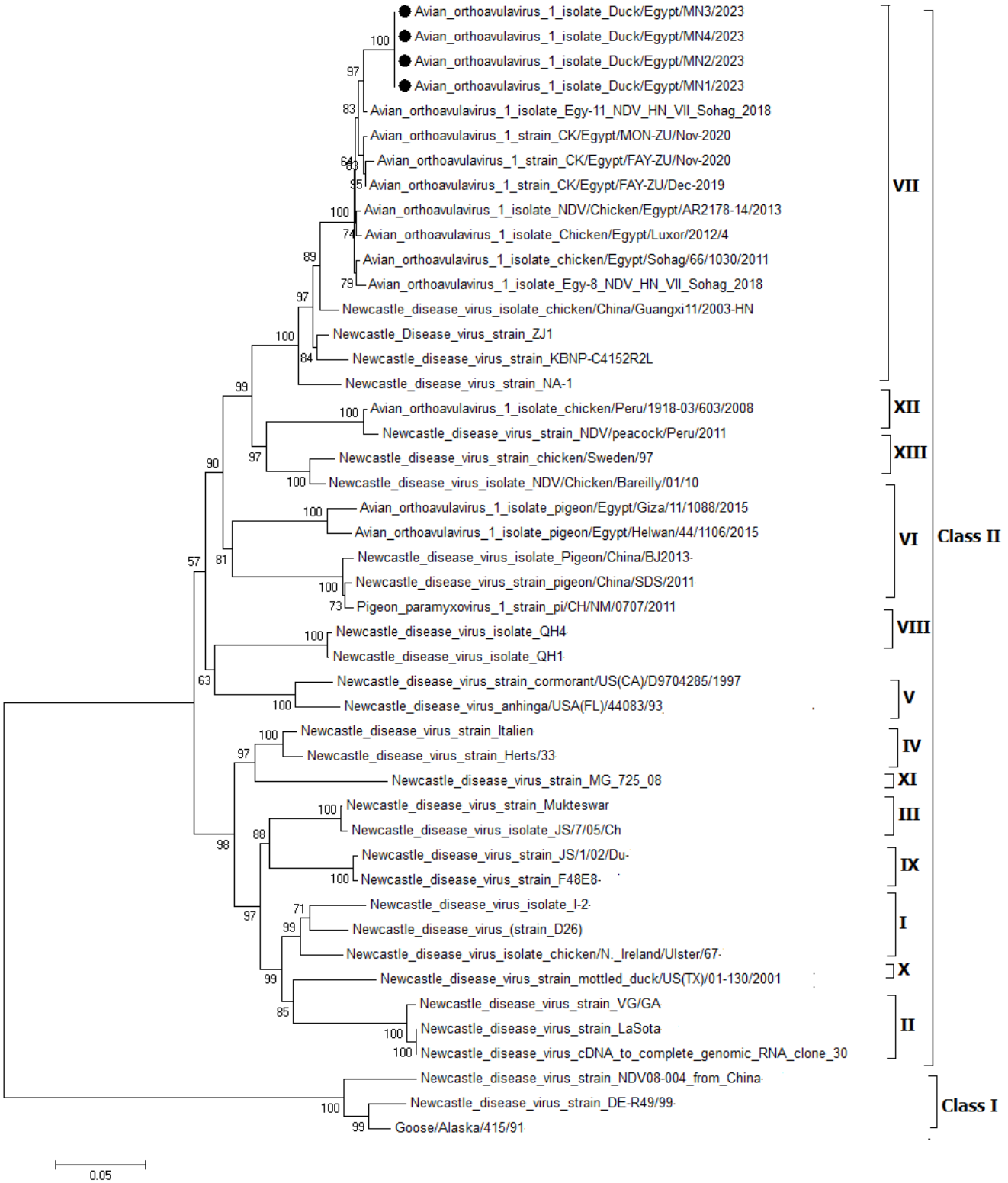

Figure 4. Phylogenetic tree based on nucleotide sequences of the hemagglutinin-neuraminidase gene using reference sequences representing different genotypes. The black bullets indicates the mallard duck Newcastle disease virus strains isolated in this study

