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Molecular Detection and Characterization of Virulent Newcastle Disease Viruses from Different Avian Species in Egypt

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ABSTRACT

Newcastle disease caused by infection with the Newcastle virus is one of dangerous and important diseases that affect poultry and other avian species. It is a disease that affects all ages with and affects broiler as well as laying flocks, causing heavy losses as a result of infection, deaths and lack of egg production. This work was performed to assess the molecular and epidemiological status of the NDVs in different domesticated birds in Egypt. One hundred sixty-eight samples were obtained from distinct avian species, including turkeys, chickens, ducks, quail, pigeons, and ostriches during 2018-2020. After sample propagation on 9-days-old SPF ECE, NDVs presence were confirmed using conventional serological methods (HI) and molecular detection (RT-PCR) using primers targeting the Matrix and Fusion genes. The NDVs were confirmed in 20 samples (12.27%) out of 168 tested samples. Pathotyping of four selected positive isolates representing different investigated avian species revealed velogenic NDVs with ICPI was 1.67 to 1.96. Further characterization via using partial F gene phylogenetic and sequencing analysis revealed that the six tested isolates were had ^{112}GRRQKR↓F^{117} at the cleavage site of Fusion protein. All six isolates were clustered with genotype VII1.1 NDVs via phylogenetic analysis. The identified nucleotide sequences among the six NDV isolates ranged from 94.9% to 100%. This study revealed the continuous circulation of velogenic NDVs in different avian species although the intensive use of vaccines against ND so regular monitoring and surveillance must be applied to decrease its adverse economic effect on poultry sector in Egypt. The aim of this study is to know the epidemiological status of the Newcastle virus in different vaccinated and non-vaccinated avian species in Egypt.

Key words: NDV, Genetic characterization, ICPI, RT-PCR, HI.

INTRODUCTION

Newcastle disease is one of infectious and easily transmitted viral diseases in the world, affecting a diverse variety of poultry species across five continents (Ashraf and Shah 2014). And the economic losses of this virus in the endemic countries as a result of mortalities and the lack of egg production, in addition to the indirect financial losses from the cost of vaccines (Miller and Koch 2013). Newcastle disease virus represents a global threat to the poultry industry, despite the existence of vaccines massive application (Czegledi et al. 2006). Newcastle virus disease results from infection with the Newcastle virus, which has the ability to infect many different bird species, as there are more than 200 avian species can be infected by NDVs (OIE 2012). The Newcastle virus can be classified with means of the virulence into the highly pathogenic, low pathogenic and A pathogenic strains based on the pathogenesis index relevant to OIE guidelines. The highly pathogenic strains are velogenic or mesogenic strains. While the low pathogenic is, lentogenic strains, and a pathogenic strain with no virulence (Alexander 2000).

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The pathogenic APMV-1 viruses to chickens has the sequence 112R/K-R-Q/K/R-K/R-R116 at the C-terminus of the F2 protein **(**Kim et al. 2008a; Choi et al. 2010) with F (phenylalanine) at residue 117 (the N-terminus of the F1 protein), but the low virulence viruses having in the same region sequences of 112G/E-K/R-Q-G/E-R116 with L (leucine) at residue 117 although, there are variant viruses of the pigeon having high ICPI having the 112G-R-Q/K-K-R-F117 sequence (Peeters et al. 1999; Meulemans et al. 2002). Another classification depends on the genetic characterization of F gene and phylogenic analysis into 2 classes; class 1 and class 2, class 1 contains only one genotype (1.I), and class 2 contains 21 genotypes (2.I-2.XXI) (Dimitrov et al. 2019). Genotype 2.VII represents the majority of velogenic NDVs and has 2.VII.1.1 sub-lineages that, encompassing viruses that in the 1990s have been emerged in the Middle East, the Far East, Asia and Europe, while 2.VII.1.2 viruses evolved in China, and circulated in East Africa and South, as well as in Israel and Pakistan (Dimitrov et al. 2019; Ibrahim et al. 2020; Mahmood and Sabir 2021).

Because of many outbreaks in vaccinated commercial or backyard birds or not vaccinated birds. In Egypt, ND is considered a perpetual issue. The epidemiology of these outbreaks' virulent NDV isolates remains unknown. As a result, the current research was carried out to isolate and molecular characterize the current circulating NDV strains from a new epidemic from domestic birds in Egypt.

