Isolation and Molecular Identification of Respiratory Diseases Viruses in Poultry During 2020

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Abstract

Poultry production has affected by multiple respiratory disease triggering serious economic losses in Egypt. In this study, the situation and genetic evolution of respiratory disease in Egypt during 2020 were studied. We collected 53 samples from infected flocks suffered from respiratory signs and variable mortality rate from nine governorates in Egypt during 2020. The collected samples were examined for detection of respiratory disease viruses (Avian influenza virus (AIV), Infectious bronchitis virus (IBV), and Newcastle disease virus (NDV)) by rRT-PCR. The single infection was confirmed in 90.6% (37.7% I. B, 30.2% AIV (H5N8), 9.4% I. B and 5.7% NDV) and co-infection of HPAIV (H5N8) + I.BV and LPAIV (H9N2) +IBV were detected in 3.8% in nine governorates. The HA gene of HPAIV (H5N8) were cluster to clad 2.3.4.4.1b in new branch with characteristic specific mutations especially in T140A in antigenic site A and R72S in the receptor binding site when comparing with A/duck/Egypt/F446/2017 with low A.A identity percent with vaccinal strains (H5N1 and H5N2) reach to 91.9-94% and 84.6% respectively. The HA gene of AIV (H9N2) were belong to A/quail/Hong Kong/G1/97-like virus clustered with group B with specific mutation (212I) that may be effect on human transmission of the virus. The HVRs of S1 gene of IBV cluster to GI23 (Egy Var I) clad with multiple mutation in HVR1, HVR2 when comparing with IBV/CU/4/2014 and low identity percent (68.3%-78.8%) with vaccine strains (H120, M41, 4/91). In conclusion, the respiratory disease continues circulate and rapidly evolved in Egypt during 2020.

Introduction

The poultry industry is one of the most significant industries impacting the national economy in Egypt by promoting investment and jobs availability. Moreover, the primary sources of inexpensive animal protein for poor people in Egypt are poultry meat and eggs (Abdelwhab and Hafez 2011). Regrettably, mainly viral respiratory diseases have affected the poultry industry in Egypt for several years (Awad et al. 2016; Haghighat-Jahromi et al. 2008; Hassan et al. 2021; ME 2014). The inability to control of this disease results in significant economic losses in the poultry sector. The avian influenza (highly pathogenic, Low Pathogenic), Newcastle disease virus (NDV) and infectious bronchitis virus (IBV) alone or with mixed infection with each other or with other bacterial infection are the main cause of respiratory disease affect poultry farm with high mortality rate (Hassan et al. 2016; Radwan et al. 2013; Samy and Naguib 2018). Avian influenza (AI) was the most severe respiratory disease in Egypt. The highly pathogenic avian influenza (HPAI) H5N8 was first recorded in 2016 from wild bird (Selim et al. 2017) then observed in domestic birds causing severe losses in poultry production (Anis et al. 2018; Salaheldin et al. 2018; Yehia et al. 2018). Until now is still circulating as single or co-infection with other respiratory viruses (Hassan et al. 2021; Shehata et al. 2019; Yehia et al. 2018). The low pathogenic avian influenza (LPAI) H9N2 was first recorded in 2010- 2011 and it is belonging to G1 lineage (El-Zoghby et al. 2012). As mainly observed co-infection with H9N2 and I.B viruses, it has immunosuppressive effects that lead to increased opportunism of the infection by other viral and bacterial diseases (Hassan et al. 2017). In addition, the co-infection with other bacterial infection as *Escherichia coli* and *Haemophilus paragallinarum* contribute to increase the virulence of H9N2 because it has endo proteases enzyme that cleavage