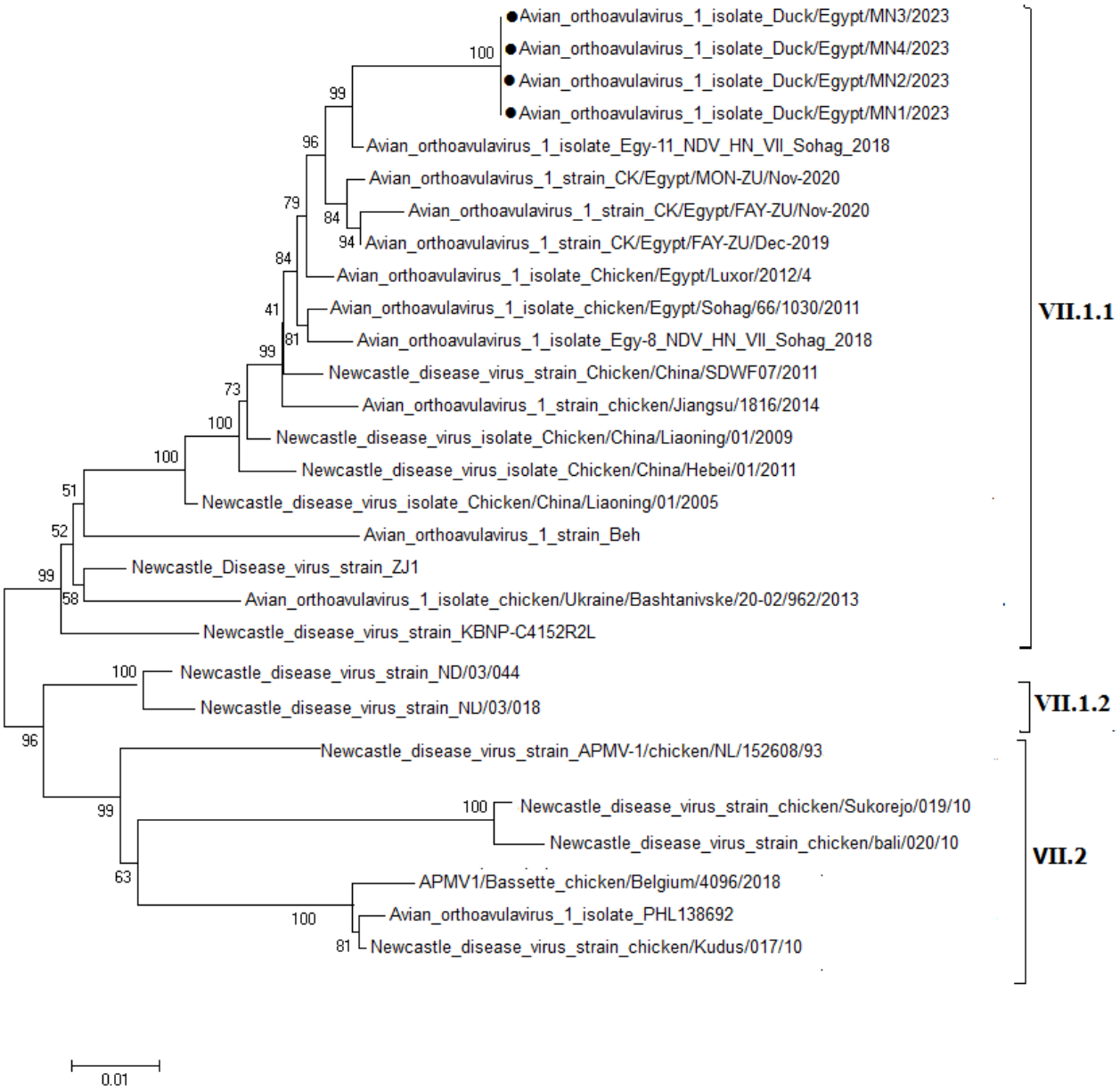

Figure 5. Phylogenetic tree based on nucleotide sequences of the hemagglutinin-neuraminidase gene using the strains and reference strains for Newcastle disease virus GVII. The black bullets indicate the mallard duck Newcastle disease virus strains isolated in this study