MATERIALS AND METHODS

Ethical Statement

The protocol and procedures employed were reviewed and approved by ZU-IACUC committee with approval number of ZU-IACUC/2/F/27/2021 and all procedures were conducted strictly in accordance with the Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize animal suffering.

Sample Origin

One hundred sixty-eight samples were collected as following: 40 chicken farms, 35 duck flocks, 27 pigeon flocks, 25 quail flocks, 30 turkey farms and eleven ostrich camp house not all birds used in sample collection were vaccinated. The samples were collected from diseased birds during 2018 - 2020. Homogenization of organs samples adding 1:10 w/v phosphate-buffered saline with pH 7.0–7.4, then the homogenates were centrifuged at 3000×g/5min after three cycles of thawing and freezing, collected supernatants were stored at−80°C for virus isolation and molecular identification.

Virus Isolation

SPF embryonated chicken eggs of 9-11 days old were obtained from Koom Oshiem, Fayoum, Egypt for virus isolation and propagation via allantoic route according to (OIE 2012), allantoic fluid harvested were checked for HA activity, using 10% chicken RBCs. The allantoic fluid was used for nucleic acid extraction for molecular identification and characterization.

Biological Pathotyping

The intracerebral pathogenicity indices (ICPI) for the isolatenad NDVs were calculated according to procedure of OIE (2018). The diluted isolates (4 isolates from chicken, pigeon, turkey, duck only have been checked for ICPI) were injected intracerebrally into 24hours aged SPF chicks (10 in Number). The chicks were checked every day for 8 days, if the result of ICPI is closer to the maximum score of 2, the virus can be considered as highly virulent, but if it is ICPI close to 0.0 it indicted lentogenic strains (OIE 2018).

Molecular Detection

The RNA of the isolates was extracted using Thermo Scientific Gene JET Viral DNA and RNA purification (Thermo Fisher Scientific Inc, and USA) and the procedure was carried out according to the manufacturer's instructions then the RNA was kept at−80°C. The Primers (M2 and F2) used in the PCR assay (Mase et al. 2002) were manufactured by Macrogen, Korea as shown in Table 1. The extracted RNA was examined for the Newcastle virus presence via using Maxime RT-PCR premix kit (INTRON, and Korea). Extracted RNA (5µL) was added to 20µL of the final reaction volume with 1µL of each forward and reverse primer set (10pmol). Thermo-cycling conditions were Reverse Transcription Step at 45°C for 30min, then Inactivation of RTase at 94°C for 5min, denaturation at 94°C for 45sec, Annealing 52°C for 60sec and Extension at 72°C for 1min by 35 cycles and the last step was the final extrusion at 72°C/10min, then the PCR product was injected into 1.2% agarose gel contained ethidium bromide stain and examined by ultraviolet gel documentation system to determine the presence of the Specific bands (BIORAD®, California, and USA).

Fusion Gene Sequencing and Phylogeny

The six selected isolates (one isolate from chicken, one isolate from duck, one isolate from turkey, two isolates from pigeon, one isolate from quail) for gene sequence were amplified using another specific primer set F300 and R700, targeting the fusion gene cleavage site (Table 1). The specific bands were cut from agarose gel and purified by QIAquick Gel Extraction kit (Qiagen, Valencia, CA), the sequence was done by Bigdye Terminator V3.1 cycle sequencing kit. (Foster city, USA). The Clustal W method was used for alignment of the obtained nucleotides sequence and using GenBank BLAST web tool to compare it with GenBank available corresponding sequences, via neighbour joining method in MEGA6 Phylogenetic analysis were done (Tamura et al. 2013), The studied gene sequences of the NDVs isolates were submitted to the GenBank database with the accession numbers (Table 2).

Table 1: Oligo nucleotide primers used for amplification of partial Matrix and Fusion genes

Primer	Sequence	Segment size	Reference
NDV-F330	AGGAAGGAGACAAAACGTTTTATAGG	400 (bp)	Selim et al. (2018)
NDV-R 700	TCAGCTGAGTTAATGCAGGGGAGG		

RESULTS

Clinical Examination

The investigated birds showed depression, greenish diarrhea, respiratory and/or neurological signs. Macroscopically birds showed congestion and inflammation of cecal tonsils (Fig. 1), trachea and lungs as well as petechial hemorrhage on proventriculus (Fig. 2). The mentioned signs and lesions were suggestive for NDV infection.

