Received: 25 November 2020 | Revised: 12 April 2021 | Accepted: 20 April 2021

DOI: 10.1111/tbed.14121

ORIGINAL ARTICLE

Transboundary and Emerging Diseases WILEY

Comparison of genomic and antigenic properties of Newcastle Disease virus genotypes II, XXI and VII from Egypt do not point to antigenic drift as selection marker

Mahmoud M. Naguib[1,2] | Dirk Höper[3] | Magdy F. Elkady[4] | Manal A. Afifi[5] | Ahmed Erfan[2] | Hassanein H. Abozeid[5] | Wafaa M. Hasan[2] | Abdel-Satar Arafa[2] | Momtaz Shahein[2] | Martin Beer[3] | Timm C. Harder[3] | Christian Grund[3]

[1]Zoonosis Science Center, Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden

[2]Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Agriculture Research Center, Giza, Egypt

[3]Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Diagnostic Virology, Greifswald Insel-Riems, Germany

[4]Department of poultry Diseases, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt

[5]Department of Poultry Diseases, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt

Correspondence
Christian Grund, OIE, FAO and National Reference Laboratory for Newcastle disease, Suedufer 10, 17493 Greifswald Riems, Germany.
Email: Christian.Grund@fli.de

Abstract

Newcastle disease (ND), caused by avian orthoavulavirus type-1 (NDV), is endemic in poultry in many regions of the world and causes continuing outbreaks in poultry populations. In the Middle East, genotype XXI, used to be present in poultry in Egypt but has been replaced by genotype VII. We investigated whether virus evolution contributed to superseding and focussed on the antigenic sites within the hemagglutinin-neuraminidase (HN) spike protein. Full-length sequences of an NDV genotype VII isolate currently circulating in Egypt was compared to a genotype XXI isolate that was present as co-infection with vaccine-type viruses (II) in a historical virus isolated in 2011. Amino acid differences in the HN glycoprotein for both XXI and VII viruses amounted to 11.7% and 11.9%, respectively, compared to the La Sota vaccine type. However, mutations within the globular head (aa 126–570), bearing relevant antigenic sites, were underrepresented (a divergence of 8.8% and 8.1% compared to 22.4% and 25.6% within the protein domains encompassing cytoplasmic tail, transmembrane part and stalk regions (aa 1–125) for genotypes XXI and VII, respectively). Nevertheless, reaction patterns of HN-specific monoclonal antibodies inhibiting receptor binding revealed differences between vaccine-type viruses and genotype XXI and VII viruses for epitopes located in the head domain. Accordingly, compared to Egyptian vaccine-type isolates and the La Sota vaccine reference strain, single aa substitutions in 6 of 10 described neutralizing epitopes of HN were found. However, the same alterations in neutralization sensitive epitopes were present in old genotype XXI as well as in newly emerged genotype VII isolates. In addition, isolates were indistinguishable by polyclonal chicken sera raised against different genotypes including vaccine viruses.

These findings suggest that factors other than antigenic differences within the HN protein account for facilitating the spread of genotype VII versus genotype XXI viruses in Egypt.

KEYWORDS
antigen sites, Egypt, evolution, genotypes, Newcastle disease, Newcastle disease virus

1 | INTRODUCTION

Newcastle disease (ND) is a highly contagious disease with significant clinical impact in poultry and serious economic losses worldwide. The causative agent, Newcastle disease virus (NDV) or avian paramyxovirus type-1 (APMV-1), is a species within the genus Avian *orthoavulavirus 1*, a member of the family *Paramyxoviridae* (Rima et al., 2019). The genome is composed of a negative-sense, single-stranded RNA of approximately 15 kb coding for six structural proteins, in the 3' to 5' order nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and large RNA-dependent polymerase protein (L) (Steward et al., 1993). The HN protein recognizes and attaches to sialic acid receptors on the surface of permissive cells and mediates the fusion activity of the F protein at the cell membrane for release of the nucleocapsid complex into the cytoplasm (Lamb & Parks, 2007).

Based on its pathogenicity in chickens, NDV is categorized into four groups with increasing virulence from apathogenic, to lentogenic, mesogenic and velogenic (Alexander, 2000). The molecular basis for NDV pathogenicity is associated with the amino acid sequence motif of the protease cleavage site of the fusion protein and the abilities of specific cellular proteases to cleave this protein. Based on phylogenetic analysis, NDV can be distinguished into two distinct classes, 1 and 2, with a single (1.I) and, currently, 21 genotypes, respectively (2.I-2.XXI) (Dimitrov et al., 2019). Whereas the majority of class 1 viruses are avirulent and have their natural reservoir in aquatic wild birds (Kim et al., 2007), class 2 comprises virulent viruses evolving over time, that are responsible for the outbreaks in poultry, as well as in pigeons and cormorants (Miller et al., 2010; Suarez et al., 2020). Within class 2, lentogenic and mesogenic pathotypes, in addition to velogenic ones, are only found in genotype II (Czegledi et al., 2002,2003), and vaccines are derived from lentogenic strains of this genotype (Seal et al., 1996). Phylogenetic evidence suggests that in the decades prior to the 1970s, genotypes II, III and IV were predominant in North America, Asia and Europe, respectively. Genotype VI viruses emerged in epizootics in the Middle East and Asia in the 1960s and (Herczeg et al., 2001; Wehmann et al., 2003) gave rise to strains adapted to pigeons, the so-called "pigeon type paramyxovirus" (PPMV-1) (D. J. Alexander et al., 1985). The new NDV nomenclature proposal (Dimitrov et al., 2019) classified the previous subgroups VIai and VIaii, encompassing the early strains circulating in poultry but not in pigeons (Chong et al., 2013), into the new genotypes XX and XXI, whereas PPMV-1 remained in genotype VI with several sub-genotypes. Genotype VII is considered to have emerged in the Far East and spread to further geographic areas (Herczeg et al., 1999). Currently, genotype VII is predominant among velogenic NDV and can be further subdivided (Dimitrov et al., 2019), that is into sub-lineage VII.1. encompassing viruses that emerged in the 1990s in the Far East, Europe and Asia, and the Middle East and were responsible for the fourth NDV panzootic and VII.2 viruses which emerged in Indonesia, affecting Asia, the Middle East, Europe, and Africa, and were responsible for the fifth NDV panzootic (Miller et al., 2015).

In Egypt, ND is endemic and, despite extensive routine vaccination programmes implemented in the commercial poultry farms, new cases occur continuously, posing a threat to the national poultry industry. In Egypt, ND was first identified in 1948 (Daubney & Mansy, 1948), and in the last few years, NDV genotypes II and VII have been reported from the country (Radwan et al., 2013). Also, co-infections with infectious bronchitis and avian influenza viruses have been recorded in Egypt (Moharam et al., 2019; Naguib et al., 2017; Samy & Naguib, 2018). This has raised concerns regarding the efficacy of ND vaccination programmes applied in the country. It has been hypothesized that, due to accumulated mutations as seen previously with highly pathogenic avian influenza virus (Grund et al., 2011), new NDV antigenic variants have arisen that have an advantage to spread within a vaccinated population. In consequence, viruses that supersede previous virus populations should have a gain in fitness, here, mutations within antigenically relevant sites. To test this hypothesis, four poultry NDV isolates were compared genetically and antigenically. The viruses were chosen as they represent genotype II vaccine strains ($n = 2$), the rare genotype XXI (former VIa; $n = 1$), and genotype VII.1.1 ($n = 1$) that apparently superseded other genotypes in poultry and currently is the dominating genotype in Egypt. Here we report on the history of the isolates focussing our analysis on changes within the viral glycoproteins that facilitate receptor-mediated virus uptake and are the main target for protective immune responses. In addition, we hit upon the phenomenon of co-infections of vaccine and virulent NDV strains in a single sample causing diagnostic problems.

2 | MATERIAL AND METHOD

2.1 | Sample collection and virus identification

Samples (oropharyngeal and cloacal swabs, trachea or kidneys) were obtained from outbreaks of systemic disease in three different commercial chicken farms in Egypt during 2011–2013 as shown in table 1. Samples were collected by the Reference Laboratory for Quality Control on Poultry production (RLQP)-Animal Health Research Institute (AHRI) and Beni-Suef University, Egypt, and shipped to the Friedrich–Loeffler-Institut (FLI), Germany. Identification of NDV was based on real-time reverse transcriptase polymerase chain reaction (RT-qPCR) using the Biorad CFX1000 real-time PCR system. Briefly, RNA was extracted from collected samples by QiaAmp viral RNA extraction kit (Qiagen, Hilden, Germany) as recommended by the manufacturer's instructions. NDV nucleic acid was detected by

TABLE 1 Egyptian NDV isolates included in the study

RT-qPCR[1]									
country/	species/	registration ID	/year	M	F-patho	F-lento	F0-cleavage site	ICPI	Genotype	Accession No.
EGY/	chicken/	R1954/2011[a]	/2011	12,07	20.18	15.15	RRQKR[b]F	0.91		
EGY/	chicken/	R1954/2011/cl–1[b]	/2011	12,16	neg	15.79	GRQGR[b]L	0.64	II	MW285786[p]/MW285793[f]
EGY/	chicken/	R1954/2011/cl–6[b]	/2011	16,58	27.96	neg	RRQKR[b]F	1.81	XXI	MW285787[p]
EGY/	chicken/	R1973/2011[a]	/2011	15,76	25.4	22.15	GRQGR[b]L	1.88	II	
EGY/	chicken/	R1973/2011/cl–2[b]	/2011	15,66	neg	21.93	GRQGR[b]L	0.75	II	MW285788[p]/MW285791[f]
EGY/	chicken/	R1973/2011/cl–4[b]	/2011	20,32	32.39	neg	RRQKR[b]F	1.70	XXI	MW285789[p]/MW285790[f]
EGY/	chicken/	AR 2178/2014[b]	/2013	16,93	25.59	neg	RRRKR[b]F	1.64	VII.1.1	MW285792[f]

Note: 1 cq-values for RT-qPCR tests detecting the M-gene (M), and the proteolytic cleavage site resembling meso,-velgenic (F-patho) or apthogenic/ lentogenic (F-lento) pathotypes.

