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Original Research Article

PREVALENCE OF NDV-VII.1.1, LPAI-H9N2 AND HPAI-H5N8 IN CHICKENS IN 2 EGYPTIAN GOVERNORATES DURING LATE 2020

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Abstract: Newcastle disease virus (NDV) and Avian Influenza (AIV) incriminated in many respiratory disease problems and egg production decline affecting both broilers and laying hens worldwide**.** Isolation and identification of NDV, AIV were carried out on 50 diseased chicken (43 broiler & 7 layer) flocks collected from Damietta and Dakahlia Governorates, Egypt. Thirty eight out of 50 examined flocks (76%) showed respiratory signs [37 broiler (74%) and 1-layer (2%) flocks]. The other 12 examined flocks exhibited general signs of illness with greenish diarrhea. Samples from the examined flocks were inoculated into ECE via allantoic sac route and the collected allantoic fluids were tested for hemagglutination activity (HA) which revealed that 21 out of 50 flocks were HA positive (42%); 19 broiler (90.47%) and 2-layer (9.52%) flocks. The twenty-one HA positive samples were subsequently subjected to real-time reverse-transcriptase polymerase-chain-reaction (rRT-PCR). Fifteen samples were positive for Velogenic NDV (71.42%); 3 samples were positive for low pathogenic avian influenza (LPAI) subtype H9 (14.28%); one samples was positive for highly pathogenic avian influenza (HPAI) subtype H5 (4.76%) and 2 samples showed mixed infection for both NDV and H9N2 (9.52%). The Phylogenetic analysis identified the Velogenic NDV genotype VII.1.1 with the amino acid sequence of the three isolates fusion protein cleavage site carries motif ^{112}RRQKRF^{117}. The partial HA gene sequence classified the one selected LPAI-H9N2 isolate as a member of G1 lineage within group B with dibasic amino acid cleavage site motif of ^{333}PARSSR/GLF^{341} which is distinctive to LPAI. While the other selected isolates of HPAI-H5 belonged to sub type H5N8 clade 2.3.4.4b with multibasic amino acid cleavage site motif of ^{73}PLREKRRKR/GLF^{84} which is characteristic to HPAI. Our data highlighted the vital role of these viruses in the respiratory disease in poultry flocks. Further continuous studies are necessary for assessing new emerging strains to AIV and NDV and to discuss the impact of the co-infections of these viruses in order to learn more details for their prevention and control.

Key words: Newcastle disease virus, NDV-VII.1.1, Avian Influenza virus, HPAI-H5N8, LPAI-H9N2; chicken flocks

Introduction

Mortality rates and respiratory signs in Egyptian commercial poultry flocks have a continuous drawback on poultry industry. A wide range of viruses, including Newcastle disease virus (NDV), Avian Influenza virus (AIV), infectious bronchitis virus (IBV) and laryngeotracheitis virus (ILT) causing respiratory infections in Egyptian poultry farms (1).

The Newcastle disease (ND) and avian influenza (AI) are the two principal viral infections

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that cause significant losses in the poultry industry due to respiratory ailments, high mortalities and decreased egg production (2). *Avian Orthoavulavirus*-1 (AOAV-1) or Newcastle disease virus (NDV) strains are classified into two classes based on the sequencing of the (F) gene into (class I & II). Class II includes all virulent NDV strains recovered from domestic birds which are grouped into at least 21 genotypes, ranged from I to XXI (3, 4). In Egypt, the majority of Newcastle outbreaks were still caused by genotype VII.1.1 in chicken flocks (5).

Avian influenza is a highly contagious respiratory disease that affects millions of birds each year, incurring enormous losses in the poultry industry and putting human health at risk. In Egypt, low pathogenic influenza virus (LPAI) subtypes H9N2 and highly pathogenic avian influenza (HPAI) virus subtypes (H5N1, H5N8 and H5N2) have been found (6).

The low pathogenic avian Influenza H9N2 virus is found all across the world, especially in Asia, Europe, and Africa, as well as the Middle East (7). Many outbreaks, especially those including additional viruses, results in significant economic losses due to H9N2 infection (8). The infection with H9N2 causes respiratory symptoms and a decrease in egg production in layer and breeder chickens (9). The infected broiler flocks, on the other hand, appear to be in good health, with no clinical indications (10). According to the hemagglutinin sequence, the Egyptian H9N2 virus belongs to the G1-like lineage group B (11, 12). The Egyptian H9N2 strain was discovered to have a genetic composition, implying that the virus is capable of acquiring basic amino acids in the HA connecting the peptide sequence required for increased pathogenicity (13).

HPAI viruses of the H5N8 subtypes were originated in China and presumably in Asia and Europe passed through several stages of transmission. They were first founded in Egyptian birds in 2016 and the number of cases jumped from 23% in 2017 to 66.6 percent in 2018 (14, 15). At least four different H5N8 HPAI Group B clade 2.3.4.4 reassortant viruses were imported into Egypt and isolated from poultry (15). A novel re-assortment virus of subtype H5N8 clade 2.3.4.4 was discovered in Russia and quickly spread throughout Europe, Asia, and the Middle East (16, 17). The transmission of the HPAIV H5N8 strain has been connected to the overlapping flyways of migrating wild birds from multiple continents because Egypt is one of the most important migration destinations for migratory birds passing via Europe, Asia, and Africa (18).

The goal of this study is to assess the prevalence of viral infections caused by dual infection with AIV subtypes (H5N8- H9N2) and Velogenic NDV genotype VII.1.1 in Egyptian broiler and layer chickens flocks in the Domiatte and Dakahlia governorates in order to update the current information on the status of these viruses to provide a more scientific approach for enhancing disease management strategies.