Isolation and Identification

Suspected samples were propagated on ECE and results showed that 56 samples out of 168 investigated samples showed positive direct hemagglutination activity using 10% RBCs. To eliminate other possibility of HA agents, the HI test was carried out using specific NDV antibodies and the results showed that out of 56 samples there are 20 positive HI agents were NDV isolates. For molecular identification, the 20 isolates were positive for RT-PCR using primers targeting Matrix and Fusion genes of NDV, six isolates out of 20 positive samples for Newcastle were selected for further genetic analysis, collected from birds had only live vaccine or unvaccinated birds (Table 2).

Biological Pathotyping

Four isolates representing different bird species suspected to be velogenic were selected based on the seriousness of clinical signs and lesion then subjected for biological Pathotyping. These isolates were identified as velogenic NDVs with ICPI was 1.67 to 1.96 (Table 2).

Molecular Characterization

The investigated isolates were obtained from birds presenting clinical signs and lesions suggesting ND infection. From the 20 confirmed NDV isolates by RT PCR, six isolates were selected for partial Fusion gene sequence. The amplified amplicon was approximately 400bp. This segment was purified, sequenced and aligned with corresponding reference NDVs strains representing different genotypes at different geographical locations. The analysis of the selected partial F gene showed the six isolates have polybasic deduced amino acid sequence at cleavage site and the motif was 112 GRRQKR↓F 117 of Fusion protein, which is a characteristic property for velogenic NDVs. The phylogenetic analysis indicated that all investigated isolates were clustered with class II genotype VII1.1 NDVs (Fig. 3). The lowest identity between the investigated isolates was 96.1% between Quail/Egypt/Giza-ZU-16/2019, Pigeon-Sharkia-ZU-9-2020 and Chicken-Qalubeya-ZU-10-2020. The nucleotides

Fig. 1: Hemorrhagic and inflamed cecal tonsils in white turkey naturally infected with velogenic NDV.

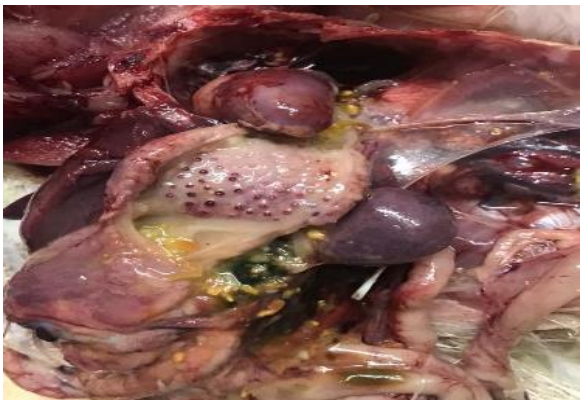

Fig. 2: Hemorrhages at proventriculus tips and congested spleen of chicken carcass naturally infected with velogenic NDV.

Table 2: Molecular identified strains with their accession number

Isolate ID	Genotype	Host	Vaccine regime	ICIP	Cleavage site	Accession No.
Turkey/Egypt/Sharkia-ZU-10/2019	VII 1.1	Turkey	Live vaccine (LaSota 3x)	1.90	^{112}GRRQKR↓F^{117}	MW824548
Duck/Egypt/Giza-ZU-8/2020	VII 1.1	Duck	Not vaccinated	1.67	^{112}GRRQKR↓F^{117}	MW824549
Quail/Egypt/Giza-ZU-16/2019	VII 1.1	Quail	Not vaccinated	NA	^{112}GRRQKR↓F^{117}	MW824550
Pigeon-Sharkia-ZU-9-2020	VII 1.1	Pigeon	Live vaccine (LaSota 2x)	NA	^{112}GRRQKR↓F^{117}	MZ029051
Chicken-Qalubeya-ZU-10-2020	VII 1.1	Chicken	Live vaccine (Hitchner 1x)	1.96	^{112}GRRQKR↓F^{117}	MZ029052
Pigeon/Egypt/Cairo-ZU-19/2019	VII 1.1	Pigeon	Not vaccinated	1.79	^{112}GRRQKR↓F^{117}	MZ029053

All the isolates were virulent. NA=not available. (x) mean frequency.

