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Original Research Article

Characterization of Avian Influenza H9N2 and Newcastle Disease Virus Isolated from Vaccinated Chickens in Upper Egypt

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ABSTRACT

In this study, 50 vaccinated broiler flocks and one layer flock from Beni Suef, Fayoum and Minia Governorates were investigated. Necropsy lesions were suggestive of LPAI-H9N2 or NDV. Samples including tracheal swabs and organs were subjected for viral isolation and molecular characterization. Specific RT-PCR for the F-gene of NDV and the HA gene of the LPAI-H9N2 viruses was used. Virus isolation and primary identification using HI test revealed 37.5 and 43.3-46.2% prevalence for LPAI-H9N2 and NDV viruses, respectively. Phylogenetic analysis of partial sequences of the F gene showed that NDV viruses belong to genotype II and VII-1.1. as indicated by the F0 protein proteolytic cleavage site motifs (aa112-117) of the NDV strains F-gene. The vNDV isolates were 98.7-99.3% and 96.6-98.9% identical to each other based on nucleotide and amino acid identities, respectively. Compared to their counterpart isolates; the lentogenic strains shared 98-99.2% and 96.3-98.1% nucleotide and amino acid identities to the LaSota reference strain. The LPAI-H9N2 phylogeny of the HA gene showed that the 2 isolates obtained in this study are related to each other and related to recent 2016-2018 Egyptian H9N2 strains. Notably, the 2 strains showed higher identity (≥99%) to recent Israeli 2018 isolates with several amino acid changes. The current study revealed widespread of both NDV and LPAI-H9N2 viruses. The vaccine failure and the mismatch between the vaccine and circulating NDV viruses is the most probable cause of current outbreaks. LPAI-H9N2 viruses are divergent from their ancestral viruses in Egypt indicating continuous circulation and vaccine pressure-induced mutations.

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Introduction

Poultry production constitutes one of the main sources of protein in Egypt. However, respiratory disease challenges have been rising in Egyptian commercial flocks during the last decade (Hassan et al., 2016). Considering the non-pathognomonic characteristics of respiratory diseases, they are frequently misdiagnosed (Peyre et al., 2009). Mixed and secondary infections may interfere with the diagnosis of the primary cause due to the absence of perceiving signs and lesions (Karimi-Madab et al., 2010; Samy and Naguib, 2018). Different pathogens such as avian influenza (AI), Newcastle disease (ND), and infectious bronchitis (IB) viruses are involved either in single or multiple respiratory infections (Nili and Asasi, 2002; Hassan et al., 2017; Setta et al., 2018).

Influenza A viruses are negative-sense, single-stranded RNA viruses of the family *Orthomyxoviridae* with 18 hemagglutinins (HA) and 11 neuraminidase (NA) subtypes. The low pathogenic avian influenza H9N2 (LPAI-H9N2) viruses first recorded in turkeys in Wisconsin in 1966 (Swayne and Brown, 2015) cause mild to severe respiratory signs, but they are mainly associated with a reduction in egg production in breeders and layers (Shehata et al., 2015). Serologically, the virus antibodies were found in poultry between 2009 to 2012 in Egypt (Afifi et al., 2012). However, virus isolation was confirmed in Egypt in December 2010 (El-Zoghby et al., 2012).

Most respiratory outbreaks associated with LPAI-H9N2 viruses are reported during winter months (Hassan et al., 2016), however, recent reports showed that the virus is circulating all over the year, especially in the Nile Delta (Nagy et al., 2017). The isolated viruses were genetically related to the viruses circulating in the Middle East region and to those isolated from the migratory birds. Moreover, the infected flocks were distributed along with the migratory birds' flyways suggesting their introduction via wild migratory birds (Abdelwhab and Abdel-Moneim, 2015).

Newcastle disease (ND) is recently classified according to The International Committee on Taxonomy of Viruses (ICTV) to be a member of family *Paramyxoviridae* , subfamily Avulavirinae, genus Orthoavulavirus, species Avian orthoavulavirus 1 (Dimitrov et al., 2019). NDV strains are separated into two clades, class I and class II, within a single serotype (Czeglédi et al., 2006). Class I viruses comprise a single avirulent genotype and class II viruses are further divided into 18 genotypes (Afonso et al., 2012; Miller, 2010). Class II strains include most virulent NDVs (Czeglédi et al., 2006), of which genotypes I to IV represent early sub-lineages (Ewies et al., 2017; Mohamed et al., 2011; Radwan et al., 2013). Though intensive NDV vaccination programs applied in Egypt, many outbreaks of NDV by genotype VII 1.1. have been recorded (Ewies et al., 2017; Mohamed et al., 2011; Radwan et al., 2013).

In this study, LPAI H9N2 and NDV viruses were from recent outbreaks in broiler chickens from Upper Egypt isolated and molecularly characterized (Fayoum, Beni Suef, and Minia Governorates).

Material and Methods

Field samples

Fifty vaccinated broiler-type and one layer-type flocks from Beni Suef (8 flocks), Fayoum (13 flocks), and Minia (30 flocks) Governorates were included in this study. Investigated flocks suffered from respiratory distress and nervous signs were reported in some flocks (Table 1). Post-mortem lesions were suggestive for either LPAI H9N2 or NDV. Collected samples included tracheal swabs, trachea and lungs, and cecal tonsils.

Table 1. History of the collected field samples.