precursor HA proteins (Haghighat-Jahromi et al. 2008; Hassan et al. 2017; Pan et al. 2012). IBV that causing air sacculitis in chickens is one of the major significant respiratory infections (S. Hofstad 1984). It is primarily recorded in Egypt in 1950s (Sheble et al. 1986) that related to the Dutch variant D3128 then D274, Mass, 4/91 and D-08880 (Eid 1998). Novel endemic genotypes associated with Israeli variant 2 and mass serotypes were detected in 2006 (Abdel-Moneim et al. 2006b). In 2011, the Egyptian virus mutated and classified into variant I and variant II (Abdel-Moneim et al. 2012). The both types of IBV virus circulated in Egypt causing outbreaks despite of vaccination program led to high economic losses (Abd El Rahman et al. 2015; Zanaty et al. 2016). The most severe respiratory disease is Newcastle disease (ND) that causing high mortality in poultry flocks (OIE 2012). In Egypt, the NDV first recorded in 1948 (Daubney and Mansy 1948) then spread rapidly in Egypt despite of vaccination programs (Abdel-Moneim et al. 2006a; El-Bagoury et al. 2015). Virus isolation is the fundamental diagnostic test for avian respiratory viruses, but it appears to be expensive, slow, and labor-intensive (Suarez et al. 2007). Recently, reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR (rRT-PCR) were rapid diagnostic tests used to detect viral nucleic acid (Spackman et al. 2002). The aim of our research is to explore the situation of respiratory disease outbreaks in Egypt during 2020 using rRT-PCR and genetically characterized the selected circulating respiratory viruses.

Materials And Methods

Collection and preparation of specimens

In this study, AIV (H5N8 and H9N2), NDV and IBV were examined for 53 infected poultry flocks exhibiting severe respiratory manifestation and high mortality during 2020. The oropharyngeal swabs were obtained from disease or freshly dead birds in nine provinces of Egypt including Alexandria, Giza, Dakhalia, El-sharqia, Qualiobia, El-Monofia, El-Behira, Domiat and Cairo (Table 1, Fig. 1). 45 chicken flocks were included in the samples (36 broilers, 5 commercial layers and 4 breeder farms), two flock of turkeys and 6 duck farms. From each flock, 10 to 15 swabs were pooled in 2 ml of phosphate buffer saline (PBS) (Naguib et al. 2017; OIE 2014).

RNA extraction, identification and subtyping of viruses

The QIAamp Viral RNA Mini Kit (Qiagen) using for extraction of viral RNA from pooled oropharyngeal swabs as directed by the manufacturer, then eluted into 50µl of nuclease-free water. All samples were tested for detection of M gene of influenza type A viruses by rRT-PCR (Fereidouni et al. 2012) using the AgPath Real-time Kit (Ambion) and the real-time PCR step one plus System (Applied Biosystems, Foster City, CA, USA). Then subtyping using rRT-PCR target HA and NA genes as described by Hoffmann et al. (2016). In addition, all samples were tested for detection of NDV, IBV using rRT-PCR target matrix gene of avian paramyxovirus⁻¹ (Wise et al. 2004) then the positive samples were tested for the detection of NDV of genotype VII d (velogenic strain) (Moharam et al. 2019). Also, rRT-PCR was used for detection IBV RNA as described by Naguib et al. (2017).

Virus isolation

The positive samples were isolated into SPF embryonated chicken eggs (ECEs) 10 days old in allantoic fluid with daily observation. The mortalities were recorded and allantoic sac was collected and tested by rapid slide hemagglutination (HA) test (OIE 2008).

Sequencing of the viral genome

The HA gene of AI (H5, H9) and HVRs of S1 gene of IBV amplification were carried out by PCR using specific primers as described by Höper et al. (2009); Naguib et al. (2015); Suarez et al. (2007) and high fidelity Phusion® DNA polymerase (Thermo Fisher Scientific, MA, USA). Using QIA quick Gel Extraction Kit, the PCR products were separated and purified from gel (Qiagen). Cycle sequencing reactions have been performed on PCR products using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Using centri-sep columns (Macherey-Nagel GmbH & Co.) the sequence products were purified then sequenced on an ABI PRISM3130 Genetic Analyzer (Life Technologies). For each sequence the blast search was performed (<http://www.ncbi.nlm.nih.gov/BLAST>). Mutation and phylogenetic analysis were conducted using MEGA version 6 program using bootstrap of 1,000 trials of clustal W alignment algorithm (Tamura et al. 2013). The amino acid identity was carried out using DNA star software (DNASar, Madison, WI). Then published by the national center for Biotechnology Information (NCBI) with accession number provided in Table 2.