Table 3: Nucleotides substations among the tested isolates

	Position	330	384	423	444	519	558	582	594	609	612	618	633
	Majority	A	A	G	T	A	C	G	C	A	G	C	C
Isolates	Pigeon-Sharkia-ZU-9-2020	G	.	.	C	-	-	A	T	G	A	T	T
	Duck/Egypt/Giza-ZU-8/2020	-	-	.	-	-	-	-	-	-	-	-	-
	Chicken-Qalubeya-ZU-10-2020	G	.	-	C	.	-	A	T	G	A	T	T
	Turkey/Egypt/Sharkia-ZU-10/2019	.	.	.	-	R	Y	-	.	.	.	-	-
	Quail/Egypt/Giza-ZU-16/2019	.	G	A	.	G	-	-	.	.	.	.	.
	Pigeon/Egypt/Cairo-ZU-19/2019	.	.	.	.	.	-	-	.	.	.	.	.

Dot (.) means the same nucleotide while dash (-) indicates this site was not included in partial genome sequence.

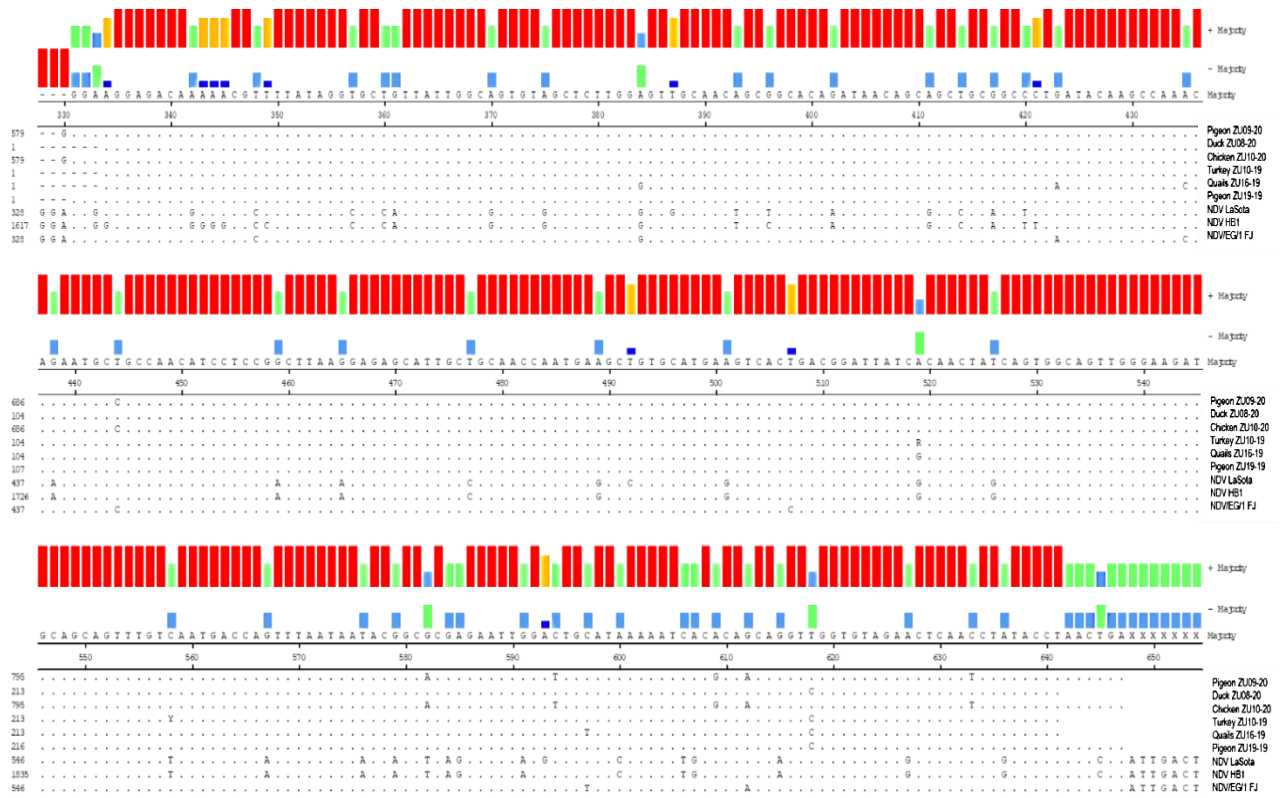

Fig. 3: Phylogenetic analysis of Newcastle disease virus using a 400-bp fragment of the fusion gene with the ML method using 1000 bootstrap replicates Using MEGAX version 10.2.4. Eighty-five sequences selected for phylogenetic analysis representing different genotypes of NDVs. The roman numerals on the right represent the genotypes of the isolates belong to. The investigated isolates marked with small black triangle clustered with NDVs belongs to Genotype VII.

substitutions among the different investigated isolates as well as reference strains including vaccines strains were summarized (Table 3; Fig. 4). A single aa substitution (K145N) was found in Quail/Egypt/Giza-ZU-16/2019. Based on nucleotides sequence the similarity between the investigated isolates and Genotype II isolates was 84.2 to 86.7% while, similarity between the investigated isolates and LaSota strain (commonly used vaccine strain) was 84.0 to 85.8%.