Sample	Age (days)	Flock size	Production type	Postmortem lesions	Vaccination program	Sampling date	
AA005	27	3000	broiler	CRD, hemorrhage at cecal tonsils	Day7: IBV+ND HB1- Day17: ND LaSota	Dec-2015	Fayoum
AA012	20	6500	broiler	Bloody intestine- congested trachea and liver			Fayoum
AA006	28	7000	broiler	Hemorrhage at cecal tonsils			Fayoum
AA007	15	4000	layer	Hemorrhage at tips of the proventriculus	Day1: IB primer Day7: Killed ND	Mar-2016	Fayoum
AA010	26	2sa024 000	layer	Hemorrhages at tips of the proventriculus. Severe congestion in carcass	Day6: IBV+ND HB1- Day17: ND LaSota	May-2016	Fayoum
AA011	13	3500	broiler	CRD. Hemorrhagic intestine	Day1: IBV+ND HB1- Day7: Killed ND Day17: ND LaSota		Fayoum
AA009	7	5000	broiler	Liver congestion, minor hemorrhages at the caecum	Day6: IBV+ND HB1	Dec-2016	Fayoum
SA001	27	6000	broiler	CRD, Ascites, congested heart, liver, spleen	Day6= IBV+ND HB1- Day9: Killed H5+ND- Day11: IBD intermediate plus- Day17: ND LaSota- Day19: IBD intermediate plus	Jan-2017	Minia
SA002	27	5000	broiler	Congested trachea with mucous exudate	Day7: Killed H5- Day8: Killed ND vaccine- Day17: ND LaSota		Minia
AA008	9	1500	broiler	General Congestion	Day1: IBV+ND HB1	Feb-2017	Fayoum
SA006	25	9000	broiler	CRD, cecal coccidiosis, tracheitis	Day8: IB and ND Colone30- Day10: Killed H5+ND-Day12: IBD intermediate plus-Day16: ND Clone30	Mar-2017	Minia
SA007	31	2500	broiler	CCRD, air saculitis, bronchitis	Day1: IBV+ND HB1- Day16: ND Clone30		Minia
SA008	39	2000	broiler	CRD, clostridial enteritis, cecal core	Day6: IBV+ND HB1- Day19: ND LaSota	Apr-2017	Minia

SA011	31	3000	broiler	CRD, enteritis, ascites	Day7: ND Colone30+ IB MA5- Day10: KilledH5+ND, Day13: IBD intermediate plus		Minia
SA012	30	3000	broiler	CRD, hydropericardium, ascites, air saculitis	Day6: IBV+ND HB1- Day11: IBD intermediate plus-Day18: ND LaSota		Minia
SA015	29	2000	broiler				Minia
SA017	32	5000	broiler	Greenish diarrhea, air saculitis, pericarditis	Day1: IBV- Day7: Killed H5N2 and ND- Day17: ND LaSota		Minia
AA004	11	6000	broiler	Hemorrhages at tips of the proventriculus	Day6: IBV+ND HB1- Day6: Killed ND vaccine		Fayoum
SA009	27	2000	broiler	CRD, air saculitis, bronchitis	Day1: IBV- Day7: ND HB1-Day12: IBD intermediate plus		Minia
SA010	29	4500	broiler	CRD, air saculitis, bronchitis, greenish diarrhea			Minia
SA013	30	5000	broiler	CRD, air saculitis, bronchitis	Day7: ND HB1 and IBD (VAXXITEC)- Day10: Killed H5+ND- Day17: ND Clone30		Minia
SA014	29	6000	broiler	CRD, air saculitis, bronchitis, greenish diarrhea, cecal coccidiosis, general septicemia	Day7: ND HB1- Day14d: IBD intermediate plus- Day18: ND Clone30		Minia
SA016	26	4000	broiler	Air saculitis, fibrinous pericarditis, hemorrhagic bronchitis	Day7: ND HB1- Day8: Killed ND- Day13: IBD intermediate plus		Minia
SA024	29	1000	broiler	Whitish diarrhea, mild clostridial enteritis, bronchitis	Day6: ND HB1- Day12: IBD intermediate plus	May-2017	Minia
SA018	22	4000	broiler	Air saculitis, greenish diarrhea, septicemia	Not available		Minia
SA019	32	1200	Sasso	pericarditis, cecal core, enteritis	Not available		Minia
SA030	32	4000	broiler	CRD, greenish diarrhea, ascites	Day8: ND HB1- Day12: IBD intermediate plus- Day16: ND Clone30		Minia
SA027	24	5000	broiler	CRD, IBD postvaccinal reaction, air saculitis	Day6: ND HB1- Day8: Killed H5+ND- Day12: IBD intermediate plus		Minia
SA021	25	40000	broiler	CRD, greenish diarrhea	Day8: ND HB1- Day12: IBD intermediate plus- Day17: ND		Minia

					Clone30		
SA022	30	3500	broiler	Bronchitis, greenish diarrhea, air saculitis	Day8: ND HB1- Day12: IBD intermediate plus- Day18: ND LaSota		Minia
SA023	18	2500	broiler	CRD, ascites, nephrosis, greenish diarrhea	Day8: ND HB1- Day13: IBD intermediate plus		Minia
SA026	35	1700	broiler	CRD, greenish diarrhea, enteritis	Not available		Minia
SA028	45	5000	broiler	CRD, greenish diarrhea, cecal coccidiosis	Day6: ND HB1- Day7: Killed H5+ND- Day10: IBD intermediate plus		Minia
SA025	30	4000	broiler	CRD, nephrosis, air saculitis	Day7: ND HB1- Day11: IBD intermediate plus- Day15: ND LaSota	Jun-2017	Minia
SA035	21	2750	broiler	perihepatitis, air saculitis, bronchitis	Not available		Minia
SA003	35	2000	Sasso	CRD, greenish diarrhea	Day8: ND HB1 - Day18: ND Clone30		Minia
SA004	32	5000	broiler	CCRD, mild clostridia, nephrosis, mycotoxicosis	Day8: Killed H5N2+ND- Day10: IBV+ND HB1- Day12: IBD intermediate plus- Day18: ND LaSota	Oct-2017	Minia
SA005	32	8000	broiler	CRD, cecal coccidiosis			Minia
SA020	32	6500	broiler	CRD, nephrosis, greenish diarrhea	Day7: ND HB1-Day8: KilledH5N2+ND-Day12: IBD intermediate plus	Dec-2017	Minia
AA001	10	2000	broiler	hemorrhages at cecal tonsils	Day1: IBV+ND HB1- Day7: Killed ND	Jan-2018	Fayoum
AM001	34	13000	broiler	Tracheal cast, severe congestion in trachea and kidney	Day1: IBV+ ND Clone30- Day7: Killed H9+ND- Day13: IBD intermediate plus		Beni-Suef
AA002	18	650	broiler	Hemorrhages at tips of the proventriculus	Day7: ND LaSota- Day17: Killed ND		Fayoum
AA003	9	2500	broiler	Tracheal cast, liver congestion	Day7: IBV+ ND HB1-Day7: Killed ND	Mar-2018	Fayoum
AM002	33	4000	broiler	Severe congestion in trachea and kidney, mild CRD	Day1: IB and ND Colone30- Day7: Killed H9+ND-Day13: IBD intermediate plus		Fayoum
AM003	33	5000	broiler				Beni Suef
AM004	23	3500	broiler	Tracheal cast, congested trachea, nephritis, hemorrhage in thigh	Day5: Killed ND- Day7: IBV+ ND HB1- Day13: IBD intermediate plus-		Beni Suef