Material and methods

Samples collection

During the months of November and December 2020, 250 tissue samples (lung, trachea, liver, spleen, and brain) were gathered from fifty flocks. Forty three out of the 50 examined flocks were broiler of different breeds [Arbor Acres (n: 19); Ross (n: 9) and Cobb (n: 7); and Sasso native breed (n: 8)] with different ages ranged from (24-42 days). Additionally, 7-layer flocks were examined (4 Hyline Brown and 3 Hisex) aged 200-300 days from different localities in Damietta and Dakahlia Governorates as shown in (Table 1). All these flocks had a history of respiratory signs and mortality rates ranged from 2-45% in broiler and 2- 15% in layer. The flocks were vaccinated with La Sota Live attenuated vaccine of NDV and inactivated vaccines of AI virus in 14 broiler flocks & 3 layer flocks) while 8 broiler flocks with inactivated AI vaccines only. All samples were brought to the lab on ice and kept at 80°C until they were examined.

Viral isolation and hemagglutination (HA) activity

Commercial embryonated chicken eggs (ECE) obtained from E-Mesalmia commercial hatchery in Sharkia Governorate, Egypt was used to isolate the suspected viruses. A pool of (trachea, lung, liver, spleen and brain) from each sample was prepared according to OIE guidelines (19), with 0.2 ml per egg, supernatant fluid was injected via the allantoic sac into 9-day-old ECE. The implanted eggs were kept at 37°C for 7 days and candled daily. Using 10 %c leaned chicken red blood cells, the allantoic fluid was collected and evaluated for haemagglutination (HA) activity (19).

Table 1: Incidence of avian influenza using RT-PCR subtypes H5, H9 genes and Newcastle F gene in Damietta and Dakahlia province

Locality	Flock type	breed	No of flocks	Flocks with respiratory manifestation	%	RT-PCR positive	%	Flock Number*	Results of RT- PCR positivity rate (H5, H9 and F) genes		
									H5	H9	F
Damietta	Broiler	Arbor Acres	3	3/3	100	1/3	33.33	13	1/1		
		Cobb	2	2/2	100	1/2	50	43			1/1
	Layer	Hyline Brown	2	0/2	0	0	0				
		Hisex	1	0/1	0	0	0				
Faraskour	Broiler	Sasso native breed	1	0/1	0	0	0				
		Cobb	3	2/3	66.66	1/3	33.33	46			1/1
		Arbor Acres	1	1/1	100	0	0				
		Ross	2	2/2	100	2/2	100	41,45		1/2 (41)*	1/2(45)*
	Layer	Hisex	2	1/2	50	2/2	100	44,49		1/2 (44)*	2/2(44,49)*
Karm and Razok	Broiler	Arbor Acres	4	4/4	100	3/4	75	8, 22,48		2/3(8, 22)*	2/3(22,48)*
		Ross	1	1/1	100	1/1	100	50			1/1
EL-Zarka	Broiler	Sasso native breed	2	0/2	0	0	0				
EL-Roda	Broiler	Ross	1	1/1	100	0	0				
Meet Abo Ghalb	Broiler	Arbor Acres	2	0/2	0	1/2	50	2			1/1
Kafr Sad	Broiler	cobb	2	2/2	100	0	0				
		Arbor Acres	1	1/1	100	0	0				
		Ross	1	1/1	100	0	0				
	Layer	Hyline Brown	2	0/2	0	0	0				
Kafr-EL-ghab	Broiler	Ross	1	0/1	0	0	0				
		Arbor Acres	1	1/1	100	0	0				
El-Manzla	Broiler	Sasso native breed	4	4/4	100	2/4	50	1, 47			2/2
		Arbor Acres	3	3/3	100	2/3	66.66	19,28			2/2
		Ross	2	2/2	100	1/2	50	29			1/1
Gamssa	Broiler	Arbor Acres	2	2/2	100	2/2	100	23,31			2/2
		Sasso native breed	1	1/1	100	0	0				
		Ross	1	1/1	100	1/1	100	42			1/1
Sherbeen	Broiler	Arbor Acres	2	2/2	100	1/2	50	24		1/1	
	43 broiler 7 layer		50	38/50 (37 broiler) (1 layer)	76	19/43 (44.18%) 2/7 (28.57%)		21	1	5(3 H9 2(H9+ND)	17 (15 ND 2 mixed ND+H9)

*mean the flock number

To ensure that the HA negative samples were negative, they were passaged at least three times in embryonated chicken eggs. The HA-positive allantoic fluids were then analyzed for NDV and AIV subtypes H5 and H9 using rRT-PCR.

RNA extraction and real time (rRT-PCR) examination

Following the manufacturer's instructions, the viral RNA was extracted from 21 HA-positive allantoic fluid samples the QIAamp Viral RNA Mini Kit (QIAGEN, Germany, catalogue No.52904). Finally, the RNA-containing elution buffer was kept at -80°C until use in rRT-PCR. Primers and probes for HPAI-H5 gene (20), LPAI- H9 gene (21) and NDV fusion gene (F) (22) were supplied by Metabion (Germany) as shown in (Table 2). Quanti Tect Master Mix (Qiagen, Germany), was used according to the manufacturer's instructions.

Sequence and Phylogenetic analysis

Five positive samples NDV (n: 3), H9 (n: 1), and H5 ((n: 1) were submitted for partial gene sequence. Their extracted RNA was transcribed to CDNA by Revert Aid H Minus First Strand CDNA Synthesis Kit Fermentas Inc., Walthan, MA, USA, according to manufacture instructions (Table 3).