DISCUSSION

ND stays a major challenge of poultry production, either in commercial or backyard production systems. ND is a contagious disease affecting more than 250 birds' species including domestic and wild birds (Dimitrov et al. 2019). Several periodical outbreaks of ND in Egypt were recorded and causes severe losses among susceptible birds over the last few years including poultry (Mohamed et al. 2011; Selim et al. 2018; Abd El-Hamid et al. 2020), pigeons (Samy et al. 2018) and ducks (Elbestawy et al. 2019). In this study, 20 NDV isolates were identified from birds showing one or more of the typical signs and lesions related to ND infection (Alexander and Senne 2008). Four NDV isolates representing different birds' species under investigation during the period 2018-2020 were selected for Pathotyping and molecular analysis. The ICPI is a trustful method for NDV biological Pathotyping (OIE 2012). The ICIP value of the investigated isolates ranged from 1.67 to 1.96, revealed that all are velogenic NDVs as the highest ICPI value the more virulent viruses (Ganar et al. 2014). The F gene is the major virulence and genotype determinant of NDVs (Aldous et al. 2003; Dimitrov et al. 2019). Kim et al. (2013) reported that Fusion protein of Newcastle disease viruses is the key of providing a protective immunity in development of genotype-matched vaccine. For molecular pathotyping the related amino acids sequence of the 6 selected isolates were aligned, the comparison of the deduced amino acid sequence at the cleavage site of fusion protein at position 112 to 117 revealed the presence of multiphasic amino acids at position 112-116 aa "^{112}GRRQKR^{116}↓F^{117} "and phenylalanine aa (F) at position 117, which is the characteristic motif for velogenic Newcastle disease viruses (OIE 2015). Dimitrov et al. (2019) based on genomic analysis stated that all strains of APMV-1 are phylogenetically divided into two classes (class I and class II) and further class II strains are classified into genotypes (I to XXI) based on genetic differences., the partial F gene sequence of the six isolate were successfully determined and the nucleotide sequences were aligned along with other corresponding sequences representing different genotypes and phylogenetic analysis indicated that all the six investigated isolates were clustered in class II Genotype VII1.1 clade with many of the Egyptian isolates were reported during the last decade (Radwan et al. 2013; Selim et al. 2018; Samy et al. 2018; Abd El-Hamid et al. 2020). Genotype VII viruses have been linked with many recent NDV outbreaks in Middle East, Eastern Europe, Asia, and South Africa (Miller et al. 2015; Manar et al. 2020).