				muscle	Day19: ND LaSota		
AM007	30	5500	broiler	Severe congestion in trachea, nephritis, dehydration, severe congestion in kidney	Day4: H9+IBD Galimune- Day9: KilledH5+ ND Colone30+ IBV MA5- Day12: IBD intermediate plus- Day18: ND LaSota		Beni Suef
AM010	27	3000	broiler	Congested trachea and liver, ascites, bloody diarrhea	Day6: IBV+ND Clone30- Day9: Killed H9+ND-Day13 IBD intermediate plus		Beni Suef
AM008	32	2000	broiler	Severe congestion in trachea, nephritis, severe congestion in kidney	Day5: IB+Clone30- Day9: Killed H9- Day13: IBD intermediate plus-Day16: IB MA5+Clone30	Apr-2018	Beni Suef
AM005	30	7000	broiler	Severe congestion in trachea, dehydration, severe congestion in the kidney, hemorrhage in thigh muscle	Day7: IB+Clone30 and Killed H5- Day9: Killed H9- Day13: IBD intermediate plus-Day16: IB MA5+Clone30		Beni Suef
AM009	32	2500	broiler		Day7: IB+ ND Hitchner IB- Day12: IBD intermediate plus-Day19: IB+ ND Hitchner		Beni Suef

Sample Processing, Virus Detection, and Virus Isolation

Tissue and/or swab samples were pooled and processed in sterile phosphate buffer saline pH 7.0–7.4 containing gentamycin (50µg/ml) (Ewies et al., 2017). Supernatants were inoculated into the allantoic sac of 9-day-old specific pathogen free embryonated chicken eggs (SPF-ECE) (Fouchier et al., 2000). Inoculated eggs were incubated at 37°C for 72 hours and candled daily for embryo viability. The collected allantoic fluids from inoculated eggs were tested for hemagglutination using 1% washed chicken RBCs (Swayne and Brown, 2015).

RT-PCR and Gene Sequencing

The viral RNA was extracted from harvested allantoic fluids by Viral Gene-Spin™ viral DNA/RNA extraction kit (iNtRON Biotechnology Inc., China) according to the manufacturer's instructions. RT-PCR was used for the detection of the F-gene of vNDV and the hemagglutinin (HA) gene of the LPAI H9N2 viruses using Specific primers (Table 2). A single step RRT-PCR assays using TOPscript™ One-Step RT-PCR Kit (Enzyomics Inc., China) was used according to the manufacturer's instructions. The final reaction volume was 20 µL, including 5 µL RNA template, 5 µL TOPscript™ One-Step RT-PCR Kit, 1 µL of each forward and reverse primers (20 pmol), and 8 µL RNase-free water. Thermal cycling RT-PCR conditions included a reverse transcription at 50°C for 30 min, then an inactivation of reverse transcription enzyme and initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 30 sec., 45 sec at 47-51°C (Table 2), and 2 min at 72°C. The addition final extension was performed at 72°C for 10 min.

Table 2. Oligonucleotide primers for amplification of the NDV F gene and LPAI H9N2 HA gene.

Virus		Primer	Annealing temperature (°C)	Size (bp)	Reference
Virulent NDV		F-5' ATGGGCTCCAAACCTTCTA-'3 R-5'GGAAACCTTCGTTCCTCAT-'3	50	1600	(Nagy et al., 2020)
LPAI h9N2	**Seg1**	F-5' TATTCGTCTCAGGGAGCAAAAGCAGG-'3 R-5'ATTACGTCTC-TGTGGAAAGGTAATGTACTG-'3	47	913	(Hoffmann et al., 2001) (Shany, 2015)
	Seg	F-5' ATTACGTCTC-TCCACAATATCAGTAAATAT-'3 R-5'ATATCGTCTCGTATTAGTAGAAACAAGG-'3	51	754	(Shany, 2015) (Hoffmann et al., 2001)

The RT-PCR products of the target bands were purified after gel electrophoresed using the MEGAquick-spin™ Plus total fragment DNA purification kit (iNtRON Biotechnology Inc., China) according to the manufacturer instructions and the DNA was shipped for sequencing at Macrogen, Korea. Sequence comparisons and phylogenetic relationships were determined with the MEGA X software the Clustal W alignment algorithm (Kumar et al., 2018). Nucleotide and deduced amino acid sequence analysis of F gene of NDV and HA gene of LPAI-H9N2 were conducted in comparison with vaccinal and virulent Egyptian NDV isolates using Geneious® 7.1.3, Copyright © 2005-2014 Biomatters Inc.

Results

Virus Isolation

The mortality in infected chicken ranged between 15-20% and necropsy revealed tracheitis with petechial hemorrhage on proventriculus. Results of virus isolation and primary identification using HI test are summarized in table 3.

Table 3. Isolation rates of NDV and LPAI-H9N2 viruses from the flocks under investigation

Location		Beni Suef	Fayoum	Minia
No of samples		8	13	30
RT-PCR Result	**NDV**	3 (37.5%)	6 (46.1%)	13 (43.3%)
	H9N2	0 (0%)	1 (7.7%)	2 (6.7%)
	Neg	5 (62.5%)	6 (46.1%)	15 (50.0%)
Isolation (out of positive samples)	**NDV**	2 (66.7%)	1 (16.7%)	4 (30.8%)
	H9N2	0 (0%)	1 (100%)	1 (50.0%)

Phylogeny and Genetic Analysis of the vNDV strains sequences

The isolated NDV belong to wither genotype II or genotype VII 1.1. (Figure 1). Both genotypes retained the previously characterized amino acid sequences of the F0 protein cleavage site motifs (^{112}GRQGRL^{117} motif and ^{112}RRQKRF^{117} motif for lentogenic and velogenic genotype, respectively). Few amino acid substitutions were observed (Figure 2). The vNDV isolates in this study were 98.7-99.3% and 96.6-98.9% identical to each other based on nucleotide and amino acid identities, respectively.

Compared to their counterpart isolates; the lentogenic strains shared 98-99.2% and 96.3-98.1% nucleotide and amino acid identities to the LaSota reference strain. While the velogenic strains shared 97.8-98.9% and 96.4-98.8% nucleotide and amino acid identities with the recent Egyptian strains isolated during 2016-2018 (table 4).