Table 2: Oligonucleotide Primer and probes used for detection of AIV H gene and NDV F gene

Virus	Gene	Primer/ probe sequence 5'-3'	Amplified Segment (bp)
H5	H	H5LH1 ACATATGACTAC CCACARTATTCA G	151
		H5RH1 AGACCAGCT AYC ATGATTGC	
		H5PRO [FAM]TCWACA GTGGCGAGT TCCCTAGCA[TAMRA]	
H9		H9F GGAAGAATTAATTATTATTGGTCGGTAC	182
		H9R GCCACCTTTTTCAGTCTGACATT	
		H9 Probe [FAM]AACCAGGCCAGACATTGCGAGTAAGATCC[TAMRA]	
Velogenic and mesogenic	F	F+4839 TCCGGAGGATACAAGGGTCT	121
		F-4939 AGCTGTTGCAACCCCAAG	
		F+4894 [FAM]AAGCGTTTCTGTCTCCTTCCTCCA[TAMRA]	

Table 3: Primers used in Reverse Transcriptase-Polymerase Chain Reaction (onestep RT-PCR) and Sequance reaction of HA of H5N8, H9N2 and F gene of NDV

Prime ID	Primer Sequence for HA gene amplification and F gene
F1-6	5' TAG CAA AAG CAG GGG AAT TTC TT 3'
H9- Rev	5' GCC ACC TTT TTC AGT CTG ACA TT 3'
H9-For	5'GGA AGA ATT AAT TAT TAT TGG TCG GTA C 3'
HT7R	5' TAA TAC GAC TCA CTA TAA GTA CAA ACA AGG GTG 3'
HGGT+ -H5	CTC TTC GAG CAA AAG CAG GGG T
KH3 -H5	TAC CAA CCG TCT ACC ATK CCYTG
NDV-F330	AGG AAG GAG ACA AAA ACG TTT TAT AGG
NDV-R700	TCA GCT GAG TTA ATG CAG GGG AGG

A purified RT-PCR product of the 5 isolates were sequenced using an applied Biosystem 3130 genetic analyzer (HITACHI,Japan)by using Bigdye TerminatorV3.1 cycle sequencing Kits (Perkin-Elmer, Foster city, USA catalogue Number 4336817 that use for performing gene sequencing using primers (Table 3) (23) ABLAST^{R} analysis (Basic local Allignment search tool) (http:// www .ncbi .nim.nih.gov/BLAST) was performed to establish the sequence identity and Gene Bank accessions. A comparative analysis of sequences was performed (24). The Phylogenetic tree was done by neighbor-Joining method in MEGA version 7 (http://www.megasoftware.net). The tree topology was evaluated by 1,000 bootstrap analyses.

Results

Thirty eight out of 50 examined flocks (76%) showed respiratory manifestation. Out of them, 37 broiler flocks (74%) and 1 layer flock (2%). Out of them, 37 broiler flocks (74%) and one layer flock (2%). The other 12 examined flocks showed general signs of illness with greenish diarrhea.

Clinical manifestation of the examined broiler flocks ranged from very mild disease to high morbidity and mortality which ranged from (5-45%). Huddling, despair, ruffled feathers, decreased feed and water consumption, weight loss are all common symptoms and edema of face and head

in most flocks, greenish diarrhea was also recorded with mild to severe respiratory signs in form of sneezing, coughing, tracheal rales, difficult breathing and occulonasal discharge were recorded. Greenish diarrhea was also recorded. While in layer flocks cyanosis in comb &wattles, respiratory symptoms, greenish watery diarrhoea, shell-less eggs, and soft eggs were described, along with a decline in egg production of 15-20% and mortality rates ranging from (2-15%).Post mortem lesions in broiler flocks were sinusitis, mild tracheitis, pneumonia, air sacculitis pancreatitis, petechial haemorrhages on proventriculus and in some cases there were elliptical raised ulcers in intestine as well as pericarditis and perihepatitis While in layer flocks cyanosis in comb and wattles, respiratory symptoms, greenish watery diarrhea, shell-less eggs, and soft eggs were described, along with a decline in egg production of 15-20% and mortality rates ranging from (2-15%). Post-mortem lesions of congested trachea, pneumonia, and air sacculitis, petechial haemorrhages in the proventriculus and pectoral muscle, hemorrhagic enteritis, egg peritonitis, and ovarian follicle haemorrhages were all observed in layer flocks.

Virus isolation, HA and Molecular identification

Following HA test, 21 HA positive allantoic fluids out of fifty samples (42%) [nineteen (90.47%) broiler and two (9.52%) layer flocks] after first passage in ECE were recorded as shown in (Table 4). The positive HA allantoic fluids were subjected to rRT-PCR which revealed 14 positive NDV (73.68%); 3 positive LPAI-H9 (15.78%); one positive HPAI-H5 (5.26%) and, one sample showed mixed infection with NDV and LPAI-H9 (5.26%) in the examined samples of broiler flocks. While, 2-layer flock samples were positive [1 for NDV (50%) and 1 (50%) had mixed infection of NDV and LPAI-H9 as shown in (Table 5).

Sequencing and phylogenetic analysis

The established partial sequence's identity was submitted to the GenBank with accession numbers of MZ668300, MZ668301 and MZ668302 for NDV-VII.1.1, MZ314936 for LPAI-H9N2 and MZ314932 for HPAI-H5N8. The isolated viruses correspond to genotype VII.1.1, according to phylogenetic analysis of partial sequences from three NDV isolates named (AOAV-1/Chicken/Egypt/31/2020, AOAV-1/Chicken /Egypt/42/2020, and AOAV-1/ Chicken /Egypt/45/2020) as in (Fig. 1). The sequence alignment of the acquired isolates with the NDV-Chicken-China-SDWF07-2011) reference strain from the GenBank demonstrated that the obtained 3 isolates amino acid sequence are showing (99.1-99.8%) identity with genotype VII as in (Fig. 2). The amino acid sequence of fusion protein cleavage site of the isolates carries motif ^{112}RRQKRF^{117} that is consistent with velogenic NDV viruses.

The Phylogenetic analysis of partial sequences of HA gene to the one selected isolate of AIV subtype H9N2 was found to show that the isolated viruses belongs to G1 lineage within group B (Fig. 3).The obtained sequences alignment with A-Quail-Hong-Kong-G1-97 reference strain from the gene bank revealed that the obtained isolate A.A sequence identity was 94.9% (Fig. 4) .The di-basic amino acid motif at cleavage site of the isolate carries motif ^{333}PARSSR/GLF^{341} which is characteristic to LPAI.