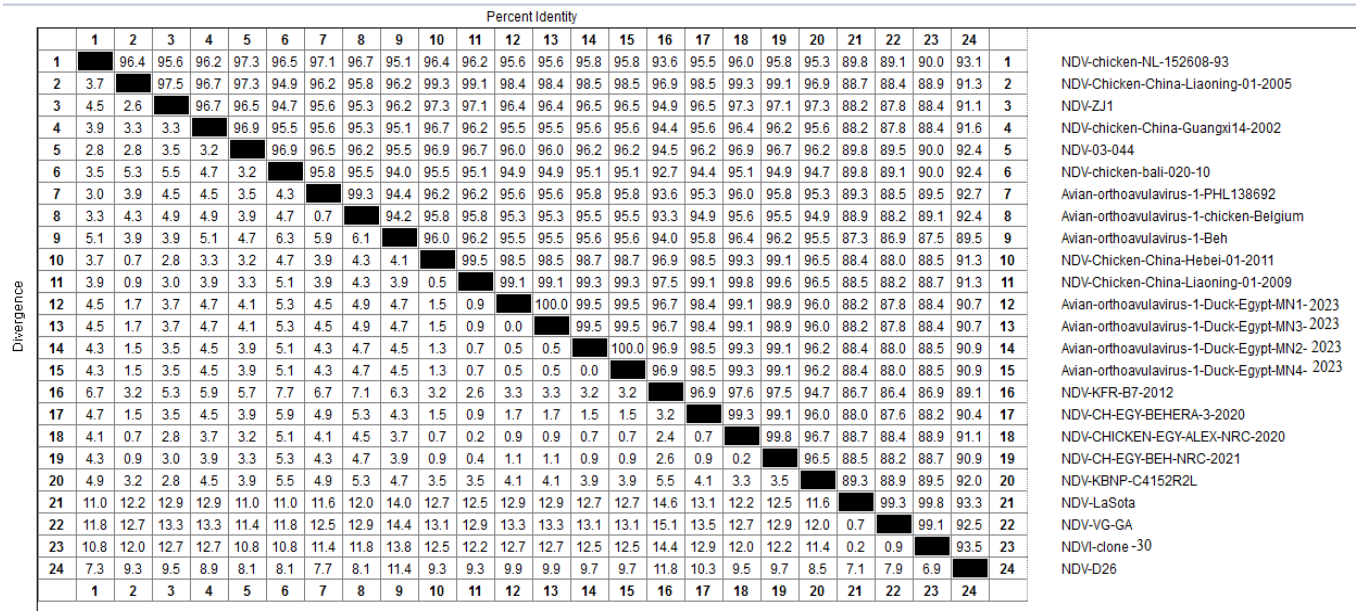

Figure 6. Amino acid identity percent of the obtained sequences of fusion gene from mallard duck and representative global Newcastle disease virus strains plus the commercial vaccinal strains