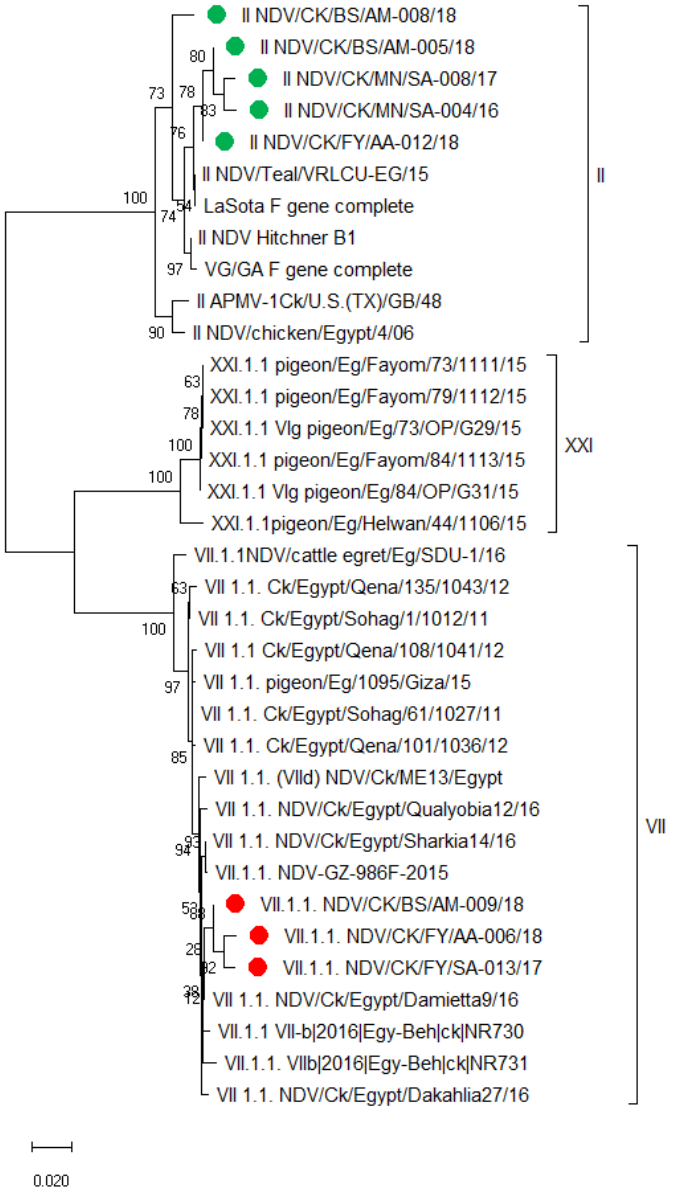

Fig. 1. Phylogenetic analysis of the partial F-gene sequence of isolated lentogenic NDV (green dots) and vNDV strains (red dots). Abbreviations: (EG, Egypt; CK, chicken). Representative strains from different genotypes were included. Phylogenetic relationships through a bootstrap trial of 1000 were determined with the MEGA version 6 using the Clustal W alignment algorithm and neighbor-joining method for tree construction.

Table 4. Nucleotide and amino acid identities between the isolated NDV strains, reference vaccinal strains and genotype VII 1.1. strains. Genetically related strains to both lentogenic and vNDV strain are gray shaded.

Virus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. II NDV/CK/MN/SA-008/17		96.7	98.6	97.6	96.6	96.3	96.1	95.4	89.9	86.1	81.9	84.3	85.9	85.5	85.7	85.9
2. II NDV/CK/MN/SA-004/16	98.7		96.7	96.7	94.2	96.7	96.7	95.4	85.4	85	81.9	84.3	85.4	85	85	85.4
3. II NDV/CK/FY/AM-006/18	99.3	98.6		97.6	96.6	98.1	97.8	97.1	91.5	88	81.9	84.3	87.8	87.3	87.6	87.8
4. II NDV/CK/BS/AA-012/18	99.3	98.7	99.2		96.5	97.6	97.3	96.5	90.5	87.8	81.9	84.3	88.1	87.5	87.8	88.1
5. II NDV/CK/BS/AM-008/18	98.8	97.6	98.9	98.6		96.8	96.6	95.7	89.4	88.4	82.6	85.5	88.5	88.2	88.5	88.5
6. LaSota F gene complete	98	98.7	99.2	99.2	98.7		99.8	99.3	93.1	88.4	81.9	84.3	88.6	88.1	88.1	88.6
7. II NDV Hitchner B1	97.3	98.2	98.4	98.6	98.3	99.2		99.5	93	88.7	81.9	84.3	88.8	88.3	88.3	88.8
8. VG/GA F gene complete	96.9	97.5	98	98.2	97.8	98.9	99.7		92.4	88	79.7	82.5	88.3	87.7	87.7	88.3
9. II NDV/chicken/Egypt/3/2006	91.1	87.8	92	91.6	91.3	93.6	93.7	93.4		89.8	84.8	86.1	89.7	89.5	89.5	89.7
10. VII.1.1. NDV/CK/BS/AM-009/18	81.8	81.4	82.8	83.2	84.3	83.3	83.5	83.3	84.8		98.6	97	98.9	98.9	99.1	98.9
11. VII.1.1. NDV/CK/FY/SA-013/17	79.5	79.5	79.2	79.5	80.4	79.5	79.5	78.7	82.6	99		96.3	97.1	97.8	97.8	97.1
12. VII.1.1. NDV/CK/FY/AA-006/18	81.5	81.5	81.3	81.5	82.1	81.5	81.5	80.9	84.7	98.8	98.8		95.8	96.4	96.4	95.8
13. VII 1.1. NDV/Ck/Egypt/Dakahlia27/16	81.6	81.3	82.5	83	84	83.3	83.4	83.2	84.7	99	98.1	98		99.5	99.5	100
14. VII 1.1. NDV/Ck/Egypt/Qualyobia12/16	81.9	81.7	82.9	83.4	84.4	83.4	83.5	83.3	84.7	99	98.1	98	99.1		99.3	99.5
15. VII 1.1. pigeon/Eg/1095/Giza/15	81.9	81.6	82.9	83.4	84.3	83.4	83.5	83.3	84.8	98.8	97.8	97.6	98.8	99		99.5
16. VII 1.1. (VIId) NDV/Ck/ME13/Egypt	82	81.7	83	83.6	84.5	83.7	83.8	83.6	85.1	98.9	97.8	97.8	99.2	99	99.1	

