Original Research Article

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

Safaa A. A. Abdel-Latif¹#, Asmaa Atef²#, Ahmed M. A. Abdel-Aleem³, AL-Hussien M. Dahshan⁴, Ahmed Ali⁴*

¹Directorate of Veterinary Medicine in Minia, General Organization of Veterinary Services, Ministry of Agriculture, Egypt
²General Administration of Student Housing Facilities, Fayoum University, Egypt.
³General Administration of student housing facilities, Beni Suef University, Beni Suef 62511 Egypt.
⁴Poultry Diseases Department, Faculty of Veterinary Medicine, Beni Suef University, Beni Suef 62511 Egypt.

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.

Corresponding author: D. Ahmed Ali. Poultry Diseases Department, Faculty of Veterinary Medicine, Beni Suef University, Beni Suef 62511 Egypt Email: ahmed.ali1@vet.bsu.edu.eg
**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 | | |
| AA006  | 28         | 7000       | broiler         | Hemorrhage at cecal tonsils | | |
| AA007  | 15         | 4000       | layer           | Hemorrhage at tips of the proventriculus | Day1: IB primer Day7: Killed ND | Mar-2016 Fayoum |
| AA010  | 26         | 2sa024000 | 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 | |
| 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 | |
| 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 sacculitis, bronchitis | Day1: IBV+ND HB1- Day16: ND Clone30 | |
| SA008  | 39         | 2000       | broiler         | CRD, clostridial enteritis, cecal core | Day6: IBV+ND HB1- Day19: ND LaSota | Apr-2017 Minia |
| Code  | Number | Age  | Gender | Outcome | Description                                                                 |
|-------|--------|------|--------|---------|-----------------------------------------------------------------------------|
| SA011 | 31     | 3000 | broiler| Day7:  | ND Colone30+ IB MA5- Day10: Killed H5+ND, Day13: IBD intermediate plus      |
| SA012 | 30     | 3000 | broiler| Day6:  | IBV+ND HB1- Day11: IBD intermediate plus-Day18: ND LaSota                  |
| SA015 | 29     | 2000 | broiler| Day1:  | IBV- Day7: Killed H5N2 and ND- Day17: ND LaSota                            |
| SA017 | 32     | 5000 | broiler| Day1:  | IBV- Day7: Killed H5N2 and ND- Day17: ND LaSota                            |
| AA004 | 11     | 6000 | broiler| Day6:  | IBV+ND HB1- Day6: Killed ND vaccine                                         |
| SA009 | 27     | 2000 | broiler| Day1:  | IBV- Day7: ND HB1-Day12: IBD intermediate plus                              |
| SA010 | 29     | 4500 | broiler| Day7:  | ND HB1 and IBD (VAXXITEC)- Day10: Killed H5+ND- Day17: ND Clone30          |
| SA013 | 30     | 5000 | broiler| Day7:  | ND HB1- Day14d: IBD intermediate plus-Day18: ND Clone30                   |
| SA014 | 29     | 6000 | broiler| Day7:  | ND HB1- Day8: Killed ND- Day13: IBD intermediate plus                      |
| SA016 | 26     | 4000 | broiler| Day7:  | ND HB1- Day8: Killed ND- Day13: IBD intermediate plus                      |
| SA024 | 29     | 1000 | broiler| Day6:  | ND HB1- Day12: IBD intermediate plus                                       |
| SA018 | 22     | 4000 | broiler| Day8:  | ND HB1- Day12: IBD intermediate plus                                       |
| SA019 | 32     | 1200 | Sasso  | Day8:  | ND HB1- Day12: IBD intermediate plus                                       |
| SA030 | 32     | 4000 | broiler| Day6:  | ND HB1- Day8: Killed H5+ND- Day12: IBD intermediate plus                   |
| SA027 | 24     | 5000 | broiler| Day6:  | ND HB1- Day8: Killed H5+ND- Day12: IBD intermediate plus                   |
| SA021 | 25     | 40000| broiler| Day8:  | ND HB1- Day12: IBD intermediate plus                                       |
| Code  | Days | Weight | Age  | Gender | Symptoms                          | Pathogens and Schedule                        | Location |
|-------|------|--------|------|--------|-----------------------------------|----------------------------------------------|----------|
| 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 | 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 | Oct-2017 |
| SA005 | 32   | 8000   | broiler | CRD, cecal coccidiosis                     | Day7: ND HB1-Day8: KilledH5N2+ND-Day12: IBD intermediate plus | Minia    |
| SA020 | 32   | 6500   | broiler | CRD, nephrosis, greenish diarrhea          | Day7: ND HB1-Day8: Killed H5N2+ND-Day12: IBD intermediate plus | Dec-2017 |
| AA001 | 10   | 2000   | broiler | hemorrhages at cecal tonsils               | Day1: IBV+ND HB1- Day7: Killed ND            | Jan-2018 |
| 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 |
| AM002 | 33   | 4000   | broiler | Severe congestion in trachea and kidney    | Day1: IB and ND Colone30- Day7: Killed H9+ND-Day13: IBD intermediate plus | Fayoum   |
| AM003 | 33   | 5000   | broiler | Severe congestion in trachea and kidney, mild CRD | Day1: IB and ND Colone30- Day7: Killed H9+ND-Day13: IBD intermediate plus | Beni Suel |
| AM004 | 23   | 3500   | broiler | Tracheal cast, congested trachea, nephritis, hemorrhage in thigh | Day5: Killed ND- Day7: IBV+ND HB1- Day13: IBD intermediate plus | Beni Suel |
| AM007 | 30 | 5500 | broiler | Severe congestion in trachea, nephritis, dehydration, severe congestion in kidney | Day19: ND LaSota | Day4: H9+IBD Galimune- Day9: Killed H5+ 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 |
| 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 (Enzymics 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’ ATGGGCTCCAACCTTCTA-3     | 50                          | 1600      | (Nagy et al., 2020)         |
|                | R-5’GGAAACCTTCTCGTCTCAT-3     |                             |           |                             |
| LPAI h9N2      | F-5’ TATTCGTCTCAGGGAGCAAAGGCAGG-3 |                             |           | (Hoffmann et al., 2001)     |
|                | R-5’ATTACGTCTCTGAGAAATGATCTGA-3 | 47                          | 913       | (Shany, 2015)               |
|                | F-5’ ATTCGTCTCAGGCTAATATCTG-3 |                             |           | (Shany, 2015)               |
|                | R-5’ATATCGTCTCGTATAGTAGAAACAAGG-3 |                             |           | (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 either 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 (GRQGRL motif and RRQKRF 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| 88.6| 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| 81.6| 80.9| 84.7| 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| 99| 99.5|   | 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. (VIIId) 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 |
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 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 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/16855/2016 | 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| 98.3| 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| 97.2| 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|    |
Fig. 4. Amino acid alignment of the isolated LPAI-H9N2 strains.

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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

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