While the other selected isolate of AIV subtype was H5N8 strain for HA gene it belongs to clade 2.3.4.4b (Fig.5). The obtained sequences alignment with A-tuffed-duck-Germany-AR84 59-L01988-201 reference strain from the gene bank revealed that the obtained isolate A.A sequence are 97.8% identity with H5N8 clade 2.3.4.4b (Fig. 6). The multibasic amino acid motif at cleavage site of the isolate carries motif PLREKRRKR/GLF which is characteristic to HPAI .

Table 4: History and rRT-PCR results of NDV-VII.1.1, LPAI-H9N2 and HPAI-H5N8 in examined flocks in Damiette and Dakahlia governorates

Gover-norate	Locality	Chicken type	Breed	Age (day)	Mortality % (last 7 days before sampling	Vaccination				rRT-PCR results		
						NDV		LPAI-H9N2	HPAI-H5	NDV	LPAI-H9N2	HPAI-H5
						Live	Killed					
Damietta	Damietta	Arbor Acres	Broiler	24	45	+	+	-	+	-	-	+
	Damietta	Cobb	Broiler	30	20	+	-	-	-	+	-	-
	Farskour	Ross	Broiler	30	2	+	+	-	+	-	+	-
	Farskour	Ross	Broiler	35	10	+	+	-	+	+	-	-
Damietta	Farskour	cobb	Broiler	25	15	+	+	-	-	+	-	-
Damietta	Farskour	Hisex	Layer	250	2	-	-	-	-	+	-	-
Damietta	Farskour	Hisex	Layer	250	15	-	-	-	-	+	+	-
Damietta	Karm &Razok	Arbor Acres	Broiler	20	5	+	+	-	+	-	+	-
Damietta	Karm &Razok	Arbor Acres	Broiler	31	15	+	+	-	+	+	-	-
Damietta	Karm &Razok	Arbor Acres	Broiler	25	20	+	+	-	+	+	+	-
Damietta	Karm &Razok	Ross	Broiler	25	17	+	+	-	+	+	-	-
Damietta	Meet-Abo-Ghalb	Arbor Acres	Broiler	35	35	+	+	-	+	+	-	-
Dakahlia	Sherbeen	Arbor Acres	Broiler	30	7	+	+	-	-	-	+	-
Dakahlia	EL-Man-zala	Sasso native breed	Broiler	42	25	+	+	-	+	+	-	-
Dakahlia	EL-Man-zala	Sasso native breed	Broiler	45	15	+	+	-	+	+	-	-
Dakahlia	EL-Man-zala	Arbor Acres	Broiler	25	9	+	+	-	+	+	-	-
Dakahlia	EL-Man-zala	Arbor Acres	Broiler	35	10	+	+	-	+	+	-	-
Dakahlia	EL-Man-zala	Ross	Broiler	35	25	+	+	-	-	+	-	-
Dakahlia	Gamasa	Arbor Acres	Broiler	25	10	+	+	-	+	+	-	-
Dakahlia	Gamasa	Arbor Acres	Broiler	32	15	+	+	-	-	+	-	-
Dakahlia	Gamasa	Ross	Broiler	31	20	+	-	-	-	+	-	-

+ mean positive presence +ve vaccinated
- mean negative presence -ve (not vaccinated)

Table 5: Prevalence of NDV-VII.1.1 , LPAI-H9N2 and HPAI-H5N8 in examined chicken flocks

Types of flocks	Number of flocks	Result of RT-PCR positive rates (H5, H9 and F gene) primers set			
		H5	H9	NDV-VII.1.1	Mixed H9 and F gene
		Rate			
Broiler	19	1/19	3/19	14/19	1/19
Layer	2	0/2	0/2	1/2	1/2
Total	21	1/21	3/21	15/21	2/21