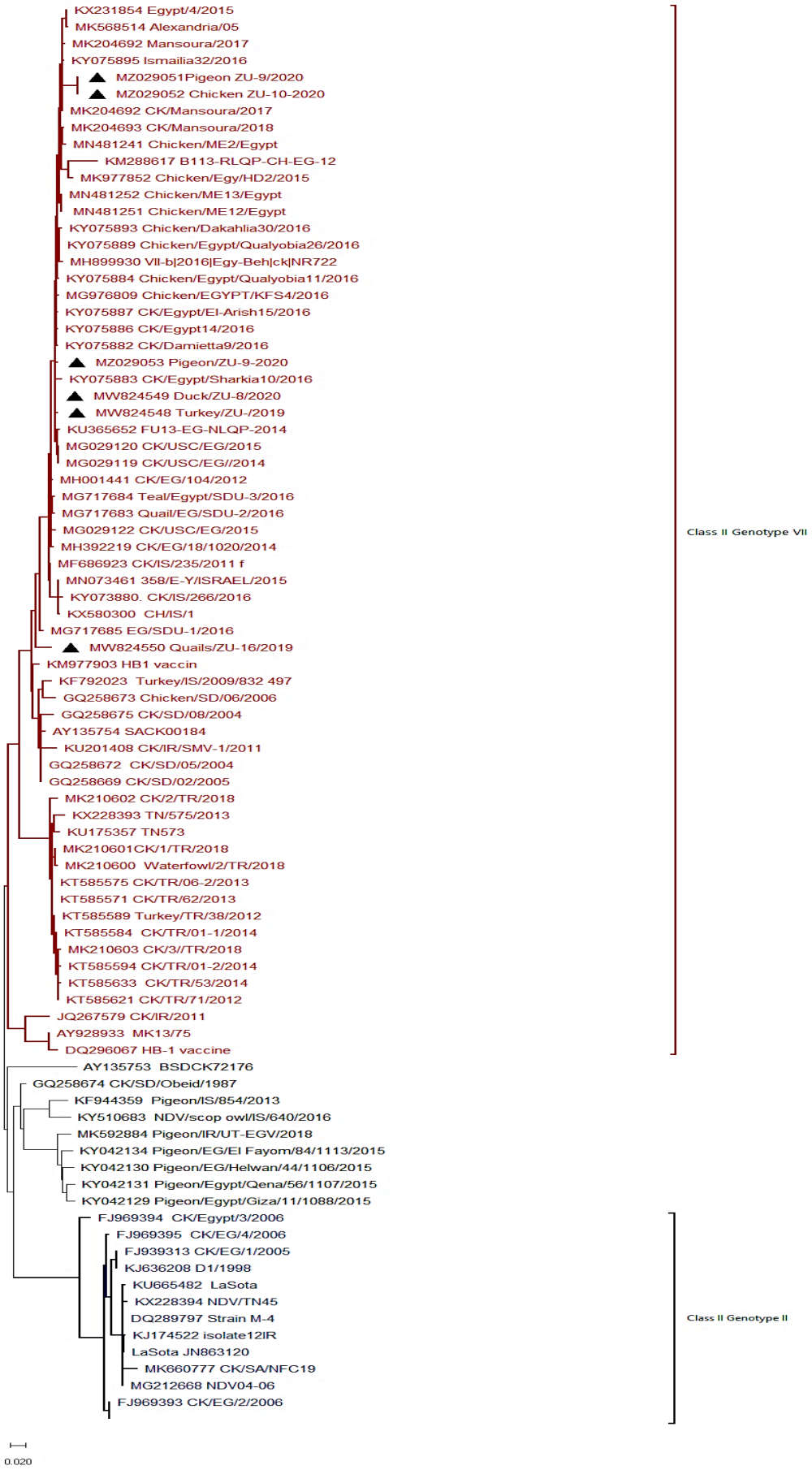

Fig. 4: Alignment and comparison of not sequences of investigated isolates showed several nucleotides substitution among them. The obtained sequences were aligned by the Clustal W and compared with corresponding sequences available in GenBank by BLAST web tool of the GenBank.

The nucleotides similarity between among the investigated isolates ranged from 96.1% to 100%. The high nucleotide similarity between NDV isolates from different avian species support the possibility of virus exchange between avian species and the appropriate vaccinal strain could be used safely and effectively in different avian species towards the control of NDVs (Dimitrov et al. 2017). Ellakany et al. (2019) and Elbestawy et al. (2019) stated the ability of ducks and pigeons in transmission of NDVs Genotype VII to chickens. The identity between the 6 isolated NDVs and LaSota NDV strain (belong to genotype II) was quite low and ranged from 84.0 to 85.8%, that may result in severe economic losses (Sattar et al. 2016; Sultan et al. 2020). The results of the genetic analysis of the isolates revealed that they are highly virulent Newcastle viruses, while the vaccine regime for isolate birds' origin were either not vaccinated or vaccinated with live vaccine only (Table 2). Hence, the vaccines are very important in combating the Newcastle virus to get best result live and inactivated genotype-matched vaccine should be used.

Conclusion

Moreover, need to update the vaccinal seed strain. Depending on the partial F gene sequence and pathotyping we stated the prevalence of NDVs genotype VII1.1 among different avian species with high possibility of virus exchange between different avian species. Continuous molecular surveillance is recommended for early detection of any changes in the circulating viruses. Using Lentogenic vaccines like LaSota NDV vaccine may results in severe losses due to the genetic divergence with the currently circulating viruses, we thought the use of genotype VII1.1 vaccinal seed strain will be more immunogenic and protective against the field viruses.

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Author contribution

ML: Conceptualization, Data curation, writing review and editing. LT: Conceptualization, Data curation, Formal analysis and resources. MM: Formal analysis, Methodology, Software, Visualization, Writing - original draft. ME: Data curation, formal analysis and Methodology. MH, AE: Data curation, Methodology, Resources. MEM: Conceptualization, Data curation, Methodology, analysis, resources, funding and editing.

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