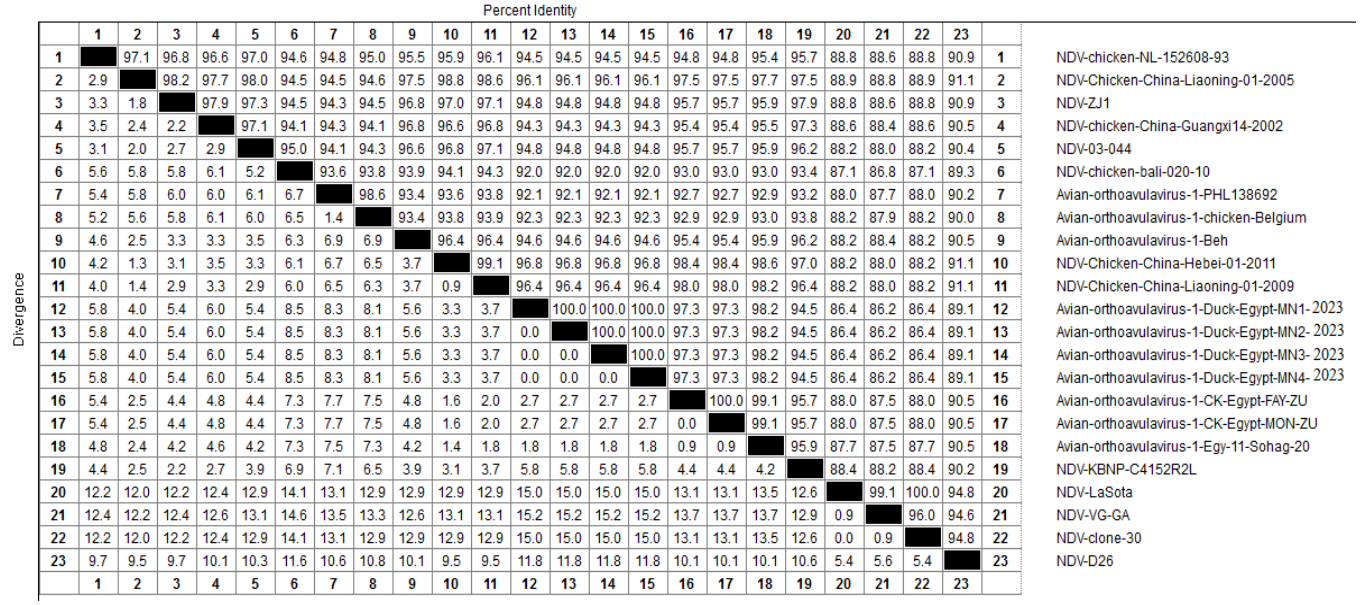

Figure 7. Amino acid identity percent of the obtained sequences of the hemagglutinin-neuraminidase gene from mallard duck and representative global Newcastle disease virus strains plus the commercial vaccinal strains

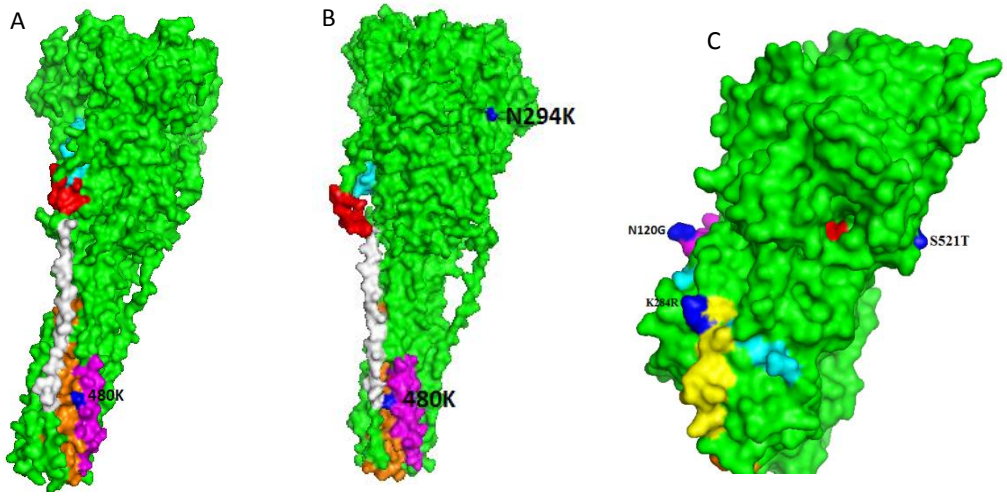

Figure 8. Three-dimensional structure of fusion and hemagglutinin-neuraminidase protein of the mallard duck Newcastle disease virus. **A** and **B**: Three-dimensional structure for fusion protein showing cleavage site (Red), fusion peptide (gray), HRa (orange), HRb (cyan), and HRc (Magnet). The mutation in the HR region (blue). The template protein was obtained by submitting the amino acid sequence of the fusion protein of Avian-orthoavulavirus-1-Duck-Egypt-MN1-2023 and Avian-orthoavulavirus-1-Duck-Egypt-MN2-2023, respectively. **C**: Three-dimensional structure for hemagglutinin-neuraminidase protein showed IDE1 (red), IDE2 (yellow), IDE3 (magnet), IDE4 (cyan), and IDE5 (orange). The mutation in the neutralizing epitopes (blue). The template protein was obtained by submitting the amino acid sequence of the hemagglutinin-neuraminidase protein of Avian-orthoavulavirus-1-Duck-Egypt-MN1-2023

Mutation analysis of fusion gene

All strains in this study had more than 52 amino acid mutations compared with Lasota strains in the F gene as that had been detected in the Egyptian strains. Compared with the lasota strain, The F gene of all strains had specific characteristics of 14 nucleotide new mutations with sense amino acid mutations at A11T in the signal peptide and T324A. Also, they had 480K in the HRc as lasota strains but different than other strains of genotype VII.1.1. The NDV-Duck-Egypt-MN1-2023 and NDV-Duck-Egypt-MN3-2023 that isolated from trachea isolates had specific addition mutations; L15F in the signal peptide and D97E in the F2 subunit. The NDV-Duck-Egypt-MN2-2023 and NDV-Duck-Egypt-MN4-2023 that were isolated from the brain had N294K in the hydrophobic heptad repeat-b (HR_b).

The distinctive characteristics of genotype VII were found in the conserved amino acids in the F gene of all isolates at positions K101 and V121 of the fusion (F) protein, and the cleavage site resembled NDV (RRQKR/F) as NDV genotype VII. Comparing them to the Lasota strain, the fusion protein's functionality depends on seven neutralizing epitopes, which were preserved at locations D72, E74, A75, R78, A79, 157SIAATNEAVHEVT171, and L343. Additionally, 10 amino acid cysteine sites were preserved at positions C76, C199, C338, C347, C362, C370, C394, C399, C401, and C424.