Abbreviations: f, Full genome sequences used for Figures 1 and 2: p, Partial F-sequence, see Figure S1.

[a]Parental virus.

[b]Representative plaque cloned viruses from corresponding parental virus stock.

SuperScript® III one-step RT-PCR using primers and probe specific to the M gene (Wise et al., 2004). Positive samples were subjected to another RT-qPCR to differentiate lentogenic from velogenic viruses based on the sequence of the fusion protein cleavage site (FPCS) using primers and probes specific for the F genes (Moharam et al., 2019).

2.2 | Virus isolation

Hepatocellular carcinoma epithelial cells (LMH) (Kawaguchi et al., 1987) were cultured with 2 μg TCPK Trypsin/mL in T25 culture flasks and infected with 0.5 ml of the NDV-positive samples. Cells were incubated at 37°C until giant cell formation was observed or for up to seven days before initiating a second passage following a freeze/thaw cycle. Further confirmation using hemagglutination (HA) and hemagglutination inhibition (HI) tests were conducted on the supernatant culture medium (OIE, 2012). Isolated viruses were aliquoted and kept at −80°C until further use.

2.3 | Plaque purification

Plaque assay was used to obtain purified virus clones for selected samples showing unequivocal results with pathotype-specific RT-qPCRs. Briefly, different virus dilutions were incubated on confluent LMH cell monolayers cultured in six-well plates overnight at 37°C. Then, virus inoculum was removed, cell layers washed carefully twice with sterile PBS and overlaid with modified Eagle's medium containing 1.8% agar. Infected cells were incubated at 37°C and 55% humidity for three days. Finally, selected plaques were picked and propagated on LMH cells as described above for further characterization.

2.4 | Genetic characterization and phylogenetic analysis

Amplification of a 698 bps fragment spanning part of the M and the F2 gene of NDV was performed using SuperScript III One-Step RT-PCR system (Invitrogen) following published protocols (Aldous et al., 2003). Amplicons were size-separated by agarose electrophoresis, excised and purified from gel using the QIAquick gel extraction kit (Qiagen). Purified PCR products were used directly for cycle sequencing reactions using BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems). The sequencing reaction products were purified using NucleoSeq kit (Macherey-Nagel) and sequenced on an ABI PRISM® 3,100 genetic analyzer (Life Technologies).

Assembly of the obtained sequences was performed using the Geneious software, version 9.0.5 (Kearse et al., 2012). Alignment and identity matrix analyses were conducted using MAFFT (Katoh & Standley, 2013) and BioEdit (Hall, 1999). Sequences generated in this study were submitted to the GenBank NCBI (National Center

for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/) and assigned accession numbers are shown in Table 1. Reference sequences of NDV genotypes (Diel et al., 2012; Snoeck et al., 2013) required for further analyses were retrieved from the GenBank database. Phylogenetic analyses were performed and trees were constructed using the IQ-tree software version 1.1.3 (Minh et al., 2013; Nguyen et al., 2014) based on maximum likelihood analysis of phylogenetic relationship after selection of the best fit substitution model (GTR + F + I + G4). Finally, trees were viewed and edited using FigTree version 1.4.2 software (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape 0.51.

2.5 | Full Genome Sequencing

Full genome sequences of four successfully isolated NDV strains, namely R1954/2011/cl-1, R1973/2011/cl-2, R1973/2011/cl-4, and AR2178-14, representing three genotypes previously reported in Egypt, were carried out. Briefly, RNA was purified from isolates using TRIzol LS reagent (LifeTechnologies) and RNeasy mini kit (Qiagen,) with on-column DNase digestion according to the manufacturer's instructions. Conversion of RNA into double-stranded DNA was performed using a cDNA synthesis system (Roche). Library construction was done as previously described (Juozapaitis et al., 2014). Sequencing was performed on an Illumina MiSeq instrument using the MiSeq reagent kit version 3 (Illumina).

Assembly of the sequence data was done using the Genome Sequencer version 2.6 software suite (Roche), and NDV-related contigs were identified with BLASTn (BLASTn; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained full-length NDV sequences were submitted to GenBank (Table 1). Further, open reading frame (ORF) analysis and annotation of the genome were carried out using the Geneious software.

Phylogenetic analyses were carried out for both of F gene (partial/full) and the whole genome sequence separately using reference viruses as mentioned in the previous section.

2.6 | Pathotyping

Intracerebral pathogenicity index (ICPI) was determined following European guidelines (CEC, 1992). Ten one-day-old SPF chickens were inoculated intracerebrally with 0.05 ml of 10^{-1} diluted virus stock (HA >32) and examined daily for eight days, and the ICPI is calculated as described. Based on this score NDV isolates are considered lentogenic (−0.7), mesogenic (>0.7–1.3) or velogenic (>1.5) (Alexander, 1998). Animal experiments were carried out in accordance with a legally approved protocol (MV-LALLF- /7221.3–1.1-030/13). Specific pathogen-free White leghorn chickens purchased from Lohmann Animal Health, Cuxhaven, Germany, were raised in isolation units at FLI until the age of inoculation. Infection and immunization experiments were performed in separated cages at animal biosafety level 3 (BSL-3). Feed and water were provided daily.

2.7 | Preparation of polyclonal anti-NDV chicken antisera

Four reference NDV strains, Ulster, clone30, R151/94, and R1468/12, representing the avian orthoavulavirus-1, I, VI (PPMV-1) and VII.1.1, respectively, were selected for preparation of polyclonal anti-NDV chicken antiserum in the FLI animal facility. Viruses were first inactivated by betapropiolactone (Sigma; 0,05%,) treatment, and 0.5 ml of each virus were injected in four 4 weeks-old-SPF White Leghorn chickens via the subcutaneous route after mixing with Freund's complete adjuvant (1:1). A second immunization shot was given after 4 weeks. Collection of serum was performed at seven days after the booster immunization, followed by inactivation at 56°C for 30 min and stored at −20°C until further use. Immunizations were carried out in accordance with the legally approved protocol (MV-LALLF- 7,221.3–2.5-010/10).

2.8 | Antigenic characterization

Antigenic analysis of three Egyptian NDVs AR2178/2014, R1973/2011/cl-4, and R1954/2011/cl-6, as well as of another five reference strains was performed by hemagglutination inhibition (HI) assay as described (CEC, 1992). In addition to a panel of four polyclonal sera (refer the previous section) five HN-specific monoclonal antibodies were used (mAb 617/161, mAb U85, mAb 7D4 were a generous gift from AVLA-Weybridge and mAb 39–2, mAb 10 were a gift from Bernd Köllner, FLI).

3 | RESULTS

3.1 | History of NDV isolates

Genotype VI.1.1 virus AR2178/14 was recovered from a diagnostic sample obtained in Egypt in 2013. In contrast, genotype II viruses and XXI virus were present as mixtures within ND virus isolates obtained in 2011 (R1954/11, R1973/11) that were submitted to FLI for further characterization. The initially obtained consensus sequence of the proteolytic cleavage site within the F_2 protein of isolate (R1954/11) represents a polybasic recognition motive, characteristic for virulent pathotypes. This would be in agreement with the obtained ICPI of 0.91, representing a virulent NDV with intermediate/mesogenic pathogenicity for chicks. In contrast, the sequence of the second isolate (R1973/11) represents a cleavage site of a lentogenic vaccine-type virus but had an ICPI of 1.88, clearly identifying the isolate as virulent NDV with pronounced/velogenic pathogenicity for chicks. Testing by pathotype-specific RT-qPCR assays using F gene-directed primers and probes specific for avirulent and virulent pathotypes (RT-F-qPCR) revealed that both Egyptian NDV isolates of 2011 were positive for both avirulent as well as virulent pathotypes, indicating mixed infection (Tab. 1). By plaque purification, clones with mono- (R1954/11/cl-1; R1973/11/cl-2) or polybasic

cleavage sites (R1954/11/cl-6; R1973/11/cl-4) were finally obtained from both samples proving the suspected co-infection. For the purified clones, unambiguous reactivity patterns in pathotype-specific RT-qPCRs, sequences of the proteolytic cleavage site, and ICPI values were obtained even though the ICPI value of R1973/11/cl-2 was at the border of 0.7 for lentogenic pathotypes.