Nucleotides Identity %

Amino acid identity %

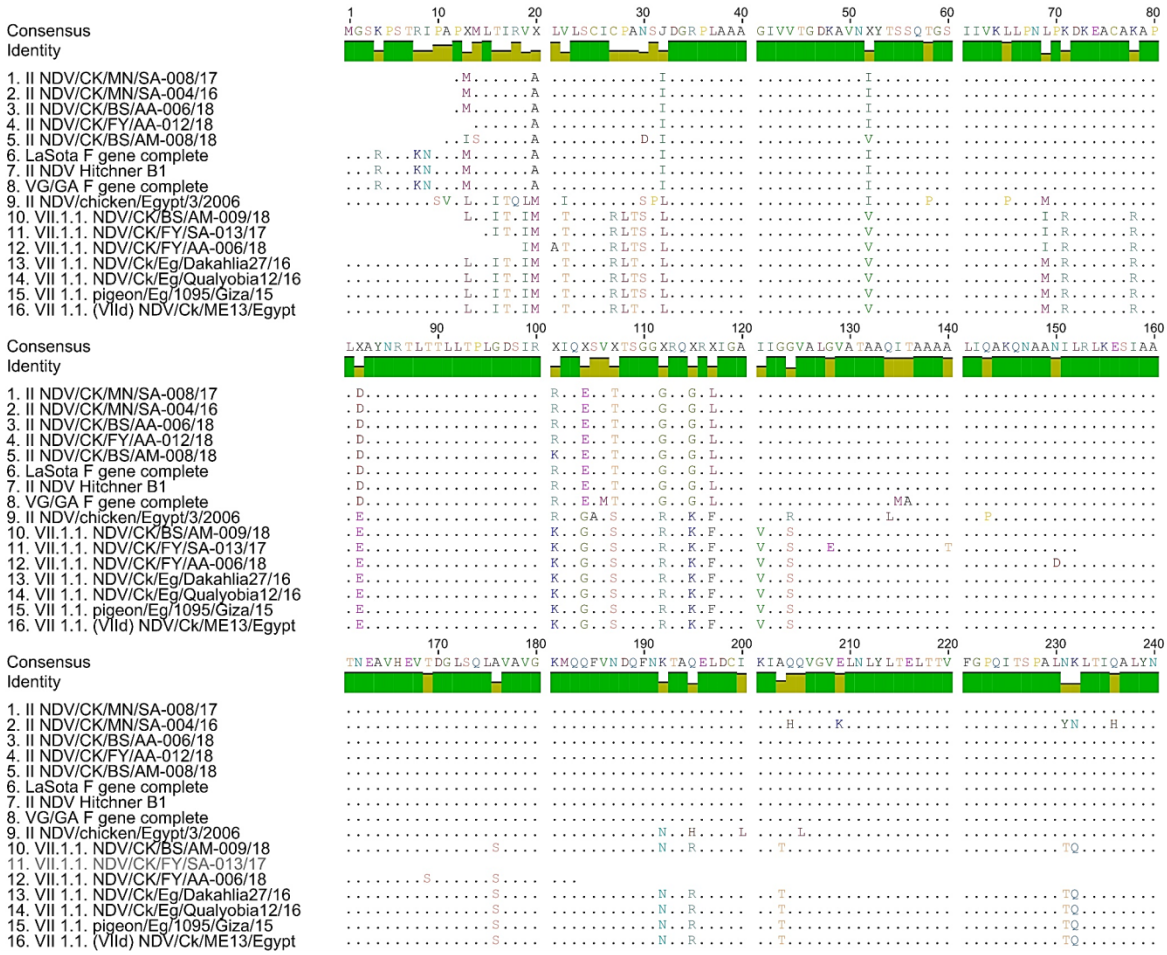

Fig. 2. Amino acid alignment of the isolated NDV strains. Grey shadow box indicate lentogenic cleavage site and red shaded box indicates velogenic cleavage site

Phylogeny and Genetic Analysis of the LPAI H9N2 strains sequences

The LPAI-H9N2 phylogeny of the HA gene showed that the 2 isolates obtained in this study are related to each other and related to recent 2016-2018 Egyptian and Middle East circulating H9N2 strains, which belonged to G1-like lineage (Figure 3). Results also showed that LPAI- H9N2 isolates share 98.4% and 98.9% nucleotide and amino acid identities in between, while they share 97.3 to 99.1 % nucleotide and 97.4 to 97.7 % amino acid identities with recent Egyptian strain. Notably, the 2 strains showed higher identity (≥99%) to recent Israeli 2018 isolates (Table 5). Multiple amino acid changes were observed in the HA gene (figure 4).