Results

Real time-PCR

The 48 samples/ 53 were positive for RT-PCR for different respiratory disease (twenty samples were positive for IBV, sixteen samples for HPAI (H5N8), five samples for LPAI (H9N2), three samples for NDV (velogenic strain), two samples for HPAI (H5N8) +IBV and two samples for LPAI (H9N2) + I.BV as mentioned in Table.1.

Genetic characterization of the HPAI (H5N8) Virus HA gene

Phylogenetic analysis of full HA sequence of a four selected AI (H5N8) isolate was named A/ CHICKEN/EGYPT/FN1/2020, A/ CHICKEN/EGYPT/FN2/2020 from co-infected farms and A/ CHICKEN/EGYPT/FN3/202, CHICKEN/EGYPT/FN4/2020 from other infected flocks were belong to clade 2.3.4.4b. They cluster with Egyptian viruses isolated in 2019 making new branch with bootstrapping 95 as showed in Fig.1. They have 5 nucleotide mutations when comparing with A/duck/Egypt/F446/2017 different from Egyptian viruses isolated in 2017 and 2018. By mutation analysis, in antigenic site A, one mutation (T140A) was detected in the A/chicken/Egypt/FN1/2020 and A/chicken/Egypt/FN2/2020 similar to A/Chicken/Egypt/186FL/2018. In addition, the receptor-binding site in A/chicken/Egypt/FN3/2020 had R72S similar to A/Chicken/Egypt/185/2018 and A/Chicken/Egypt/186FL/2018. The S94R also found only in the A/chicken/Egypt/FN3/2020. The A.A.

identity of our strains and H5N1 vectoremune vaccine (A/mute_swan/Hungary/4999/2006), B.E.S.T Vaccine (A/duck/China/E319-2/03) ranged from 91.9-94% and H5N2 CEVac Flukem (A/chicken/Mexico/232/1994) was 84.6% (Fig.2).

Genetic characterization of the H9N2 Virus HA gene

The phylogenetic analysis of full HA sequence of three selected AI H9 isolate was named A/ CHICKEN/EGYPT/FW1/2020 from co-infected farms and A/ CHICKEN/EGYPT/FW2/2020 and A/ CHICKEN/EGYPT/FW3/2020 from single infected flocks was belonged to the A/quail/Hong Kong/G1/97-like virus lineage clustered with group B (Fig.3). The viruses in our study were closely related to other Egyptian strains with identities of 94.9- 98.2% (Fig.4). By mutation analysis we recorded mutation in S16N, M58K, T121I/V, I134M, T145S, N179T as specific to all Egyptian viruses and N41G, I75V, V212I, T413N resemble to Egyptian viruses in 2018-2019 when comparing with the A/quail/Hong Kong/G1/97. In addition, R180K/Q specific for viruses in this study. The amino acid sequences at the cleavage site of HA contained low pathogenic RSSR/GLF motif.

Genetic characterization of S gene of I.B virus

The phylogenetic analysis of HVRs of S1 genes of six selected viruses were named IBV/EGY/CH/AY1/2020, IBV/EGY/CH/AY2/2020 and IBV/EGY/CH/AY3/2020 from co-infected flocks and IBV/EGY/CH/AY4/2020, IBV/EGY/CH/AY5/2020 and IBV/EGY/CH/AY6/2020 from single infected flocks. The viruses in this study were cluster to clad GI23 (Egy Var I) (Fig.5).

By mutation analysis of HVRs, we recorded mutation in comparing with the reference strain IBV/CU/4/2014. The HVR1 had IBV-EGY-CH-AY1-2020, IBV-EGY-CH-AY2-2020, IBV-EGY-CH-AY3-2020 had 3 A.A. and IBV-EGY-CH-AY4-2020, IBV-EGY-CH-AY6-2020 had 4 A.A. and IBV-EGY-CH-AY5-2020 had 2 A.A. The HVRII had 7A.A. mutation in all viruses except IBV-EGY-CH-AY2-2020 had 6 amino acid mutations and IBV-EGY-CH-AY1-2020, IBV-EGY-CH-AY5-2020 had 4 A.A. as shown in Fig.6. The S1 gene identity of amino acids revealed that our viruses relate to vaccine seeds used commonly in Egypt (M41, H120, 4/91) ranging from 68.3%-78.8% as shown in Fig.7.