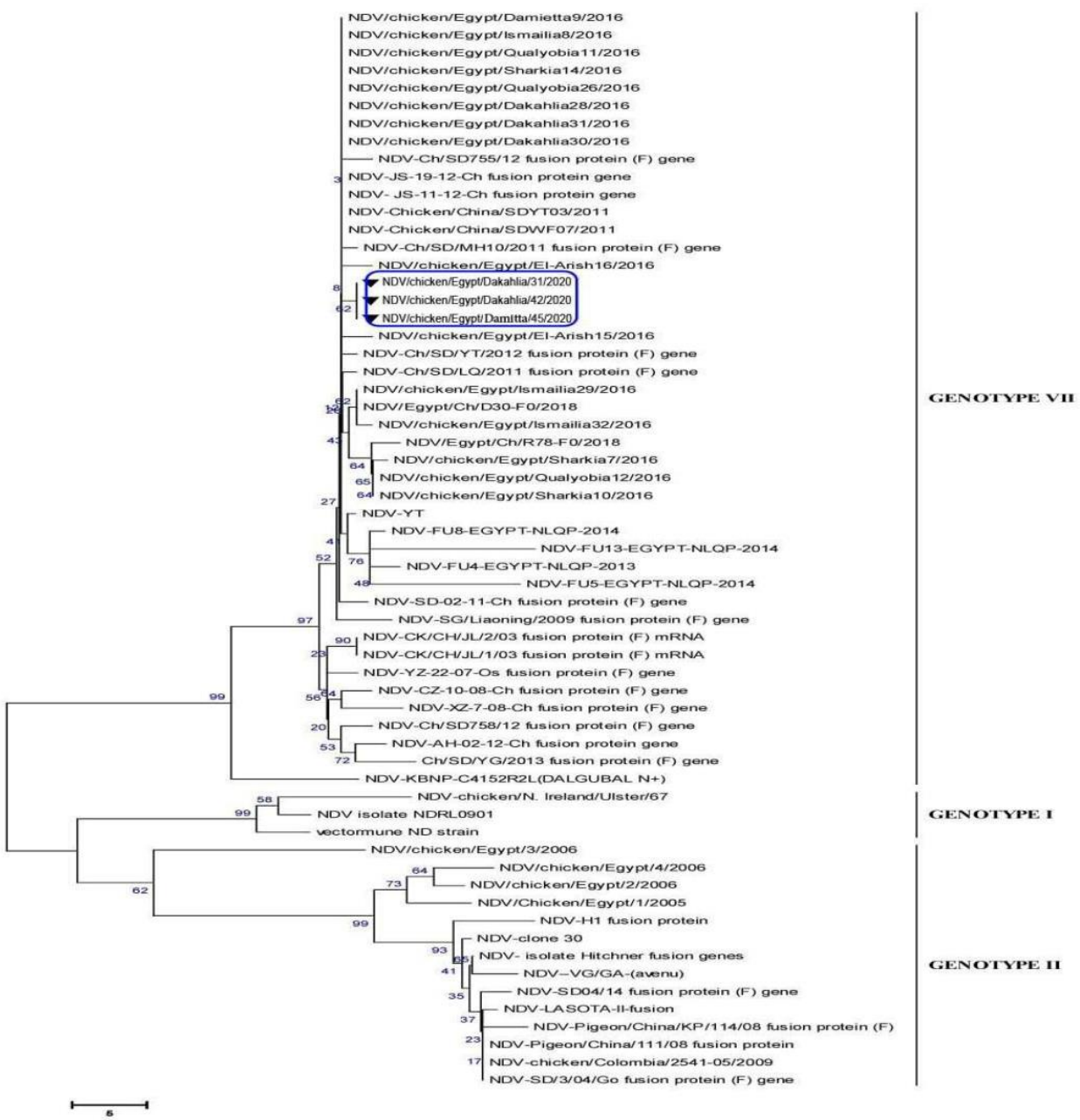

Figure 1: Phylogenetic tree of selected three isolates of NDV genotype VII.1.1 indicated by black triangle and compared with other related reference strains of NDV

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
1		87.9	88.2	88.6	88.7	88.1	96.7	90.8	89.5	98.7	98.9	97.3	88.2	88.6	99.8	91.0	99.1	99.5	99.8	1
2	13.2		98.7	98.7	98.7	98.2	88.4	92.2	92.0	87.0	87.3	86.1	97.1	96.4	88.1	91.9	87.5	87.5	87.9	2
3	12.8	1.3		99.6	99.6	99.1	88.8	93.1	92.9	87.3	87.7	86.4	98.0	97.3	88.4	92.8	87.9	87.9	88.2	3
4	12.4	1.3	0.4		99.6	99.5	89.2	93.1	92.9	87.7	88.1	86.8	98.0	97.3	88.8	92.8	88.2	88.2	88.6	4
5	12.3	1.3	0.4	0.4		99.1	89.3	93.3	93.1	88.0	88.1	86.9	98.1	97.4	88.9	93.0	88.3	88.3	88.7	5
6	13.0	1.8	0.9	0.5	0.9		88.6	92.6	92.4	87.2	87.5	86.3	97.5	96.7	88.2	92.2	87.7	87.7	88.1	6
7	3.3	12.6	12.2	11.7	11.6	12.4		91.7	90.6	95.8	96.4	94.8	87.7	88.1	96.6	91.3	96.2	96.2	96.6	7
8	9.9	8.2	7.2	7.2	7.0	7.8	8.8		97.5	89.7	90.2	89.0	92.0	92.4	90.6	98.0	90.6	90.2	90.6	8
9	11.3	8.4	7.4	7.4	7.2	8.0	10.1	2.6		88.4	89.0	87.7	91.9	92.2	89.3	97.3	89.0	89.0	89.3	9
10	1.3	14.3	13.9	13.5	13.2	14.1	4.3	11.1	12.6		99.1	96.7	87.5	87.9	98.6	89.9	97.8	98.2	98.6	10
11	1.1	13.9	13.5	13.0	12.9	13.7	3.7	10.5	12.0	0.9		97.3	87.7	88.1	98.7	90.4	98.4	98.4	98.7	11
12	2.8	15.4	15.0	14.6	14.5	15.2	5.4	12.0	13.5	3.3	2.8		86.4	86.8	97.1	89.2	96.7	96.7	97.1	12
13	12.8	3.0	2.0	2.0	1.9	2.6	13.5	8.4	8.6	13.7	13.5	15.0		98.9	88.4	91.7	87.9	87.9	88.2	13
14	12.4	3.7	2.8	2.8	2.6	3.3	13.0	8.0	8.2	13.2	13.0	14.6	1.1		88.8	92.0	88.2	88.2	88.6	14
15	0.2	13.0	12.6	12.2	12.1	12.8	3.5	10.1	11.5	1.5	1.3	3.0	12.6	12.2		90.8	98.9	99.3	99.6	15
16	9.7	8.6	7.6	7.6	7.4	8.2	9.2	2.0	2.8	10.9	10.3	11.7	8.8	8.4	9.9		90.8	90.4	90.8	16
17	0.9	13.7	13.2	12.8	12.7	13.5	3.9	10.1	12.0	2.2	1.6	3.3	13.2	12.8	1.1	9.9		98.9	99.3	17
18	0.5	13.7	13.2	12.8	12.7	13.5	3.9	10.5	12.0	1.8	1.6	3.3	13.2	12.8	0.7	10.3	1.1		99.6	18
19	0.2	13.2	12.8	12.4	12.3	13.0	3.5	10.1	11.5	1.5	1.3	3.0	12.8	12.4	0.4	9.9	0.7	0.4		19
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	

NDV-Chicken-China-SDWF07-2011
NDV-H1-fusion-protein
NDV-LASOTA-II-fusion
NDV--isolate-Hitchner-fusion-genes
NDV-clone-30
NDV--VG-GA-(avenu)
NDV-KBNP-C4152R2L(DALGUBAL-N+)
vectormune-ND-strain
NDV-chicken-N.-Ireland-Ulster-67
NDV-FU4-EGYPT-NLQP-2013
NDV-FU8-EGYPT-NLQP-2014
NDV-FU13-EGYPT-NLQP-2014
NDV-chicken-Egypt-4-2006-
NDV-chicken-Egypt-2-2006-
NDV-Egypt-Ch-D30-F0-2018-
NDV-isolate-NDRL0901-
NDV-chicken-Egypt-Dakahlia-31-2020
NDV-chicken-Egypt-Dakahlia-42-2020
NDV-chicken-Egypt- Damitta -45-2020