Mutation analysis of the HN gene

All strains in this study had more than 52 A.A. mutations compared with lasota strains as in the Egyptian strains. Compared with the lasota strain, the HN gene had a specific 16 nucleotide new mutation with multiple sense A.A. mutations at A4V, R15K in the cytoplasmic region, A28T in the transmembrane domain, S76L in the HRa. also, it had A50T, S54R T232N, P392S, T443V. There was no difference between the strains isolated from the brain and trachea tissues.

The HN gene of all strains in this study contained seven conserved neutralizing epitopes (193 to 201 [site 23]; 345 to 353 [site 1,14], 494 [site 12], 513 to 521 [site 2,12], and 569 [site 2]) compared with Lasota strain except E347K, S494D, I514V as other Egyptian strains and S521T that specific to strains in this study. In addition, all strains in this study had five (IDEs) neutralizing epitopes, the IDE1 was conserved with Lasota strains. Compared with the Lasota strain, the IDE2 had V288T, T290V, and G293K. The IDE3 had A118E, W123C, I127V. IDE4 had

E256G and IDE5 had V329A, Y340H, D342N, E347K as other Egyptian strains. The N120G and K284R in IDE3 and IDE2 had characteristics in all strains in this study. Also, additional mutations in the neutralizing epitope were detected at F156Y, Y203H, N263K, G495E, and A155V in all Egyptian strains.

All strains of this study shared three essential residues for receptor recognition at positions 174 (R), 401 (E), 416 (R), and 526 (Y), as well as the sialic acid-binding site (NRKSCS) at positions 234–239. In addition, all strains exhibited conservation of the 13 cysteine residues located at positions 123, 172, 186, 196, 238, 247, 251, 344, 455, 461, 465, 531, and 542 when compared to Lasota strains.

Glycosylation sites

All isolates' F gene glycosylation motifs indicated that residues 85, 191, 366, 447, 471, and 541 were the six possible locations for N-linked glycosylation. A study of the glycosylation motif in the HN gene across all isolates identified five possible glycosylation sites at amino acid locations (119, 341, 433, 481, and 508).

The three-dimension structure

The three-dimensional structure of the NDV trachea and brain stains was represented by the full fusion protein, which also clarified the substitution residues in the various active domains (HR1, HR2, cleavage site, and fusion peptides region) when compared to the Lasota strain. (Figure 8 A and B). The NDV strains isolated in this study were represented by the full HN protein, whose three-dimensional structure modeling demonstrated IDE1-IDE5 antigenic epitope and clarified the substitution residues in the neutralizing epitope specific to all strains of this study when compared to the Lasota strain (Figure 8 C).

DISCUSSION

Newcastle disease was a highly contagious avian disease that generated significant financial losses for the global poultry industry. Newcastle disease virus, also referred to as orthoavulavirus-1 or avian paramyxovirus serotype 1 (APMV-1), was presently categorized under the genus Orthoavulavirus of subfamily Avulavirinae in the family Paramyxoviridae of order Mononegaviriales (Rima et al., 2019). The virus encoded six structural proteins: large polymerase protein (L), fusion protein (F), matrix protein (M), phosphoprotein (P), nucleoprotein (NP), and hemagglutinin-neuraminidase (HN). It was a single-stranded RNA virus that was enclosed and had a negative sense genome (Steward and others, 1993). Newcastle disease virus infection and antigenicity depend on surface proteins known as fusion (F) and haemagglutinin-neuraminidase (HN) proteins (de Leeuw, 2005; Kim et al., 2011).

The amino acid sequence motif of the fusion protein's protease cleavage site and the capacity of particular cellular proteases to break this protein were linked to the molecular bases of NDV pathogenicity. Phylogenetically, NDV may be divided into two classes: class 1 and class 2. Class 1 has only one genotype while Class 2 has 21 genotypes till now (2.I-2.XXI, Dimitrov et al., 2019). Major populations of class 1 viruses were apathogenic and originated in aquatic wild birds (Kim et al., 2007), while class 2 viruses were those that cause epidemics in poultry and usually were highly pathogenic with continuous evolution over time (Miller et al., 2010).