3.1.1 | Genetic and phylogenetic characterization

Sequences of the F2 fragment (358 nt), including the proteolytic cleavage site, were used for initial phylogenic characterization. BLAST tools indicated the highest homology for isolate AR2178/14, with Egyptian Avian avulavirus-1/168-2012 (Genbank accession: MN381174) with nt and aa identities each of 99%, clustering in phylogenetic analysis with genotype VII.1.1 viruses (supplementary Figure 1.). In contrast, virulent NDV clones from 2011 (R1954/2011/cl-6; R1973/2011/cl-4) showed the highest identities with EG-14/90 (Genbank accession: DQ096604) and chicken/Nigeria/2006 (Genbank accession: MH092825), respectively. The lentogenic clone R1954/2011/cl-1 matched 100% with vaccine strain La Sota (Genbank accession: AF077761), while R1973/2011/cl-2 matched with vaccine strain Hitchner B1 (Genbank accession: JN872151). When sequence collections of the genotyping group (Dimitrov et al., 2019) were used to create a first phylogenetic analysis (S1), viruses grouped into genotype VII.1.1 (AR2178/2014), XXI (R1954/2011/cl-6; R1973/2011/cl-4) and II (R1954/2011/cl-1; R1973/2011/cl-2). While genotype VII.1.1 viruses were circulating in Egypt from 2012 up to 2019 (Figure S1), no further sequences for NDV genotype XXI have been reported for chickens after 2011. It is interesting to note that together with viruses from Egypt (1990, 2006) and Nigeria (2006), both XXI viruses are not assigned to a specific sub-genotype. Instead, they form a separate branch to the outgroup of Ethiopian genotype XXI viruses. However, with an estimated divergence of 0.1213 the genetic distance to the outgroup viruses is equidistant to sub-genotpye XX.1.1, XXI.1.2 or XXI.2 with 0.1293, 0.1085 and 0.1438, respectively (Table S1). Subsequent viruses from Egyptian genotype XXI belong to sub-genotype XXI.1.1 and are derived from pigeons. These data indicate that R1954/2011/cl-6 and R1973/2011/cl-4 are descendent of genotype VI viruses circulating during the epizootics in Egypt in the 1960s, but now are apparently extinct.

3.1.2 | Full genome sequence analysis

To verify and further analyse selected NDV isolates, full genome sequences were generated for both vaccine-type clones (R1954/2011/cl-1, R1973/2011/cl-2) as well as for genotype XXI (R1973/2011/cl-4) and VII.1.1 (AR2178/2014) isolates. The genome length obtained for the lentogenic NDV clones R1954/2011/cl-1 and R1973/2011/cl-2 was 15,135 and 15,150 nt. The genomes of the virulent strains AR2178/2014 and clone R1973/2011/cl-4 were 15,179 and 15,165 nt long with a 6-nt-insertion ACCCCC or CCTCTC respectively in the untranslated region (UTR) downstream of the NP gene.

The two NDV strains of and R1973/2011/cl-2 showed highest similarity to closely related vaccine-type virus La Sota C5 (accession: KC844235; R1954/2011/cl-1(99.8%)) (Table 2) and B1 (accession AF375823; R1973/2011/cl-2 (99.8%)) respectively (details see Table S2). On the other hand, the velogenic NDV strain AR2178/2014 showed the highest nt sequence similarity of 99% to the Chinese NDV strain SD04/2011 (accession: JQ015296), whereas strain R1973/2011/cl-4 showed 94% nt similarity with the Japanese NDV strain Osaka/2440/1969 (accession: AB853926), an ancestral strain formerly assigned to genotype VIc but currently unclassified because of lack of sequence information of a sufficient number of related strains (Dimitrov et al., 2019).

Phylogenic analysis of the full F gene (Figure 1), as well as the whole genome sequence (Figure 2), confirmed the phylogrouping obtained by partial F-gene analysis (Figure S1). With respect to R1973/2011/cl-4 the virus could still be identified as genotype XXI, but again was an outgroup virus with an estimated divergence of 0.099736 to the Ethiopian viruses and 0.086579, 0.097996, 107,285 to XXI.1.1, XXI.1.2 and XXI.2, respectively. In contrast, strain AR2178/2014 mapped with other Egyptian genotype VII.1.1 viruses from poultry as well as from wild birds, a further indication that this genotype virus was widespread in avian hosts in Egypt. Clones R1954/2011/cl-1, R1973/2011/cl-2 were confirmed as vaccine-type virus closely related to La Sota and B1, respectively.

Genome organization of the four fully sequenced NDV strains was found to be identical to other Avian orthoavulavirus-1, containing six genes in the order of 3'-NP-P-M-F-HN-L-5'. Analysis of the F protein of the four NDV strains revealed that three strains, namely R1954/2011/cl-1, R1973/2011/cl-2 and AR2178/2014 (genotype II, and VII.11, respectively), possessed six potential N-glycosylation sites (NGS); five within the ectodomain at amino acid (aa) positions 85, 191, 366, 447, and 471 and one in the cytoplasmic domain at position 542. Strain R1973/2011/cl-4 (genotype XXI) possessed the same five NGS in the ectodomain and an additional one at position aa 4 (https://prosite.expasy.org/PDOC00001).

The predicted HN proteins of the two NDV strains R1973/2011/cl-4 and AR2178/2014 (genotype XXI and VII.1.1, respectively) were 571 aa long, which is 6 aa shorter than those of the NDV strains of genotype II, namely R1954/2011/cl-1 and R1973/2011/cl-4 (577 aa). The HN protein of all genotypes possessed five potential NGS. Four NGS at positions 119, 341, 433 and 481 were common in the four NDV strains, whereas NDV strains R1973/2011/cl-4 and AR2178/2014 (genotype XXI and VIIb, respectively) possessed an additional NGS at site 508, and strains R1954/2011/cl-1 and R1973/2011/cl-2 (genotype II) at position 538.

Comparing the level of homology of the different isolates to the La Sota and B1 vaccine strains (Accession No. AF077761) confirms the close relationship between the genotype II viruses. Regarding genotype XXI and VII, both genotypes accumulated mutations compared to the La Sota vaccine strain, but these changes were present for both viruses to a comparable level with homology of 81.0

TABLE 2 Similarity comparisons of nucleotide and amino acid sequences of ND viruses sequenced in this study and the LaSota vaccine strain (AF077761)

Homology (%)														
		Nucleotide							Amino Acid					
Strain	Genotype	Full	NP	P	M	F	HN	L	NP	P	M	F	HN	L
EG/R1954/2011/cl−1	II	99.6	99.5	99.3	99.8	99.9	100	99.8	98.8	98.7	99.7	100	100	98.5
EG/R1973/2011/cl−2	II	98.8	98.6	99.3	99.1	99.2	99.0	99.2	98.6	99.0	98.6	99.8	99.1	98.3
EG/R1973/2011/cl−4	XXI	83.0	84.3	81.8	85.2	84.3	82.9	86.1	92.0	80.1	89.9	88.8	88.1	91.9
EG/AR2178/14	VII	82.4	84.7	82.3	83.0	83.6	81.0	85.5	90.8	81.8	87.9	88.3	87.9	91.8
		homology (%)			100–96		95–91		90–86		85–81		80–76	

FIGURE 1 Phylogenetic tree of ND viruses based on nucleotide sequence of full F gene. Phylogenetic tree is based on maximum likelihood calculations (IQTree software) under the best fit model (model: GTR+F + I+G4) according to the Bayesian Information Criterion (BIC). Numbers at nodes represent measures of robustness based on an ultrafast bootstrap approach implemented in IQTree. The sequences of other reference strains as well as all publicly available Egyptian NDV were obtained from GenBank. Divisions and genotypes are designated according to Dimitrov et al., 2019. Beside the phylogenetic tree of class 2 viruses the particular genotypes of interest (circled) were magnified and Egyptian viruses are coloured in red with a black dot indicating the position of viruses of this study

to 85.5%. For the different genes, no striking differences are evident. Comparing the amino acid (aa) composition of genotype XXI and VII.1.1, the homology to II strain La Sota is higher than on the level of nucleotides, with 92 to 87.9% for NP, M, F, HN and L protein. It is striking that the homology of the P protein was considerably lower than the other genes with 80.1 and 81.9% for genotype XXI and VII.1.1, respectively. When focussing on the HN protein, responsible for receptor-mediated binding, our data reveal overall aa differences of 11.7% and 11.9% for XXI and VII.1.1 viruses, respectively, compared to La Sota vaccine type (Table 3). Mutations accumulated within the protein fragment encompassing the tail, transmembrane part and stalk region (aa 1–125; 22,4% and 25,6%), whereas the globular head (aa 126–570), bearing relevant antigenic sites, was underrepresented and had an aa divergence of only 8.8% and 8.1% for genotypes XXI and VII.1.1, respectively. Among the aa in the globular head, 21 of 39 and 35 substitutions were identical for both XXI and VII.1.1 viruses. These changes include six single point mutations in sites that were described to be part of neutralization sensitive epitopes (Y203H, N263K, N347K, E494D, G495E and V514E) of the HN protein (Table 3), that is site 23; site 3, site I, site12, site II, site 2 (Iorio et al., 1989,1991). In an additional six positions, both viruses had substitutions compared to La Sota (aa, 266, 288, 310, 433, 443, 509) but differed in aa composition. Unique changes were evident on 9 and 10 positions for genotype XXI and VII.1.1 viruses, respectively. None of these isolate-specific sites affected known functional sites directly. The vaccine-type isolates only R1973/2011/cl-1 had five alterations that were all located within the globular head of the HN protein. It is interesting to note that four of those mutations were identical to closely related vaccine B1 strain. From mutations within described mAb binding sites only epitope 23 (Y203H) was present in the B1 strain, whereas the NA binding site (A502V) was identical to the genotype XXI and VII.1.1 viruses.