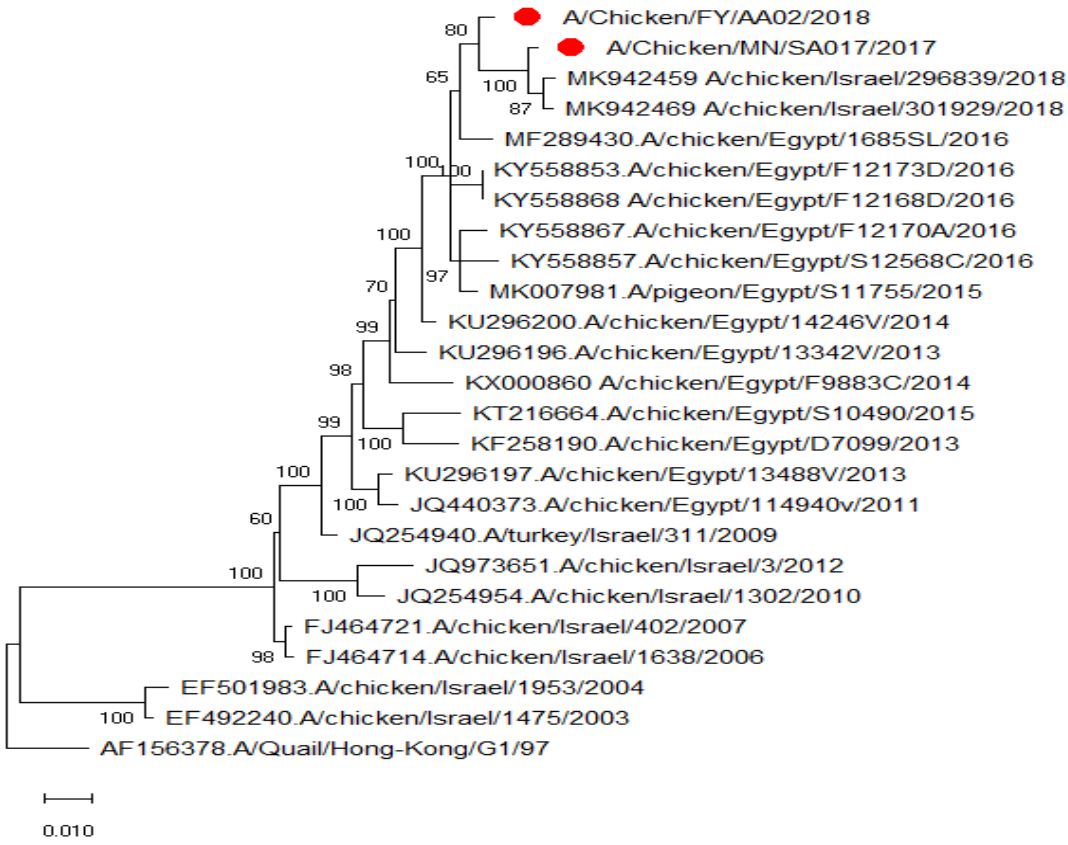

Fig. 3. Phylogenetic analysis of the HA-gene sequence of isolated LPAI-H9N2 strains (red circle ●). Phylogenetic relationships through a bootstrap trial of 1000 were determined with the MEGA version 6 using the Clustal W alignment algorithm and neighbor-joining method for tree construction.

Table 5. Nucleotide and amino acid identities between the isolated LPAI-H9N2 strains, and recent Middle East strains

Virus	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. A/Chicken/MN/SA017/2017		98.9	99.4	99.4	97.9	97.4	97.4	95.5	97.7	97	96.4	96.4	95.9	95.1
2. A/Chicken/FY/AA002/2018	98.4		99.1	98.9	98.7	98.5	98.5	96.2	98.6	98.1	96.8	96.8	97	95.6
3. MK942459 A/CK/Israel/296839/2018	99.3	98.1		99.5	97.9	97.3	97.5	95.4	97.5	96.9	94.7	96.1	96.1	95
4. MK942469 A/CK/Israel/301929/2018	99.1	98	99.3		97.9	97.5	97.7	95.5	97.5	96.9	94.7	96.3	96.1	95.2
5. MF289430.A/CK/Egypt/1685SL/2016	97.9	98.3	97.6	97.7		98.2	98.4	96.1	98.8	98.3	95.9	96.6	96.6	95.5
6. KY558868 A/CK/Egypt/F12168D/2016	97.3	98.9	96.9	97.1	98.2		98.8	96.6	99.2	98.7	95.6	97.3	97.3	96.3
7. KT216664.A/CK/Egypt/S10490/2015	94.6	95.2	94.1	94.4	95.5	95.5		96.6	98.8	98.3	95.6	97.2	97.2	96.1
8. MK007981.A/PG/Egypt/S11755/2015	97.4	98.4	97.1	97.2	98.5	98.7	95.7		96.9	96.9	97.1	97.1	97.1	96.6
9. KU296200.A/CK/Egypt/14246V/2014	97.1	98.1	96.9	96.9	98.3	98.5	96.3	98.5		99	97.5	97.7	97.7	96.5
10. KU296196.A/CK/Egypt/13342V/2013	96.3	97.1	95.9	96	97.4	97.6	96.4	97.6	98.5		97.3	97.5	97.5	96.5
11. KF258190.A/CK/Egypt/D7099/2013	95.5	95.9	93.9	94.1	95.7	95.2	97.6	95.3	96.9	97.2		95.9	95.9	94.9
12. JQ440373.A/CK/Egypt/114940v/2011	95.6	95.9	94.9	95.1	96	96.3	96.7	96.4	97.1	97.4	96.4		97.9	97.2
13. JQ254940.A/TK/Israel/311/2009	95.2	96.1	94.8	95	96	96.4	96.9	96.4	97.2	97.3	96.4	98.1		98.4
14. FJ464721.A/CK/Israel/402/2007	94.2	94.8	93.8	94.1	95.1	95.3	95.9	95.3	96.2	96.2	95.4	97.3	98.5	

Nucleotide identity %

Amino acid identity %

Fig. 4. Amino acid alignment of the isolated LPAI-H9N2 strains.

Discussion

The outbreaks of LPAI-H9N2 and *of NDV by genotype VII* viruses usually cause severe economic losses associated with reduced performance and/or high mortalities in chicken flocks in Egypt (Ewies et al., 2017; Shehata et al., 2015). Hence, continuous surveillance is needed to monitor virus evolution under field conditions. In the current study, samples from respiratory infection outbreaks were collected from broiler chickens in Beni Suef, Fayoum, and Minia Governorates.

The postmortem examination suggested LPAI H9N2 and/or NDV viral infections and included revealed tracheitis with petechial hemorrhage on proventriculus. Virus detection using RT-PCR test revealed 37.5-46.1% and 0-7.7% prevalence for NDV and LPAI H9N2 viruses, respectively. Both viruses are widely detected in Egyptian poultry since early 2011 (Radwan et al., 2013; Hassan et al., 2016; Orabi et al., 2017). No significant seasonal variation was observed with minimal elevation of the outbreaks in the winter season.

The analysis of selected strains F gene revealed the isolation of both lentogenic NDV strain and genotype VII 1.1. NDV. (Figure 1). Both genotypes retained the previously characterized F0 protein proteolytic cleavage site motifs at residues 112 to 117 (Orabi et al., 2017) with high homology to their counterpart previously identified isolates. Though NDV conventional vaccines demonstrated good efficacy to prevent clinical disease but the vaccines are unable to reduce the virus replication and shedding of currently circulating divergent virulent NDV isolates (Bello et al., 2018; Kilany et al., 2015).