Genotype VII was widely distributed globally causing serious economic losses and imposing great risk to the international poultry industry. Newcastle disease virus VII was subdivided into three subgenotypes comprise genotype VII: VII.1.1 (previously containing subgenotypes VIIb, VIId, VIIe, VIIj, and VIIl), subgenotype VII.1.2 (previously referred to as sub-genotype VIIf), and subgenotype VII.2 (previously containing subgenotypes VIIa, VIIh, VIIi, and VIIk; Dimitrov et al., 2019).

The NDV endemic situation in Egypt threatens the country's poultry industry since new cases continue to arise despite widespread routine vaccination programs that have been implemented in commercial poultry farms. Newcastle disease virus genotype VII has been reported from Egypt in the last few years (Radwan et al., 2013), in particular, Sub-genotype VII.1.1 (Nagy et al., 2020; AbdElfatah et al., 2021). Furthermore, Egyptian researchers have reported cases of co-infection with avian influenza viruses and infectious bronchitis (Moharam et al., 2019), this might affect on the effectiveness of the ND vaccination programs and complicate the ND control.

Newcastle disease virus is known to infect a minimum of 241 bird species belonging to 27 out of 50 bird orders. Waterfowl, including ducks and geese, are typically thought of as natural NDV transporters or reservoirs that exhibit few or non-existent clinical symptoms when infected with viruses, even the most pathogenic for chickens (Alexander and Senne, 2008).

This study was the first report for NDV genotype VII.1.1 isolation and characterization from mallard ducks that showed severe clinical signs and a high mortality rate in Egypt during the winter of 2023. The duck flocks showed highly pathogenic H5 avian influenza with similar

signs and lesions like nervous manifestations, respiratory and enteric signs, and a high mortality rate 55-70%, with sever congestion and swelling of parenchymatous organs like liver, spleen, and kidney (Figure 1). Testing tracheas from 16 duck flocks by rRT-PCR using NDV and AIV-specific primers, only 2 flocks tested positive for NDV, and the remaining 14 flocks were tested positive for AIV. Then NDV was successfully isolated from the trachea and brain of these two NDV-positive flocks then allantoic fluids from the first egg passage were positive for NDV by rRT-PCR and negative for influenza as previously detected in breeder duck flocks in China was reported with drop in egg production by about 70% and mortalities up to 50% (Liu et al., 2015), also high pathogenicity in experimentally infected ducks was reported (Dai et al., 2013). These observations should be considered by duck consultants and producers in Egypt to put in mind the NDV infection for differential diagnosis with HPAI H5 outbreaks in ducks and highlight the need for duck vaccination, especially with NDV-killed vaccines to control the disease.

The phylogenetic analysis of F and HN genes of NDV strains isolated in this study belonged to class II genotype VII 1.1 in new branch as previously described in other studies (Eid et al., 2022; Ragab et al., 2022; Sallam et al., 2022) with high identity percent with Chinese strains (98.5-99.3% and 96.4-96.8%) and other Egyptian strains (96.7-99.3% and 97.3-98.2%) for F and HN gene respectively. The NDV strains isolated in this study were closely related to each other, according to sequence analysis of F and HN, and there has been a modest new branch within genotype VIIj (Figures 6 and 8), this is probably because the mutations in fusion and HN genes in the strains in this study. This finding indicated the high evolution rate of NDV and reflected the complicated situation in the poultry industry with the highly increased rate of backyard rearing of multiple bird species through all Egyptian governorates.

The F protein of NDV was carrying structures with important functional roles like fusion peptide, signal peptide cleavage sites, and B-cell mediated antibody response. The fusion peptide (FP), located between positions 117 and 136 aa in the F1 subunit, three hydrophobic heptad repeat (HR) domains (HRa, 143–185 aa, HRb, 268–299 aa, and HRc, 471–500 aa), the transmembrane (TM) domain (501–522 aa), and the cytoplasmic tail (from 523–553 aa) are crucial for the pathogenicity and infectivity of the virus (Sergel-Germano et al., 1994). Newcastle disease virus strains isolated from the trachea and brain of duck had over 52 mutations in amino acids when compared with the Lasota strain as other Egyptian strains which can be reflected in the immunogenicity of these strains producing heterologous immunity (Selim et al., 2018). With specific mutations in all strains in this study in the F gene clustered in the new branch, mutations were detected in the signal peptide at A11T and T324A specific strains in this study.