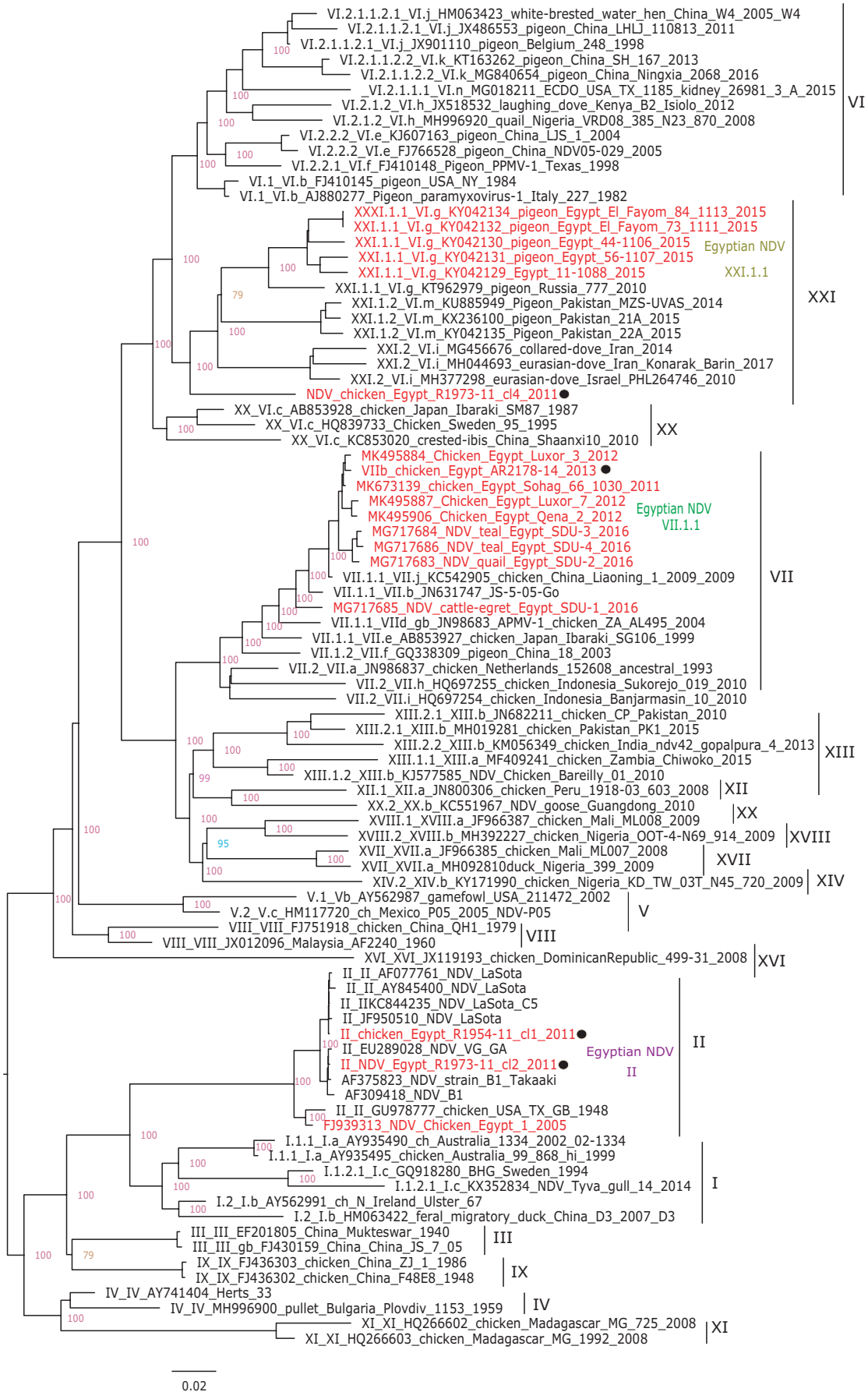
3.1.3 | Antigenic characterization

By cross hemagglutination inhibition (HI) assays (Table 4) reaction patterns of polyclonal chicken sera directed against clone 30 (II) or Ulster (I.2) and the Egyptian isolates of genotype II were indistinguishable. Reactivity even with a phylogenetically distant class 1 APMV-1 isolate (R2919/06) was in the range of 2 $\log_2$ steps. In contrast, reactivity of a serum raised against another genotype VII.1.1 virus (R1468/12) was considerably lower with genotype II and I viruses (3 $\log_2$) as well as with the class 1 virus (4 $\log_2$). However, genotype VII.1.1 specific serum did not distinguish between Egyptian genotype VI and VII.1.1 viruses. In addition, a serum raised against classical PPMV-1 virus (R151/94) assigned to genotype VI showed equal reactivity to both genotype XXI chicken isolates, but reactivity was decreased to genotype II and I and class 1 virus and to a lesser extent to genotype VII.1.1 viruses. Reactivity profiles obtained by HI with a panel of mAbs further highlighted antigenic differences between PPMV-1 and other genotype XXI strains (R1973/2011/cl-4 and R1954/2011/cl-6). A second set of mAbs, reactive with vaccine-type genotype II viruses, varied in their reactivity profile: whereas mAb U85 recognized genotype II, I and R1468/12 (VII.1.1) viruses, mAb 7D4 was specific only for vaccine type II viruses, and mAb 10 recognized an epitope present in genotype II and I viruses. These data strongly suggested that epitope-specific difference existed, but that they were of minor importance for recognition by polyclonal sera that comprise antibodies directed against multiple epitopes.

4 | DISCUSSION

NDV continues to cause outbreaks and substantial losses in the poultry industry worldwide (Brown & Bevins, 2017; Dimitrov et al., 2016). In Egypt, in spite of the routine implementation of ND vaccination, detection of NDV of genotypes II, VI, and VII.1.1 have been reported continuously over the last 10 years (Orabi et al., 2017; Saad et al., 2017; Sabra et al., 2017). Continuously growing phylogenic diversity points to the evolution of virus populations that display a genetic distance to vaccine-type NDV genotype II. This observation has been taken as an indication for antigenic vaccine mismatch and a cause for suspected vaccine failure (Dimitrov et al., 2017; Nagy et al., 2020). In this model, new NDV strains emerge because of a change in antigenic sites relevant for protection. To test this hypothesis for the situation in Egypt an NDV isolate from 2011 representing NDV genotype XXI was compared to a currently and continuously detected NDV genotype in chickens (Ewies et al., 2017; Moharam et al., 2019; Radwan et al., 2013; Saad et al., 2017)in Egypt (VII.1.1). Sequence analyses focussed on sites of the HN glycoprotein reported to be sensitive for receptor blocking and virus neutralization (Crennell et al., 2000; Yuan et al., 2011).

Based on the new classification system proposed by Dimitrov et al. (2019), viruses sequenced in the current study were grouped phylogenetically as genotype II, XXI and VII.1.1. The latter genotype originated from China and circulated extensively in the Middle East (Ewies et al., 2017; Radwan et al., 2013; Saad et al., 2017). In contrast, NDV genotype XXI has not been recorded in poultry after 2011. This group of viruses is a descendent of viruses that circulated in poultry in the 1960 and diversified into genotype XX, XXI and VI (Dimitrov et al., 2019) (Hicks et al., 2019) Evolutionary time scale analysis indicated that genotype XX appeared around 1959 in poultry (Chong et al., 2013) forming sub-lineage VIai that arose in Eastern Asia and later circulated in Europe, causing sporadic ND outbreaks in Western Europe and Bulgaria in the mid1990s (Czegledi et al., 2002; Ujvári et al., 2003). A second branch includes the new genotype XXI and the consolidated genotype VI (former sub-genotype VIb). Viruses from genotype VI were successful to establish a pandemic in pigeons and are referred to as PPMV-1 (Chong et al., 2013; Ujvári et al., 2003). Likewise, other descendants of genotype XXI established infections in pigeons. These pigeon-derived viruses are accumulated in three sub-genotypes, that is XXI.1.1; XXI.1.2 and XXI.2, respectively with genotype XXI.1.1 subsequently detected in Egypt (Sabra et al. (2017). These phylogenetic data highlight the possible