This may explain the high prevalence observed in the current study despite intensive vaccination programs using various live attenuated and inactivated NDV vaccines. The results also highlight the importance of using genotype-matched vaccines to reduce the economic losses, especially under multiple viral diseases co-circulation in Egypt (Ali et al., 2019a; Ali et al., 2019b). Additionally, vaccine efficacy problems associated with the mass administration procedures, the difficulty of cold chain maintaining of the vaccines in Egypt, as well as bacterial viral co-infections due to suboptimal biosecurity practices may hinder the efficacy of the vaccines (Ali et al., 2019c; Dimitrov et al., 2017).

On the other hand, the LPAI-H9N2 isolates obtained in this study are related to each other, and related to recent 2016-2018 Egyptian and The Middle East circulating H9N2 strains, belonging to G1-like lineage. 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 (Kandeil et al., 2017). The risk of reassortment between HPAI H5N1 and LPAI H9N2 circulating in Egypt was previously anticipated (Naguib et al., 2017). However, such event was reported with HPAI H5N8 in 2020 after their introduction to Egypt (Hagag et al., 2019; Hassan et al., 2020).

The reassortant was HPAI H5N2 virus from a commercial duck farm with the exchange of the neuraminidase segment from LPAI H9N2 (Hagag et al., 2019). Another reassortant HPAI H5N2 was detected that acquired multiple genes segments from LPAI-H9N2 isolated from both chicken and pigeon in Egypt with the HA from the HPAI H5N8 virus clade (Hassan et al., 2020). These reassortments were rather expected considering the widespread of LPAI H9N2 viruses in poultry flocks. Concurrently, the HA gene of the isolated LPAI H9N2 viruses showed multiple amino acid substitutions. These mutations are resulting from wide field circulation of the virus and/or continuous

vaccine pressure due to the use of inactivated vaccines. Passaging of LPAI H9N2 viruses in embryonated chicken eggs with maternally derived antibodies induced selective pressure of the virus leading to genetic and antigenic variation (Jin et al., 2018) that may lead to mismatching LPAI H9N2 to the vaccine strains and even may generate pandemic strains of zoonotic risk (Meng et al., 2016).

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Conflict of interest

The authors declare no conflict of interest

References

Abdelwhab, E.M., Abdel-Moneim, A.S., (2015). Epidemiology, ecology and gene pool of influenza a virus in egypt: Will egypt be the epicenter of the next influenza pandemic? Virulence 6, 6-18. 10.4161/21505594.2014.992662

Afifi, M.A.A., El-Kady, M.F., Zoelfakar, S.A., Abddel-Moneim, A.S., (2012). Serological surveillance reveals widespread influenza A H7 and H9 subtypes among chicken flocks in Egypt. Tropical animal health and production 45, 687-690.10.1007/s11250-012-0243-9

Afonso, C.L., Miller, P.J., Grund, C., Koch, G., Peeters, B., Selleck, P.W., Srinivas, G.B. (2012). Newcastle disease virus (infection with Newcastle disease virus) In The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. (OIE).

Ali, A., I. Abd El-Mawgoud, A., M. Dahshan, A.-H., A. EL-Sawah, A., A. Nasef, S., (2019a). Escherichia coli in broiler chickens in Egypt, its virulence traits and vaccination as an intervention strategy. Novel Research in Microbiology Journal 3, 415-427. 10.21608/nrmj.2019.44950

Ali, A., Safwat, M., Kilany, W.H., Nagy, A., Shehata, A.A., Zain El-Abideen, M.A., Dahshan, A.-H.M., Arafa, A.-S.A., (2019b). Combined h5nd inactivated vaccine protects chickens against challenge by different clades of highly pathogenic avian influenza viruses subtype h5 and virulent newcastle disease virus. Veterinary world 12, 97-105. 10.14202/vetworld.2019.97-105

Ali, A.M., Samy, M.M., Fasina, F.O., Hassan, M.K., Kilany, W.H., El-Mahdy, S., Saad, A., Lubroth, J., Jobre, Y., (2019c). Field evaluation of common poultry viral vaccinesin egypt: A need for reassessment of the vaccine value chain. Veterinaria italiana 55, 231-239. 10.12834/VetIt.999.5287.1

Bello, M.B., Yusoff, K., Ideris, A., Hair-Bejo, M., Peeters, B.P.H., Omar, A.R., (2018). Diagnostic and vaccination approaches for newcastle disease virus in poultry: The current and emerging perspectives. Biomed Res Int 2018, 7278459. 10.1155/2018/7278459

Czeglédi, A., Ujvári, D., Somogyi, E., Wehmann, E., Werner, O., Lomniczi, B., (2006). Third genome size category of avian paramyxovirus serotype 1 (newcastle disease virus) and evolutionary implications. Virus Research 120, 36-48. 10.1016/j.virusres.2005.11.009

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., Torchetti, M.K., Kolosov, S., Lambrecht, B., Lewis, N.S., Liu, H., Liu, H., McCullough, S., Miller, P.J., Monne, I., Muller, C.P., Munir, M., Reischak, D., Sabra, M., Samal, S.K., Servan de Almeida, R., Shittu, I., Snoeck, C.J., Suarez, D.L., Van Borm, S., Wang, Z., Wong, F.Y.K., (2019). Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus.

Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 74, 103917.10.1016/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. 10.1016/j.vetmic.2016.12.019

El-Zoghby, E.F., Arafa, A.S., Hassan, M.K., Aly, M.M., Selim, A., Kilany, W.H., Selim, U., Nasef, S., Aggor, M.G., Abdelwhab, E.M., Hafez, H.M., (2012). Isolation of h9n2 avian influenza virus from bobwhite quail (colinus virginianus) in egypt. Archives of Virology 157, 1167-1172. 10.1007/s00705-012-1269-z

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, 232-237. 10.1016/j.bjbas.2017.04.004

Fouchier, R.A., Bestebroer, T.M., Herfst, S., Van Der Kemp, L., Rimmelzwaan, G.F., Osterhaus, A.D., (2000). Detection of influenza a viruses from different species by pcr amplification of conserved sequences in the matrix gene. Journal of clinical microbiology 38, 4096-4101.