Interestingly, the amino acid mutation was observed at N294K in the HRb in the *F* protein of brain strains NDV-Duck-Egypt-MN2-2023 and NDV-Duck-Egypt-MN4-2023 compared to the NDV-Duck-Egypt-MN1-2023 and NDV-Duck- Egypt-MN3-2023 strains isolated from trachea of the same duck. The N294K in the $HR_{b\ is}$ expected to modify F protein receptor binding affinity to receptors on nervous cells, these mutations might be an important factor for the virus to cross the blood-brain barrier and adapt to brain tissue indicating the major importance of the *F* gene for NDV adaptation (Sergel et al., 2001), it needs further experimental study. In addition, the 480K in the HRc was detected in strains isolated in this study also as Lasota strain but different from other NDV GVII strains that is deemed to be characteristic to NDV genotype VII.1.1 duck strains that may alter the fusion activity of the virus (Sergel et al., 2001), this may have a role in the adaptation of the virus and its increased virulence to ducks. Additional research is necessary to examine the impact of these mutations on the virus's pathogenicity.

When compared to the Lasota strain, the entire fusion protein portrayed the three-dimensional structure of the NDV trachea and brain staining and made the substitution residues in the several active domains (HR1, HR2, cleavage site, and fusion peptides region) (Morrison, 2003). By merging the viral envelope with the plasma membrane, the host cell proteases influenced this proteolytic cleavage, which increased the virus's capacity to infect the host cell (Lamb and Parks, 2007). Two pairs of various basic amino acids were discovered when the amino acid sequences of the F protein were aligned: F phenylalanine amino acid at position 117 and R/K at locations 112 to 116. These locations matched the virulent strain RRQKRF's cleavage site motif. According to Wang et al. (2017), the presence of Q in the motif of the virulent strain RRQKRF enhanced and magnified its pathogenic potential. The conserved amino acids at positions K101 and V121 of the F protein, which were unique to genotype VII, were also present in all strains. This discovery aligned with the findings of previous studies (Lien et al., 2007).

No amino acid mutations were detected in the antigenic epitope of F protein for strains isolated in this

study, the neutralizing epitopes contained in fusion protein are necessary for antibody binding and the diversity of antigens (Qin et al., 2008). Viral glycoprotein structure and function, which impacted viral tropism, infectivity, and antigenicity, were influenced by the glycosylation process (Aguilar et al., 2006; Eichler et al., 2006). Modifications at one of these two N-glycosylation residues might be crucial for the facilitation of fusion (McGinnes et al., 2001). The NDV strains isolated in this study had conserved six glycosylation sites as previously found (Selim et al., 2018). Cysteine residues were essential for protein folding because they created disulfide bonds, which gave the protein structural stability (McLellan et al., 2013). In all strains examined in this investigation, there were ten consistent cysteine residues found in the NDV fusion as previously recorded (Selim et al., 2018).

Newcastle disease virus's surface glycoprotein HN has several functions related to viral tropism and pathogenicity (Huang et al., 2004). The stalk region, globular head, transmembrane region, and cytoplasmic domain make up the *HN* protein (Ferreira et al., 2004). It can attach to cell surface receptors that contain sialic acid and has the neuraminidase (NA) activity that is required to prevent viral self-agglomeration. Furthermore, HN stimulated the fusion activity of the *F* protein, which mediated both cell-to-cell and virus-to-cell fusion (Melanson and Iorio, 2004). What's more, during NDV infection, HN, a significant protective antigen, could stimulate the production of antibodies that neutralize the virus (Kim et al., 2009; Yan et al., 2009).

It was found that they possessed over fifty-two amino acid mutations in the HN protein when comparing the two strains isolated from the trachea and brain of domestic duck with the Lasota strain, so antibodies produced against the Lasota vaccine will be heterologous to these circulating strains that could be failed to neutralize them (Selim et al., 2018). Specific mutations in all strains in this study in the HN gene clustered them in new branches with no difference between strains isolated from the trachea and brain (Figures 7 and 8).

Although the HN protein's structure and functions could be impacted by mutations in its transmembrane and stalk domains (McGinnes et al., 1993), the cytoplasmic tail was important for replication, and the species-specific phenotypes (Kim et al., 2011). The HN in all strains detected from ducks in this study had a transmembrane mutation in A28T, also, A4V, and R15K in the cytoplasmic domain that could affect viral fusion (Kim et al., 2009). In addition, S76L in the HRa, and mutations at A50T, S54R, T232N, P392S, and T443V were also detected. These reported amino acid mutations could be considered species adaptive mutations and could have a role in increased virus virulence in ducks. We need further research to study the effect of these mutations on the pathogenicity of the virus.