interspecies transmission of genotype XXI viruses but clearly support the notion that isolates R1954/2011/cl-1 R1973/2011/cl-2 are original poultry-derived strains and not a spill-over infection from pigeon to poultry as described for PPMV-1 in the 1980s in England (D. J. Alexander et al., 1985). This notion of genotype XXI being a virus circulating in poultry is further supported by the finding that closely related viruses were detected in chickens in Ethiopia in 2011 and 2012 (de Almeida et al., 2013). However, genotype VII.1.1 viruses dominate subsequent outbreaks in Egypt. This observation indicates that genotype XXI was superseded in poultry but XXI.1.1 is still present in pigeons. It was considered that ND vaccination is a driving force for virus evolution (Chong et al., 2010) with a selection of escape variants (Cho et al., 2007; Cho et al., 2008). In this respect, genotype VII.1.1 virus (AR 2178/2014) should harbour mutations within neutralizing sites not present in genotype XXI virus (R1973/2011/cl-2). However, when analysing sites that are part of neutralizing epitopes within the HN molecule, all six mutations present in genotype VII.1.1. virus were also present in genotype XXI virus. It was remarkable that for 5 out of 6 sites mutations were associated with a change of principal properties of the amino acid, that is a change from hydrophobic to alkaline, positively charged (Y203H), from acidic, negatively charged to alkaline, positively charged (N263K, E347K), from neutral to acidic, negatively charged (G494D) or from hydrophobic to acidic, negatively charged (V495E). This indicates that mutations might be associated with a change of antigenic properties. In line with this assumption are the data from the cross HI tests. Reactivity profile with mAb revealed changes in specific epitopes and also polyclonal sera discriminated between viruses from different genotypes. However, overall our data support the notion that NDV / avian orthoavulavirus-1 is still a single serogroup (Miller & Koch, 2013) and a discrimination between genotype XX and VII was not evident. Taken together, our data do not indicate that a switch of antigenic sites were the driving selection criterion for the replacement of genotype XXI by genotype VII.1.1. This would be in line with the observation that the overall mutation rate of NDV indicates strong purifying (negative) selection for all proteins (Chong et al., 2013; Miller et al., 2009).

Strikingly, both historical XXI isolates contained also a vaccine-type virus (genotype II). Co-infections were finally revealed by pathotype-specific RT-qPCR but would have been concealed by regular pathotyping using Sanger sequencing: The F protein cleavage site of virulent strains carries a polybasic motive (^{112}R/K-R-Q-K/R-R*F^{117}), and avirulent NDV strains harbour ^{112}G/E-K/R-Q--G/E-R*L^{116} and a leucine at position 116 at the F protein cleavage site (Collins et al., 1993; de Leeuw et al., 2005; Peeters et al., 1999; Römer-Oberdörfer et al., 1999). For isolate R1954/11 the obtained polybasic cleavage site corresponded to the ICPI, however pointing to a mesogenic pathotype. Sanger sequence of isolate R1973/11 pointed to an avirulent pathotype, but the ICPI result clearly revealed a velogenic pathotype. Such apparently contradicting results have been published before (Nagy et al., 2020; Tan et al., 2008), but were not further resolved. After observing such contradicting results, we plaque cloned both isolates and were able to separate two different NDV: besides genotype XXI virus, a vaccine-type virus (genotype II) was obtained. Subsequent pathotyping of the cloned viruses by ICPI now matched with the sequence of the proteolytic cleavage site, and full genome sequence comparison of the sequenced lentogenic strains reveals a close relationship to vaccine strain Hitchner B1/JN872151 (R1973/2011/cl-2) or La Sota /AF077761 (R1954/2011/cl-1). This finding further supports the notion of re-isolated vaccine strains and is clearly distinct from described virulent genotype II viruses, detected in ND outbreaks in 2006 in Egypt (Mohamed et al., 2011). All viruses from the year 2006 (NDV/chicken/Egypt/2-4/2006; FJ969393, FJ969394, FJ969395) belong to genotype II but are of velogenic pathotype with ICPI values between 1.6 to 1.8 and have a corresponding proteolytic polybasic cleavage site (RRQKR*FIG). The re-isolation of vaccine-type virus is in line with an earlier description (Abolnik et al., 2004; Nagy et al., 2020), and it is conceivable that emergency vaccination in flocks with suspected ND might lead to such double infections. These circumstances have been considered by the OIE (OIE, 2018) when defining criteria for virulence: The definition includes the sequence information of the proteolytic cleavage site, but has the amendment "Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterization of the isolated virus by an ICPI test". In the brain, multicycle replication is restricted to virulent NDV due to the cleavability of the F protein (Nagai et al., 1976; Rott, 1979) and thus brain passage leads to enrichment of virulent NDV. In case of contradicting results as described by others (Nagy et al., 2020; Tan et al., 2008) sequencing of re-isolated virus from the brain is indicated.

Overall the failure to demonstrate antigenic escape in the genotype XXI precursor and its genotype VII.1.1 successor virus indicates that other factors contributed to the assertiveness of a specific ND virus population. Like in Influenza A virus (Neumann & Kawaoka, 2015; Suttie et al., 2019) the evolutionary fitness of NDV may also be associated with other viral genes. Studies on AIV have demonstrated that genomic factors skewing viral fitness, such as replication and transmission efficacy, do not reside exclusively in the glycoprotein-encoding genes (Pantin-Jackwood et al., 2017). Further studies will help to elucidate this hypothesis and help to better understand the epidemiology of different NDV genotypes in poultry.

FIGURE 2 Phylogenetic tree of NDV viruses based on whole genome sequences. Phylogenetic tree was based on maximum likelihood calculations (IQTree software) under the best fit model (model: GTR+F + I+G4) according to the Bayesian Information Criterion (BIC). Numbers at nodes represent measures of robustness based on an ultrafast bootstrap approach implemented in IQTree. The sequences of other reference strains as well as all publicly available Egyptian NDV were obtained from GenBank. Divisions and genotypes are designated according to Dimitrov et al., 2019. Egyptian viruses are coloured in red with a black dot indicating the position of viruses of this study

TABLE 3 Amino acid mutations of Egyptian viruses within the HN protein

HN amino acid		6	7	9	13	24	28	29	31	33	34	35	36	41	42	44	49	54	56	57	60
Virus stamm / genotype																					
LaSota	II	S	Q	A	D	I	A	I	F	T	V	V	T	V	S	L	G	S	L	V	P
B1 Takaaki (AF373823)	II	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EGY/R1954/2011/cl−1	II	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EGY/R1973/2011/cl−2	II	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EGY/R1973/2011/cl−4	XXI	R	R	V	E	V	V	A	L	I	I	M	.	A	A	V	R	R	I	.	S
EGY/AR2178/2014	VII	N	R	V	E	V	.	V	L	M	A	M	I	A	A	A	E	H	.	A	S

		Globular head (aa 126 – 570)																			
	HN amino acid	127	145	155	156	182	197	203	220	249	254	263	265	266	269	288	290	293	304	310	315
Virus stamm / genotype																					
LaSota (KC844235)	II	I	A	A	F	A	R	Y	F	M	V	N	A	V	R	V	T	G	G	S	S
B1 Takaaki (AF373823)	II	.	.	.	.	.	.	H	.	.	A	.	.	.	.	.	.	.	.	.	.
EGY/R1954/2011/cl−1	II	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EGY/R1973/2011/cl−2	II	.	.	.	.	.	.	H	.	.	A	.	.	.	.	.	.	.	.	.	.
EGY/R1973/2011/cl−4	XXI	V	I	.	Y	.	K	H	Y	I	.	K	.	T	S	S	V	K	S	D	P
EGY/AR2178/2014	VII	V	I	V	Y	T	.	H	.	.	.	K	V	A	S	T	V	K	.	G	P

changes of amino acids (aa):