Hagag, N.M., Erfan, A.M., El-Husseiny, M., Shalaby, A.G., Saif, M.A., Tawakol, M.M., Nour, A.A., Selim, A.A., Arafa, A.S., Hassan, M.K., Hassan, W.M.M., Fahmy, H.A., Ibraheem, E., Attia, M., Abdelhakim, A.M.M., Shahein, M.A., Naguib, M.M., (2019). Isolation of a novel reassortant highly pathogenic avian influenza (h5n2) virus in egypt. Viruses 11. 10.3390/v11060565

Hassan, K.E., Ali, A., Shany, S.A.S., El-Kady, M.F., (2017). Experimental co-infection of infectious bronchitis and low pathogenic avian influenza h9n2 viruses in commercial broiler chickens. Research in veterinary science 115, 356-362. 10.1016/j.rvsc.2017.06.024

Hassan, K.E., King, J., El-Kady, M., Afifi, M., Abozeid, H.H., Pohlmann, A., Beer, M., Harder, T., (2020). Novel reassortant highly pathogenic avian influenza a(h5n2) virus in broiler chickens, egypt. Emerg Infect Dis 26, 129-133. 10.3201/eid2601.190570

Hassan, K.E., King, J., El-Kady, M., Afifi, M., Abozeid, H.H., Pohlmann, A., Beer, M., Harder, T., (2020). Novel Reassortant Highly Pathogenic Avian Influenza A(H5N2) Virus in Broiler Chickens, Egypt. Emerg Infect Dis 26, 129-133.10.3201/eid2601.190570

Hassan, K.E., Shany, S.A.S., Ali, A., Dahshan, A.H.M., El-Sawah, A.A., El-Kady, M.F., 2016. Prevalence of avian respiratory viruses in broiler flocks in egypt. Poultry science 95, 1271-1280. 10.3382/ps/pew068

Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R., (2001). Universal primer set for the full-length amplification of all influenza a viruses. Archives of Virology 146, 2275-2289. 10.1007/s007050170002

Jin, H., Wang, W., Yang, X., Su, H., Fan, J., Zhu, R., Wang, S., Shi, H., Liu, X., (2018). Evolution of h9n2 avian influenza virus in embryonated chicken eggs with or without homologous vaccine antibodies. BMC veterinary research 14, 71. 10.1186/s12917-018-1391-6

Kandeil, A., El-Shesheny, R., Maatouq, A., Moatasim, Y., Cai, Z., McKenzie, P., Webby, R., Kayali, G., Ali, M.A., (2017). Novel reassortant h9n2 viruses in pigeons and evidence for antigenic diversity of h9n2 viruses isolated from quails in egypt. The Journal of general virology 98, 548-562. 10.1099/jgv.0.000657

Karimi-Madab, M., Ansari-Lari, M., Asasi, K., Nili, H., (2010). Risk factors for detection of bronchial casts, most frequently seen in endemic h9n2 avian influenza infection, in poultry flocks in iran. Preventive Veterinary Medicine 95, 275-280. 10.1016/j.prevetmed.2010.03.010

Kilany, W.H., Ali, A., Bazid, A.H.I., Zain El-Abideen, M.A., El Sayed, M., (2015). Evaluation of two inactivated newcastle disease virus vaccines (genotype ii and vii) against challenge of newcastle disease genotype vii infection in chicken. Journal of Animal and Veterinary Advances 14, 211-218. 10.3923/javaa.2015.211.218

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., (2018). Mega x: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35, 1547-1549. 10.1093/molbev/msy096

Meng, F., Xu, H., Zhang, W., Huang, D., Zhang, Z., Liu, X., Chang, W., Qin, Z., (2016). Genetic evolution and substitution frequency of avian influenza virus ha gene in chicken h9n2 subtype in china in the last 20 years. Wei sheng wu xue bao = Acta microbiologica Sinica 56, 35-43.

Miller, P.J., E. L. Decanini, and C. L. Afonso. , (2010). Newcastle disease: Evolution of genotypes and the related diagnostic challenges. Infect. Genet. Evol. .

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. 10.1186/1743-422X-8-237

Naguib, M.M., Arafa, A.-S., Parvin, R., Beer, M., Vahlenkamp, T., Harder, T.C., (2017). Insights into genetic diversity and biological propensities of potentially zoonotic avian influenza h9n2 viruses circulating in egypt. Virology 511, 165-174. 10.1016/j.virol.2017.08.028

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. Transboundary and emerging diseases.10.1111/tbed.13543

Nagy, A., Mettenleiter, T.C., Abdelwahab, E.M., (2017). A brief summary of the epidemiology and genetic relatedness of avian influenza h9n2 virus in birds and mammals in the middle east and north africa. Epidemiology and infection 145, 3320-3333. 10.1017/S0950268817002576

Nili, H., Asasi, K., (2002). Natural cases and an experimental study of h9n2 avian influenza in commercial broiler chickens of iran. Avian Pathology 31, 247-252. 10.1080/03079450220136567

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, 3069-3079. 10.1007/s00705-017-3483-1

Peyre, M., Samaha, H., Makonnen, Y.J., Saad, A., Abd-Elnabi, A., Galal, S., Ettel, T., Dauphin, G., Lubroth, J., Roger, F., Domenech, J., (2009). Avian influenza vaccination in egypt: Limitations of the current strategy. Journal of molecular and genetic medicine : an international journal of biomedical research 3, 198-204.

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, 311-316. 10.1007/s11262-013-0950-y

Samy, A., Naguib, M.M., (2018). Avian respiratory coinfection and impact on avian influenza pathogenicity in

domestic poultry: Field and experimental findings. Vet Sci 5. 10.3390/vetsci5010023

Setta, A., Salem, H.M., Elhady, M., El-Hussieny, A., Arafa, A.S., (2018). Molecular and genetic characterization of infectious bronchitis viruses isolated from commercial chicken flocks in egypt between 2014 and 2016. Journal of World's Poultry Research 8, 1-7.

Shany, S.A.S., (2015). Further studies on the current situation of avian influenza in egypt, PhD. Thesis, Poultry Diseases Department, Beni-Suef University, Egypt.

Shehata, A.A., Parvin, R., Sultan, H., Halami, M.Y., Talaat, S., Abd Elrazek, A., Ibrahim, M., Heenemann, K., Vahlenkamp, T., (2015). Isolation and full genome characterization of avian influenza subtype h9n2 from poultry respiratory disease outbreak in egypt. Virus Genes 50, 389-400. 10.1007/s11262-015-1188-7

Swayne, D., Brown, I. (2015). Chapter 3.3.4. Infection with avian influenza viruses, In: Manual of diagnostic tests and vaccines for terrestrial animals. OIE.