Figure 2: Amino acid sequence identity showing identity percent based on A.A sequence of NDV genotype VII.1.1 comparison of 121 bp of fusion gene, black squares indicate identical sequences

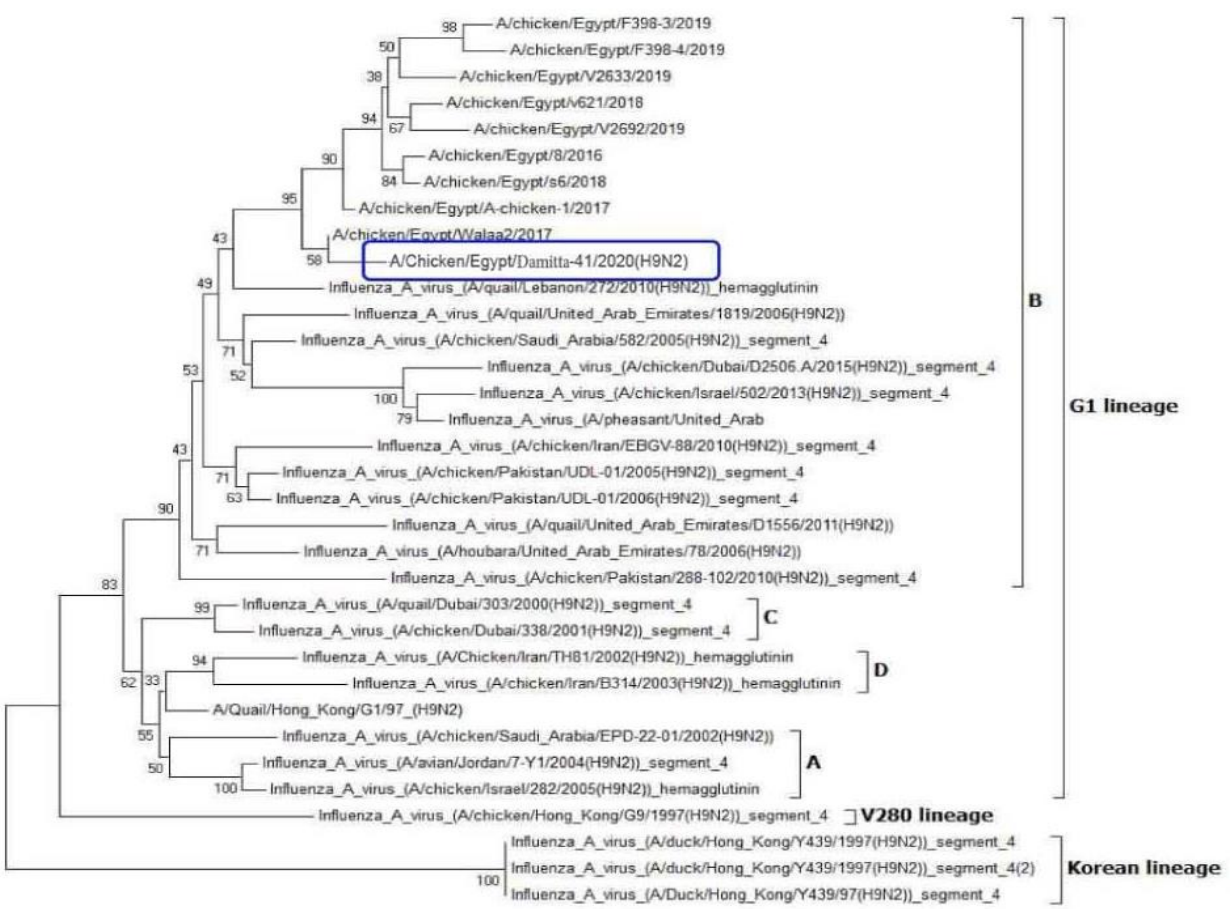

Figure 3: Phylogenetic tree of one selected H9N2 virus of G1 lineage group B isolated during 2020 and compared with other Egyptian, Middle East (Israeil) and Asian (Hong Kong) isolates

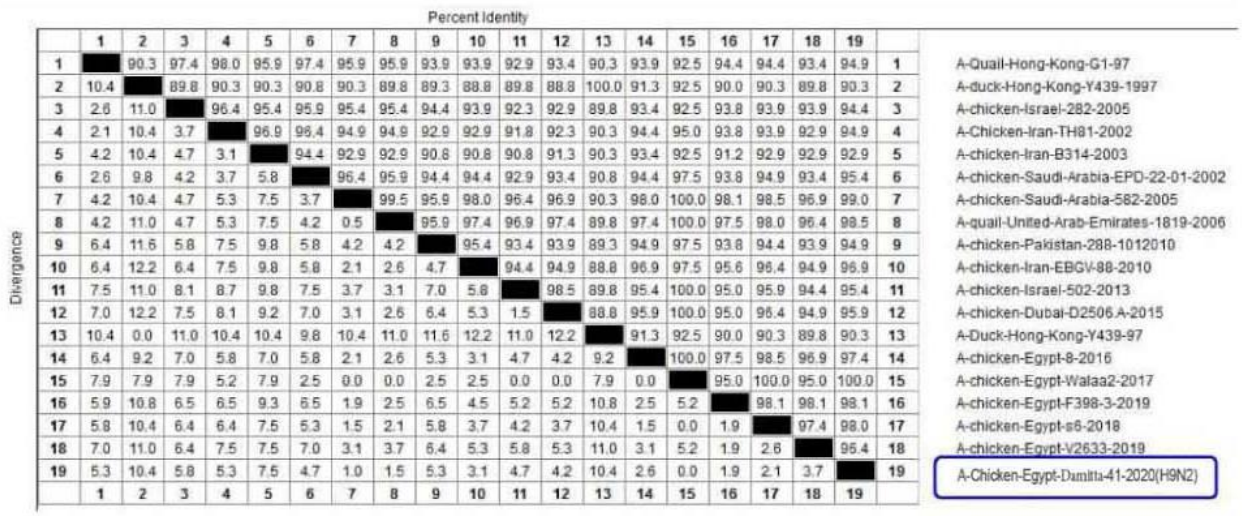

Figure 4: Amino acid sequence identity showing identity percent based on A.A sequence comparison of 182 bp of HA gene of H9N2, black squares indicate identical sequences