compared to other sequences investigated: x

within known mAb-binding site

within NA-binding site

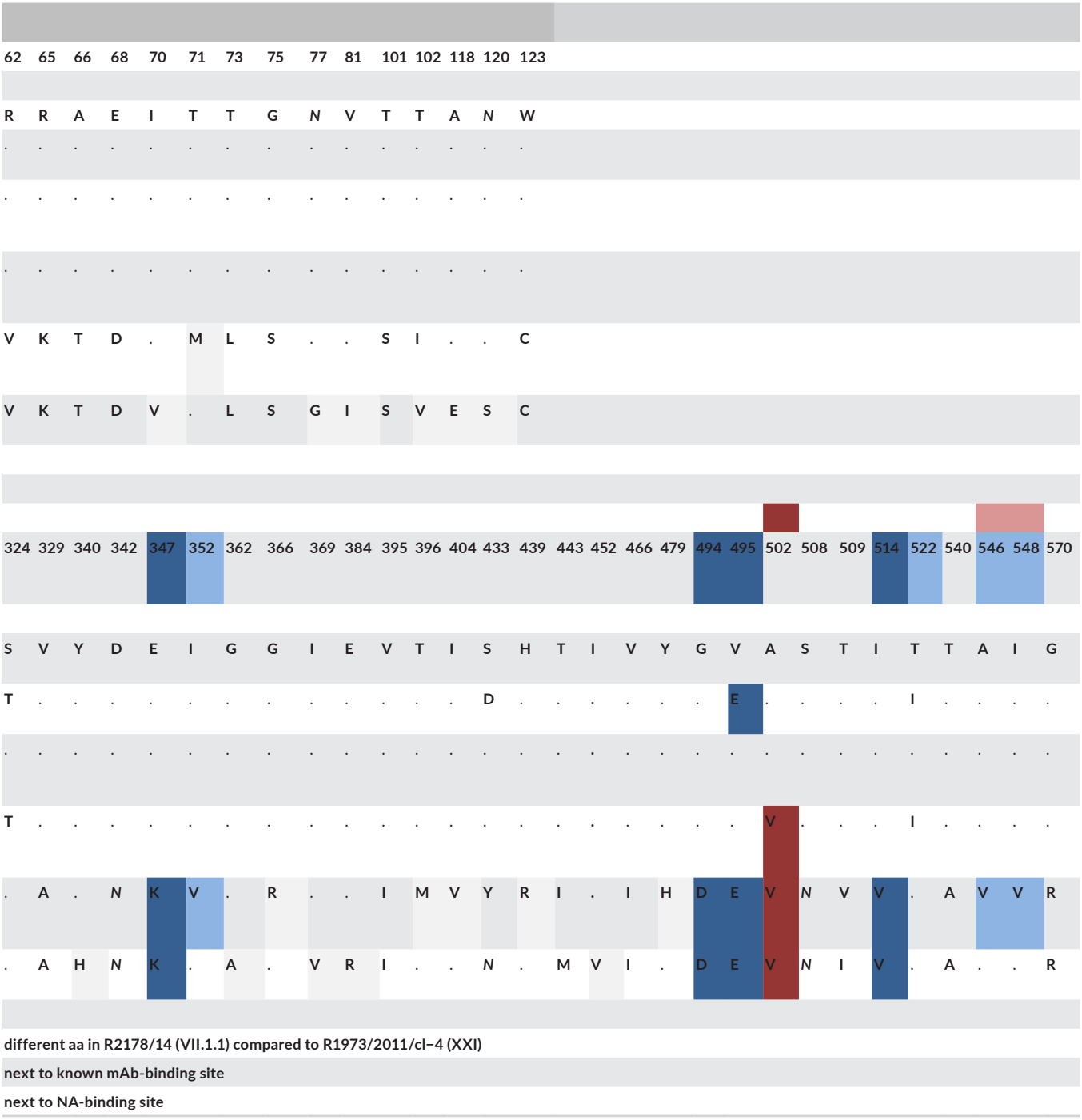


TABLE 4 Antigenic characterization of the NDV strains isolated in this study and NDV reference viruses as well as anti-F mAbs

Virus–Antigen								
	class 1	**class 2**						
	class 1	I	II	VI	XXI	XXI	VII.1.1	VII.1.1
	R2919/06	**Ulster**	**Clone 30**	**R 151/1994**	**R1954/2011/ cl−6**	**R1973/2011/ cl−4**	**R1468/2012**	**AR 2178/2014**
Serum	**HI antibody titre (log2)**							
a-Ulster (I)	10	10	11	11	11	11	10	10
a-clone30 (II)	6	7	8	7	8	9	8	8
a-R151/94 (PPMV−1)	5	6	6	8	8	9	6	7
a-R1468/12 (VII.1.1)				8	10	11	10	9
mAb 617/161	3	<1		11	1	2	<1	
mAb 39								
mAb U85		8	11	1	2		7	
mAb 7D4	3		11	1	1	2	2	
mAb 10		5	8	<1	<1	<1	<1	
ΔHI to homology virus				≤ 2				

ACKNOWLEDGEMENT

The authors thank Cornelia Illing, FLI, Germany, for excellent technical support. We are grateful to colleagues and co-workers at NLQP and Beni-Suef University, Egypt, for helping in field samples collection.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Mahmoud M. Naguib https://orcid.org/0000-0001-6876-8903
Dirk Höper https://orcid.org/0000-0001-8408-2274
Timm C. Harder https://orcid.org/0000-0003-2387-378X
Christian Grund https://orcid.org/0000-0002-3328-2193

REFERENCES

Abolnik, C., Horner, R. F., Bisschop, S. P., Parker, M. E., Romito, M., & Viljoen, G. J. (2004). A phylogenetic study of South African Newcastle disease virus strains isolated between 1990 and 2002 suggests epidemiological origins in the Far East. *Archives of Virology*, *149*(3), 603–619. https://doi.org/10.1007/s00705-003-0218-2

Aldous, E. W., Mynn, J. K., Banks, J., & Alexander, D. J. (2003). A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. *Avian Pathol*, *32*(3), 239–256. https://doi.org/10.1080/0307945031000097783

Alexander, D. J., Russell, P. H., Parsons, G., Elzein, E. M. E. A., Ballouh, A., Cernik, K., Engstrom, B., Fevereiro, M., Fleury, H., Guittet, M., Kaleta, E. F., Kihm, U., Kosters, J., Lomniczi, B., Meister, J., Meulemans, G., Nerome, K., Petek, M., Pokomunski, S., … Tumova, B. (1985). Antigenic and biological characterisation of avian paramyxovirus type I isolates from pigeons-an international collaborative study. *Avian Pathology*, *14*(3), 365–376. https://doi.org/10.1080/03079458508436238

Alexander. (1998). Newcastle disease and other avian paramyxoviruses. A laboratory manual for the isolation and identification of avian pathogens. *American Association of Avian Pathologists, Kennett: Square*, 156-163.

Alexander, Dj. (2000). Newcastle disease and other avian paramyxoviruses. *Revue Scientifique Et Technique*, *19*(2), 443–462. https://doi.org/10.20506/rst.19.2.1231

Brown, V. R., & Bevins, S. N. (2017). Correction to: A review of virulent Newcastle disease viruses in the United States and the role of wild birds in viral persistence and spread. *Veterinary Research*, *48*(1), 77. https://doi.org/10.1186/s13567-017-0485-7

CEC (1992). Council directive 92/66/EEC introducing community measures for the control of Newcastle disease. *Official Journal of the European Community.*, *L*, *260*, 1–20.

Cho, S. H., Kim, S. J., & Kwon, H. J. (2007). Genomic sequence of an antigenic variant Newcastle disease virus isolated in Korea. *Virus Genes*, *35*(2), 293–302. https://doi.org/10.1007/s11262-007-0078-z.

Cho, S. H., Kwon, H. J., Kim, T. E., Kim, J. H., Yoo, H. S., Park, M. H., Park, Y. H., & Kim, S. J. (2008). Characterization of a recombinant Newcastle disease virus vaccine strain. *Clin Vaccine Immunol*, *15*(10), 1572–1579. https://doi.org/10.1128/CVI.00156-08.

Chong, Y. L., Lam, T. T., Kim, O., Lu, H., Dunn, P., & Poss, M. (2013). Successful establishment and global dispersal of genotype VI avian paramyxovirus serotype 1 after cross species transmission. *Infection Genetics and Evolution*, *17*, 260–268. https://doi.org/10.1016/j.meegid.2013.04.025

Chong, Y. L., Padhi, A., Hudson, P. J., & Poss, M. (2010). The effect of vaccination on the evolution and population dynamics of avian paramyxovirus-1. *PLoS Pathogens*, *6*(4), e1000872. https://doi.org/10.1371/journal.ppat.1000872

Collins, M. S., Bashiruddin, J. B., & Alexander, D. J. (1993). Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. *Archives of Virology*, *128*(3), 363–370. https://doi.org/10.1007/bf01309446

Crennell, S., Takimoto, T., Portner, A., & Taylor, G. (2000). Crystal structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase.

Nature Structural Biology, *7*(11), 1068–1074. https://doi.org/10.1038/81002

Czegledi, A., Herczeg, J., Hadjiev, G., Doumanova, L., Wehmann, E., & Lomniczi, B. (2002). The occurrence of five major Newcastle disease virus genotypes (II, IV, V, VI and VIIb) in Bulgaria between 1959 and 1996. *Epidemiology and Infection*, *129*(3), 679–688. https://doi.org/10.1017/S0950268802007732

Czegledi, A., Wehmann, E., & Lomniczi, B. (2003). On the origins and relationships of Newcastle disease virus vaccine strains Hertfordshire and Mukteswar, and virulent strain Herts'33. *Avian Pathol*, *32*(3), 271–276. https://doi.org/10.1080/0307945031000097868

Daubney, R., & Mansy, W. (1948). The occurrence of Newcastle disease in Egypt. *J Comp Pathol Ther*, *58*(3), 189–200. https://doi.org/10.1016/S0368-1742(48)80019-6

de Almeida, R. S., Hammoumi, S., Gil, P., Briand, F. X., Molia, S., Gaidet, N., Cappelle, J., Chevalier, V., Balança, G., Traoré, A., Grillet, C., Maminiaina, O. F., Guendouz, S., Dakouo, M., Samaké, K., Bezeid, O. E. M., Diarra, A., Chaka, H., Goutard, F., … Albina, E. (2013). New avian paramyxoviruses type I strains identified in Africa provide new outcomes for phylogeny reconstruction and genotype classification. *PLoS One*, *8*(10), e76413. https://doi.org/10.1371/journal.pone.0076413

de Leeuw, O. S., Koch, G., Hartog, L., Ravenshorst, N., & Peeters, B. P. (2005). Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin-neuraminidase protein. *Journal of General Virology*, *86*(Pt 6), 1759–1769. https://doi.org/10.1099/vir.0.80822-0