Seven antigenic sites were previously discovered, consisting of residues 193-201 (site 23), 345-353 (sites 1 and 14), and residues 494, 513 to 521, and 569 (sites 12 and 2) in the C-terminal domain (Iorio et al., 1991). Jin et al. (2021) have employed the PepSCan technique to identify the NDV, HN protein's immuno-dominant epitopes (IDEs). These were the IDE numbers: IDE1 (554-568aa), IDE2 (283-297aa), IDE3 (119-133aa), IDE4 (242-256aa), and IDE5 (328-342aa, Jin et al., 2021). Neutralizing epitopes have been shown to undergo amino acid modification, which may result in neutralizing escape variants and help create antigenic epitopes (Hu et al., 2010). Nineteen amino acid mutations in the HN neutralizing epitopes were indicated in Egyptian strains as previously described by Naguib et al. (2021), in addition to three amino acid mutations (N120G, K284R, S521T) were detected specific to NDV strains of this study that can alter the virus antigenicity. The mutations reported in F and HN proteins overformed novel NDV strains with variable antigenicity and transmission of these viruses from ducks back to chickens can escape the immunity produced by used commercial vaccines in chickens (Elbestawy et al., 2023).

According to Chen et al. (2001), an essential membrane protein that is glycosylated through the N-link is the HN glycoprotein. The initiation and maintenance of protein folding into its physiologically active conformation, protein stability and solubility, intracellular transport of the proteins to different subcellular compartments and the cell surface, and the antigenicity and immunogenicity of the proteins were just a few of the many glycoprotein properties that were impacted by N-linked glycosylation (Quinones-Kochs et al., 2002). According to Panda et al. (2004), the loss of a single glycosylation site modified the pathogenicity of NDV. According to the findings of Cattoli et al. (2010), the Newcastle disease virus strains examined in this investigation possessed five putative glycosylation sites (residues 119, 341, 433, 481, and 508) as VII, NDV.

Comparative to vaccines used in Egypt and strains isolated in this study, we detected low similarity percent 87.8-88.5% and 86.2-86.4% between vaccines related to genotype II (Lasota, clone 30 and VG/GA) and ranged between 90.7-90.9% and 89.1% with D26/76 vaccine (genotype I) for F and HN gene respectively as previously

reported by Xue et al. (2017). The genotype VII vaccine (KBNPC415R2L) used in Egypt has an interesting nucleotide identity range of 96-96.2% and 94.5% with the F and HN genes protein sequence, suggesting a higher probability of protection with this type of vaccine as previously described (Abd El-Hamid et al., 2020). These observations indicated that genotype-matched vaccine with VII strains in NDV vaccination programs is a must now in Egypt and any country that has a similar situation.

In conclusion, this was the first report of outbreaks of NDV genotype VII.1.1 in domestic mallard ducks with severe clinical signs, nervous manifestations, respiratory and enteric signs, and high mortalities during the winter season of 2023 in Egypt. The full gene sequence for F and HN genes revealed significant evolution of NDVs isolated from ducks compared to circulating VII strains in other species specifically chickens and vaccine strains, especially of genotypes II and I. These A.A. mutations recorded in *F* and HN proteins clustered them in new branches that could be the result of the extensive circulation of the virus in different Egyptian poultry over a long period, and also, could be due to specific signature mutations for duck adaptation. Interestingly, we reported specific amino acid mutations between NDV strains isolated from brain strains compared to trachea strains from the same duck, which might play the main role in virus adaptation to brain tissue and crossing the blood-brain barrier but further experimental studies were required. Based on the results obtained in this study, vaccination of duck flocks in Egypt should be considered in future ND control strategies to reduce economic losses to duck producers, decrease shedding to other poultry flocks, especially chickens, and control NDV evolution in the country.

DECLARATIONS

Authors' contributions

Mahmoud Ibrahim contributed in collecting samples, isolation, and identification of. Mohamed Wahba performed RT-PCR for F and HN genes. Nahed Yehia made the sequence and phylogenetic analysis. M Ibrahim and N Yehia wrote the darft of the manuscript, and revised it before submission. All authors checked and confirmed all data and the last draft of the manuscript before submission to the journal.

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Ethical considerations

The authors confirm that all authors have reviewed and submitted the manuscript to this journal for the first time.

Availability of data and materials

The original contributions presented in the study are included in the article/supplementary material. For inquiries, please contact the corresponding author/s.

Conflict of interests

The authors have not declared any conflict of interest.

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