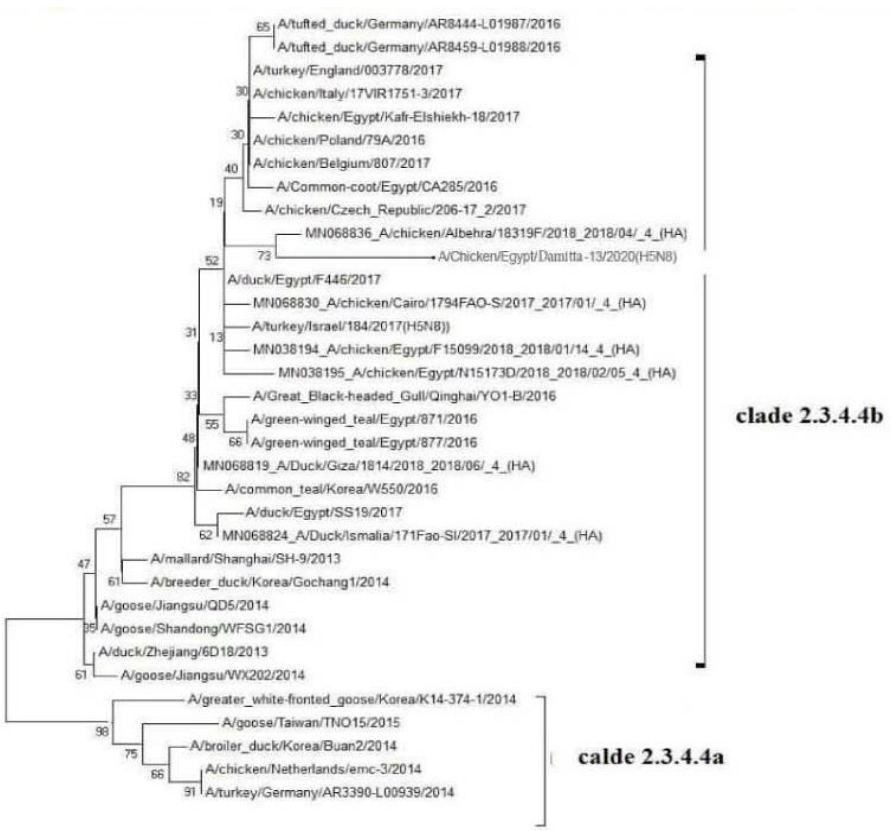

Figure 5: Phylogenetic tree of amino acid sequence of HA gene of one field HPAI-H5N8 clade 2.3.4.4b isolate with other reference strains in gene bank

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
1		100.0	96.8	98.9	97.8	96.8	95.7	96.8	100.0	100.0	100.0	98.9	98.9	100.0	97.8	1
2	0.0		96.8	98.9	97.8	96.8	95.7	96.8	100.0	100.0	100.0	98.9	98.9	100.0	97.8	2
3	3.3	3.3		97.8	96.8	100.0	98.9	100.0	96.8	96.8	96.8	97.8	97.8	96.8	97.8	3
4	1.1	1.1	2.2		98.9	97.8	96.8	97.8	98.9	98.9	98.9	100.0	97.8	98.9	97.8	4
5	1.1	1.1	2.2	0.0		96.8	95.7	96.8	97.8	97.8	97.8	98.9	96.8	97.8	96.8	5
6	3.3	3.3	0.0	2.2	2.2		98.9	100.0	96.8	96.8	96.8	97.8	97.8	96.8	97.8	6
7	4.4	4.4	1.1	3.3	3.3	1.1		98.9	95.7	95.7	95.7	96.8	96.8	95.7	96.8	7
8	3.3	3.3	0.0	2.2	2.2	0.0	1.1		96.8	96.8	96.8	97.8	97.8	96.8	97.8	8
9	0.0	0.0	3.3	1.1	1.1	3.3	4.4	3.3		100.0	100.0	98.9	98.9	100.0	97.8	9
10	0.0	0.0	3.3	1.1	1.1	3.3	4.4	3.3	0.0		100.0	98.9	98.9	100.0	97.8	10
11	0.0	0.0	3.3	1.1	1.1	3.3	4.4	3.3	0.0	0.0		98.9	98.9	100.0	97.8	11
12	1.1	1.1	2.2	0.0	0.0	2.2	3.3	2.2	1.1	1.1	1.1		97.8	98.9	97.8	12
13	1.1	1.1	2.2	2.2	2.2	2.2	3.3	2.2	1.1	1.1	1.1	2.2		98.9	98.9	13
14	0.0	0.0	3.3	1.1	1.1	3.3	4.4	3.3	0.0	0.0	0.0	1.1	1.1		97.8	14
15	2.2	2.2	2.2	2.2	2.2	2.2	3.3	2.2	2.2	2.2	2.2	2.2	1.1	2.2		15
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	

A-tufted-duck-Germany-AR8459-L01988-201
A-chicken-Poland-79A-2016
A-broiler-duck-Korea-Buan2-2014
A-goose-Jiangsu-QD5-2014
A-goose-Shandong-WFSG1-2014
A-chicken-Netherlands-emc-3-2014
A-goose-Taiwan-TNO15-2015
A-turkey-Germany-AR3390-L00939-2014
A-chicken-Czech-Republic-206-17-2-2017
A-Common-coot-Egypt-CA285-2016
A-duck-Egypt-F446-2017
A-Duck-Ismalia-Fao-SI-2017
A-chicken-Albehra-18319F-2018
A-chicken-Cairo-FAO-S-2017
A-Chicken-Egypt-Damitta-13-2020(H5N8)