Diel, D. G., da Silva, L. H., Liu, H., Wang, Z., Miller, P. J., & Afonso, C. L. (2012). Genetic diversity of avian paramyxovirus type 1: Proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. *Infection Genetics and Evolution*, *12*(8), 1770–1779. https://doi.org/10.1016/j.meegid.2012.07.012

Dimitrov, K. M., Abolnik, C., Afonso, C. L., Albina, E., Bahl, J., Berg, M., Briand, F.X., Brown, I. H., Choi, K. S., Chvala, I., Diel, D. G., Durr, P. A., Ferreira, H. L., Fusaro, A., Gil, P., Goujgoulova, G. V., Grund, C., Hicks, J. T., Joannis, T. M., … Wong, F. Y. K. (2019). Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. *Infect Genet Evol*, *74*, 103917. https://doi.org/10.1016/j.meegid.2019.103917

Dimitrov, K. M., Afonso, C. L., Yu, Q., & Miller, P. J. (2017). Newcastle disease vaccines-A solved problem or a continuous challenge? *Veterinary Microbiology*, *206*, 126–136. https://doi.org/10.1016/j.vetmic.2016.12.019

Dimitrov, K. M., Lee, D. H., Williams-Coplin, D., Olivier, T. L., Miller, P. J., & Afonso, C. L. (2016). Newcastle disease viruses causing recent outbreaks worldwide show unexpectedly high genetic similarity to historical virulent isolates from the 1940s. *Journal of Clinical Microbiology*, *54*(5), 1228–1235. https://doi.org/10.1128/jcm.03044-15

Ewies, S. S., Ali, A., Tamam, S. M., & Madbouly, H. M. (2017). Molecular characterization of Newcastle disease virus (genotype VII) from broiler chickens in Egypt. *Beni-Suef University Journal of Basic and Applied Sciences*, *6*(3), 232–237. https://doi.org/10.1016/j.bjbas.2017.04.004

Grund, C., Abdelwhab, E. S., Arafa, A. S., Ziller, M., Hassan, M. K., Aly, M. M., Hafez, H. M., Harder, T. C., & Beer, M. (2011). Highly pathogenic avian influenza virus H5N1 from Egypt escapes vaccine-induced immunity but confers clinical protection against a heterologous clade 2.2.1 Egyptian isolate. *Vaccine*, *29*(33), 5567–5573. https://doi.org/10.1016/j.vaccine.2011.01.006

Hall, T. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98.

Herczeg, J., Pascucci, S., Massi, P., Luini, M., Selli, L., Capua, I., & Lomniczi, B. (2001). A longitudinal study of velogenic Newcastle disease virus genotypes isolated in Italy between 1960 and 2000. *Avian Pathology*, *30*(2), 163–168. https://doi.org/10.1080/03079450120044000

Herczeg, J., Wehmann, E., Bragg, R. R., Travassos Dias, P. M., Hadjiev, G., Werner, O., & Lomniczi, B. (1999). Two novel genetic groups (VIIb and VIII) responsible for recent Newcastle disease outbreaks in Southern Africa, one (VIIb) of which reached Southern Europe. *Archives of Virology*, *144*(11), 2087–2099. https://doi.org/10.1007/s007050050624

Hicks, J. T., Dimitrov, K. M., Afonso, C. L., Ramey, A. M., & Bahl, J. (2019). Global phylodynamic analysis of avian paramyxovirus-1 provides evidence of inter-host transmission and intercontinental spatial diffusion. *BMC Evolutionary Biology*, *19*(1), 108. https://doi.org/10.1186/s12862-019-1431-2

Iorio, R. M., Glickman, R. L., Riel, A. M., Sheehan, J. P., & Bratt, M. A. (1989). Functional and neutralization profile of seven overlapping antigenic sites on the HN glycoprotein of Newcastle disease virus: Monoclonal antibodies to some sites prevent viral attachment. *Virus Research*, *13*(3), 245–261. https://doi.org/10.1016/0168-1702(89)90019-1

Iorio, R. M., Syddall, R. J., Sheehan, J. P., Bratt, M. A., Glickman, R. L., & Riel, A. M. (1991). Neutralization map of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus: Domains recognized by monoclonal antibodies that prevent receptor recognition. *Journal of Virology*, *65*(9), 4999–5006. https://doi.org/10.1128/JVI.65.9.4999-5006.1991

Juozapaitis, M., Aguiar Moreira, É., Mena, I., Giese, S., Riegger, D., Pohlmann, A., Höper, D., Zimmer, G., Beer, M., García-Sastre, A., & Schwemmle, M. (2014). An infectious bat-derived chimeric influenza virus harbouring the entry machinery of an influenza A virus. *Nature Communications*, *5*, 4448. https://doi.org/10.1038/ncomms5448

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, *30*(4), 772–780. https://doi.org/10.1093/molbev/mst010

Kawaguchi, T., Nomura, K., Hirayama, Y., & Kitagawa, T. (1987). Establishment and characterization of a chicken hepatocellular carcinoma cell line LMH. *Cancer Res*, *47*(16), 4460–4464.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., & Drummond, A. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*(12), 1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Kim, L. M., King, D. J., Curry, P. E., Suarez, D. L., Swayne, D. E., Stallknecht, D. E., Slemons, R. D., Pedersen, J. C., Senne, D. A., Winker, K., & Afonso, C. L. (2007). Phylogenetic diversity among low-virulence newcastle disease viruses from waterfowl and shorebirds and comparison of genotype distributions to those of poultry-origin isolates. *Journal of Virology*, *81*(22), 12641–12653. https://doi.org/10.1128/jvi.00843-07

Lamb, R. A., & Parks, G. D. (2007). Paramyxoviridae: the viruses and their replication. In B. N. Fields, D. N. Knipe, & P. M. Howley (Eds.), *Fields virology*, 5th ed. (pp. 1449–1496). Lippincott, Williams, and Wilkins. https://www.scholars.northwestern.edu/en/publications/paramyxoviridae-the-viruses-and-their-replication-2.

Miller, P. J., Decanini, E. L., & Afonso, C. L. (2010). Newcastle disease: Evolution of genotypes and the related diagnostic challenges. *Infection, Genetics and Evolution*, *10*(1), 26–35. https://doi.org/10.1016/j.meegid.2009.09.012

Miller, P. J., Haddas, R., Simanov, L., Lublin, A., Rehmani, S. F., Wajid, A., Bibi, T., Khan, T. A., Yaqub, T., Setiyaningsih, S., & Afonso, C. L. (2015). Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. *Infection, Genetics and Evolution*, *29*, 216–229. https://doi.org/10.1016/j.meegid.2014.10.032

Miller, P. J., Kim, L. M., Ip, H. S., & Afonso, C. L. (2009). Evolutionary dynamics of Newcastle disease virus. *Virology*, *391*(1), 64–72. https://doi.org/10.1016/j.virol.2009.05.033

Miller, P. J., & Koch, G. (2013). Newcastle disease. In D. E. Swayne, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez, & V. Nair (Eds.), *Diseases of Poultry*, 13th ed. (pp. 89–138). Wiley-Blackwell.

Minh, B. Q., Nguyen, M. A., & von Haeseler, A. (2013). Ultrafast approximation for phylogenetic bootstrap. *Molecular Biology and Evolution*, *30*(5), 1188–1195. https://doi.org/10.1093/molbev/mst024

Mohamed, M. H., Kumar, S., Paldurai, A., & Samal, S. K. (2011). Sequence analysis of fusion protein gene of Newcastle disease virus isolated from outbreaks in Egypt during 2006. *Virology Journal*, *8*, 237. https://doi.org/10.1186/1743-422x-8-237

Moharam, I., Razik, A. A. E., Sultan, H., Ghezlan, M., Meseko, C., Franzke, K., Harder, T., Beer, M., & Grund, C. (2019). Investigation of suspected Newcastle disease (ND) outbreaks in Egypt uncovers a high virus velogenic ND virus burden in small-scale holdings and the presence of multiple pathogens. *Avian Pathology*, *48*(5), 406–415. https://doi.org/10.1080/03079457.2019.1612852

Nagai, Y., Klenk, H. D., & Rott, R. (1976). Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. *Virology*, *72*(2), 494–508. https://doi.org/10.1016/0042-6822(76)90178-1

Naguib, M. M., El-Kady, M. F., Lüschow, D., Hassan, K. E., Arafa, A.-S., El-Zanaty, A., Hassan, M. K., Hafez, H. M., Grund, C., & Harder, T. C. (2017). New real time and conventional RT-PCRs for updated molecular diagnosis of infectious bronchitis virus infection (IBV) in chickens in Egypt associated with frequent co-infections with avian influenza and Newcastle Disease viruses. *Journal of Virological Methods*, *245*, 19–27. https://doi.org/10.1016/j.jviromet.2017.02.018

Nagy, A., Ali, A., Zain El-Abideen, M. A., Kilany, W., & Elsayed, M. (2020). Characterization and genetic analysis of recent and emergent virulent newcastle disease viruses in Egypt. *Transbound Emerg Dis*, *67*, 2000–2012. https://doi.org/10.1111/tbed.13543

Neumann, G., & Kawaoka, Y. (2015). Transmission of influenza A viruses. *Virology*, *479–480*, 234–246. https://doi.org/10.1016/j.virol.2015.03.009

Nguyen, L. T., Schmidt, H. A., von Haeseler, A., & Minh, B. Q. (2014). IQ-TREE: A fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. *Molecular Biology and Evolution*, https://doi.org/10.1093/molbev/msu300

OIE (2012). Newcastle disease (infection with Newcastle disease virus). *OIE Terrestrial Manual*, *Chapter*, *2*(3), 14.

OIE (2018). *Newcastle disease. Terrestrial Manual: Chapter 3.3.14*. Available online https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.14_NEWCASTLE_DIS.pdf. Accessed on 24.10.2020.

Orabi, A., Hussein, A., Saleh, A. A., El-Magd, M. A., & Munir, M. (2017). Evolutionary insights into the fusion protein of Newcastle disease virus isolated from vaccinated chickens in 2016 in Egypt. *Archives of Virology*, *162*(10), 3069–3079. https://doi.org/10.1007/s00705-017-3483-1

Pantin-Jackwood, M. J., Costa-Hurtado, M., Bertran, K., DeJesus, E., Smith, D., & Swayne, D. E. (2017). Infectivity, transmission and pathogenicity of H5 highly pathogenic avian influenza clade 2.3.4.4 (H5N8 and H5N2) United States index viruses in Pekin ducks and Chinese geese. *Veterinary Research*, *48*(1);33. https://doi.org/10.1186/s13567-017-0435-4

Peeters, B. P., de Leeuw, O. S., Koch, G., & Gielkens, A. L. (1999). Rescue of Newcastle disease virus from cloned cDNA: Evidence that cleavability of the fusion protein is a major determinant for virulence. *Journal of Virology*, *73*(6), 5001–5009. https://doi.org/10.1128/jvi.73.6.5001-5009.1999

Radwan, M. M., Darwish, S. F., El-Sabagh, I. M., El-Sanousi, A. A., & Shalaby, M. A. (2013). Isolation and molecular characterization of Newcastle disease virus genotypes II and VIId in Egypt between 2011 and 2012. *Virus Genes*, *47*(2), 311–316. https://doi.org/10.1007/s11262-013-0950-y

Rima, B., Balkema-Buschmann, A., Dundon, W. G., Duprex, P., Easton, A., Fouchier, R., Kurath, G., Lamb, R., Lee, B., Rota, P., & Wang, L. (2019). ICTV Virus Taxonomy Profile: Paramyxoviridae. *Journal of General Virology*, *100*(12), 1593–1594. https://doi.org/10.1099/jgv.0.001328

Römer-Oberdörfer, A., Mundt, E., Mebatsion, T., Buchholz, U. J., & Mettenleiter, T. C. (1999). Generation of recombinant lentogenic Newcastle disease virus from cDNA. *Journal of General Virology*, *80*(Pt 11), 2987–2995. https://doi.org/10.1099/0022-1317-80-11-2987

Rott, R. (1979). Molecular basis of infectivity and pathogenicity of myxovirus. *Archives of Virology*, *59*(4), 285–298. https://doi.org/10.1007/BF01317469

Saad, A. M., Samy, A., Soliman, M. A., Arafa, A., Zanaty, A., Hassan, M. K., Sultan, A. H., Bazid, A. I., & Hussein, A. H. (2017). Genotypic and pathogenic characterization of genotype VII Newcastle disease viruses isolated from commercial farms in Egypt and evaluation of heterologous antibody responses. *Archives of Virology*, *162*(7), 1985–1994. https://doi.org/10.1007/s00705-017-3336-y

Sabra, M., Dimitrov, K. M., Goraichuk, I. V., Wajid, A., Sharma, P., Williams-Coplin, D., Basharat, A., Rehmani, S. F., Muzyka, D. V., Miller, P. J., & Afonso, C. L. (2017). Phylogenetic assessment reveals continuous evolution and circulation of pigeon-derived virulent avian avulaviruses 1 in Eastern Europe, Asia, and Africa. *BMC Veterinary Research*, *13*(1), 291. https://doi.org/10.1186/s12917-017-1211-4

Samy, A., & Naguib, M. M. (2018). Avian respiratory coinfection and impact on avian influenza pathogenicity in domestic poultry: field and experimental findings. *Veterinary Sciences*, *5*(1), 23. https://doi.org/10.3390/vetsci5010023

Seal, B. S., King, D. J., & Bennett, J. D. (1996). Characterization of Newcastle disease virus vaccines by biological properties and sequence analysis of the hemagglutinin-neuraminidase protein gene. *Vaccine*, *14*(8), 761–766. https://doi.org/10.1016/0264-410x(95)00252-v

Snoeck, C. J., Owoade, A. A., Couacy-Hymann, E., Alkali, B. R., Okwen, M. P., Adeyanju, A. T., Komoyo, G. F., Nakoune, E., Le Faou, A., & Muller, C. P. (2013). High genetic diversity of Newcastle disease virus in poultry in West and Central Africa: Cocirculation of genotype XIV and newly defined genotypes XVII and XVIII. *Journal of Clinical Microbiology*, *51*(7), 2250–2260. https://doi.org/10.1128/JCM.00684-13

Steward, M., Vipond, I. B., Millar, N. S., & Emmerson, P. T. (1993). RNA editing in Newcastle disease virus. *Journal of General Virology*, *74*(Pt 12), 2539–2547. https://doi.org/10.1099/0022-1317-74-12-2539

Suarez, D. L., Miller, P. J., Koch, G., Mundt, E., & Rautenschlein, S. (2020). Newcastle disease, other avian paramyxoviruses, and avian metapneumovirus infections In D. E. Swayne, M. Boulianne, C. M. Logue, L. R. McDougald, V. Nair, D. L. Suarez, S. D. Wit, T. Grimes, D. Johnson, M. Kromm, T. Y. Prajitno, I. Rubinoff, & G. Zavala (Eds.), *Diseases of Poultry* (pp. 109–166). https://onlinelibrary.wiley.com/doi/abs/10.1002/9781119371199.ch3.

Suttie, A., Deng, Y. M., Greenhill, A. R., Dussart, P., Horwood, P. F., & Karlsson, E. A. (2019). Inventory of molecular markers affecting biological characteristics of avian influenza A viruses. *Virus Genes*, *55*(6), 739–768. https://doi.org/10.1007/s11262-019-01700-z

Tan, L. T., Xu, H. Y., Wang, Y. L., Qin, Z. M., Sun, L., Liu, W. J., & Cui, Z. Z. (2008). Molecular characterization of three new virulent Newcastle disease virus variants isolated in China. *Journal of Clinical Microbiology*, *46*(2), 750–753. https://doi.org/10.1128/jcm.01587-07

Ujvári, D., Wehmann, E., Kaleta, E. F., Werner, O., Savić, V., Nagy, E., & Lomniczi, B. (2003). Phylogenetic analysis reveals extensive evolution of avian paramyxovirus type 1 strains of pigeons (Columba livia) and suggests multiple species transmission. *Virus Research*, *96*(1–2), 63–73. https://doi.org/10.1016/s0168-1702(03)00173-4

Wehmann, E., Czegledi, A., Werner, O., Kaleta, E. F., & Lomniczi, B. (2003). Occurrence of genotypes IV, V, VI and VIIa in Newcastle

disease outbreaks in Germany between 1939 and 1995. *Avian Pathol*, *32*(2), 157–163. https://doi.org/10.1080/03079450210000071623

Wise, M. G., Suarez, D. L., Seal, B. S., Pedersen, J. C., Senne, D. A., King, D. J., Kapczynski, D. R., & Spackman, E. (2004). Development of a real-time reverse-transcription PCR for detection of newcastle disease virus RNA in clinical samples. *Journal of Clinical Microbiology*, *42*(1), 329–338. https://doi.org/10.1128/jcm.42.1.329-338.2004

Yuan, P., Swanson, K. A., Leser, G. P., Paterson, R. G., Lamb, R. A., & Jardetzky, T. S. (2011). Structure of the Newcastle disease virus hemagglutinin-neuraminidase (HN) ectodomain reveals a four-helix bundle stalk. *Proceedings of the National Academy of Sciences U S A*, *108*(36), 14920–14925. https://doi.org/10.1073/pnas.1111691108

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How to cite this article: Naguib, M. M., Höper, D., Elkady, M. F., Afifi, M. A., Erfan, A., Abozeid, H. H., Hasan, W. M., Arafa, A.-S., Shahein, M., Beer, M., Harder, T. C., & Grund, C. (2022). Comparison of genomic and antigenic properties of Newcastle Disease virus genotypes II, XXI and VII from Egypt do not point to antigenic drift as selection marker. *Transboundary and Emerging Diseases*, *69*, 849–863. https://doi.org/10.1111/tbed.14121