Figure 6: Amino acid sequence identity showing identity percent based on A.A sequence comparison of 151 bp of HA gene of H5N8, black squares indicate identical sequences

Discussion

Since the advent of vNDV-VII.1.1 and LPAI- H9N2 in 2011, and more recently HPAI-H5N8 in late 2016, avian respiratory viruses in Egypt have undergone significant alterations (25, 26). In 2018, the most common viral infections that result in significant losses due to respiratory affections and drop in egg production to the poultry industry were Avian Influenza and NDV. These viruses have a significant impact since they can cause disease both on their own and in combination with other viral agents (9, 27, 28).

The goal of this study was to track the occurrence of various respiratory viruses in chicken flocks in Damietta and Dakahlia governorates. The recorded clinical signs and post-mortem lesions in broiler and layer chickens matched with previous studies (29, 30). After viral inoculation in ECE for isolation, the recorded HA combined with embryonic mortalities and on 2-4 days post inoculation indicated the presence of haemagglutinating virus similar to results reported by (4).

The presence of NDV and AIV was confirmed using the rRT-PCR technique with a specific set of primers for F and HA genes, respectively. In previous reports the use of molecular approaches for nucleic acid detection has become a significant, fast, and low-cost tool for detecting ND and AIV viruses (31, 32)

Our results of isolation were agreed with previous study in which the NDV had the highest detection rate from respiratory viruses (30%) followed by LPAI-H9N2 (6%) then mixed infection (4%) and HPAI-H5N8 virus (2%) (26).

The phylogenetic analysis of partial sequences from three NDV isolates designated (AOAV-1/Ch/Egypt/31/2020, AOAV-1/Ch/Egypt/42/2020, and AOAV-1/Ch/Egypt/45/2020) revealed that the isolates belong to genotype VII.1.1. The sequences obtained were compared to the NDV-Chicken-China-SDWF 07-2011 reference strain (retrieved from the GenBank), the a.a. sequences of all the three isolates showed 99.1-99.8% identity with genotype VII.1.1 and the F protein cleavage site bears motif ^{112}RRQKRF^{117}, which is consistent with viruses of velogenic strains. NDV of genotype VII were also identified to be mostly responsible for ND outbreaks in Egypt since 2012 (33, 34, 35).

Despite rigorous immunization programs, NDV was found in 14 of 21 commercial broiler flocks and one flock of laying hens. This high prevalence of vNDV-VII.1.1 infection in commercial broiler flocks could be linked to the short life duration of broiler chickens, which prevents inactivated or traditional live vaccinations from providing full protective immunity (36). Furthermore, biosecurity, mixed infections and/or the difference between circulating and vaccinating strains may be considered as evidence for these problems (37). Also, this could be owing to the recent genetic diversity among NDV strains (4) leading in breakouts in the field or the emergence of a novel pathotypes causing

severe infection (37). As a result, periodical isolation and pathotyping of NDV from poultry epidemics is crucial for NDV control and vaccination evaluation (5).

The obtained NDV-VII.1.1 strain with the cleavage site motif ^{112}RRQKRF^{117} belonged to the same genotype causing severe outbreaks in China (36) and Middle East (37)

Regarding the H9N2 selected isolate; it belonged to G1-like lineage within group B. The obtained HA sequence alignment was 94.9% identical to the LPAI-H9N2 (A-Quail-Hong-Kong-G1-97) reference strain (retrieved from the GenBank) and closely related to recent 2016-2018 Egyptian and the Middle East circulating H9N2 strains. Based on nucleotide and amino acid identities, the isolates are divergent by about 5% from the earlier isolates of 2011-2013. Recent studies showed that the Egyptian H9N2 viruses from different avian species showed several genetic markers that enhance virulence in poultry and transmission to humans and confirming that LPAI H9N2 viruses in Egypt are continuously evolving (38). The risk of reassortment between HPAI H5N1 and LPAI H9N2 circulating in Egypt was previously anticipated (39). Co-infection with other pathogens can aggravate H9N2 infections resulting in high mortality rates as two flocks were mixed infection of H9 and NDV as reported by (40) who revealed that the presence H9N2 in flock may reduce the immune response of birds, so the virus infection considered predisposing to other viral disease infection. The outbreaks of LPAI-H9N2 and of NDV genotype VII viruses usually cause severe economic losses associated with reduced performance and/or high mortalities in chicken flocks in Egypt (41).

For the phylogenetic analysis of partial HA sequence of the AIV subtype H5N8 strain (A/chicken/Egypt/13//2020), it belonged to clade 2.3.4.4b. The recovered sequence compared to the reference strain A-tuffed-duck-Germany-AR8459-L01988-201 (retrieved from the GenBank) demonstrated that the obtained isolate aa sequencing had 97.8% similarity with the H5N8 clade 2.3.4.4b with multibasic amino acid motif of PLREKRRKR/GLF at the cleavage site. Our findings were accepted (42-45).

Conclusion

The circulation of NDV-VII.1.1, LPAI-H9N2 and HPAI-H5N1 is still a major problem facing Egyptian commercial broiler and laying flocks and are continuously in genetic evolution. So, annual continuous surveillance is recommended to follow up the evolution of new subtypes of Egyptian respiratory viruses.

The authors declare that there is no conflict of interest in the current work.

Ahmed Mohamed EL-Sadek Hegazy provided guidance, technical support, conceived and designed Review. Amr Abd El-Fattah Bedair prepared figures and/or tables. Ehab M. Abdallah analyzed the data. Adel M. Abd Elaziz revised the paper and Hala MNTolba analysed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft. All authors read and approved the final manuscript.

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