Discussion

In Egypt during the last years, the outbreaks due to respiratory disease with high mortality rates has increased and effect on the poultry production in Egypt. Many avian viral pathogens were the main cause of this problem (Malik et al. 2004; Roussan et al. 2008). The avian influenza virus (HP, LP), IBV and NDV were the main detected viruses alone or as co-infected in broiler chicken (Hassan et al. 2016). The aim of this study was to identify the incidence of viral respiratory disease in broiler chicken especially AIV (HP, LP), IBV and NDV in Egypt during 2020 and study the molecular characterization and evolution of detected viruses. In this study, multiple respiratory viruses as single or mixed infection were detected. The AIV subtypes and IBV recorded in 48 out of 53 farms. Single infection represented only 90.6% (37.7% I.B, 30.2% H5N8, 9.4% I.B and 5.7% NDV). Also, co-presence of HPAI (H5N8) and IBV that is unique detection

of these co-infected flocks and LPAI (H9N2) and IBV were detected in 3.8% as previously recorded in El-Shall et al. (2019). The occurrence of IBV and HPAI (H5N8) in Egyptian poultry with high mortality rate in different vaccinated poultry flocks has previously been reported (Abd El Rahman et al. 2015; Abdel-Moneim et al. 2006a; Yehia et al. 2020).

In recent years, the avian influenza situation in Egypt has been more complicated due to detection and circulation of many serotypes HPAI (H5N1) and HPAI (H5N8) and LPAI (H9N2) (Shehata et al. 2019). The phylogenetic analysis of four selected samples of HA gene of HPAI H5N8 revealed that the four samples were cluster to clad 2.3.4.4.1b as previously recorded in (El-Shall et al. 2019; Shehata et al. 2019) in new branch with characteristic specific mutations. By mutation analysis we detect change in the (T140A) antigenic site A in two samples A/chicken/Egypt/FN1/2020 and A/chicken/Egypt/FN2/2020 as previously recorded in 43 that may be due to excessive using of avian influenza vaccine H5N1 and H5N2. In addition, the R72S in A/chicken/Egypt/FN3/2020 in the receptor binding site was detected as previously recorded in Nabil et al. (2020). We need further study to detect the effect of mutation in the virulence of H5N8 virus. The A.A. identity was very low in H5N1 and H5N2 vaccine reach to 91.9-94% and 84.6% respectively as previously recorded (Kandeil et al. 2018; Yehia et al. 2020). It was explained where the outbreaks occurred in the vaccinated flocks and causing high mortality. Further study needs for evaluation the protective efficacy of the vaccines and prepare autogenous vaccine that may be more effective against HPAI H5N8. The phylogenetic analysis of three selected strains from single and co-infected flocks reveal that all viruses in our study belong to A/quail/Hong Kong/G1/97-like virus lineage clustered with group B as previously recorded (Hassan et al. 2016; Yang et al. 2017) with six A.A. mutation specific for Egyptian viruses. In addition, I75V, N41G and T413N mutation specific to 2018-2019 Egyptian viruses and the 212I mutation that has important effect on increased the pathogenicity of the H9N2 in mice as previously recorded in Abozeid et al. (2017). We need further studied to study their effect on the pathogenicity of the virus. In this study, we recorded high number of infected vaccinated flocks with IBV viruses by RT-PCR but it does not distinguish between field and vaccine strains of IBV. So partial S1 gene sequencing is important to identify the IBV strains. Furthermore, the partial IBV S1 gene was sequenced and analyzing reveal that six selected viruses of single and co-infected I.B were related to clad GI23 (Egy Var I) clad as previously recorded (Hassan et al. 2016; Zanaty et al. 2016) with low identity percent with vaccine strains H120, M41, 4/91 ranging from 68.3%-78.8% that lead to appearance of infection in vaccinated farms as previously recorded (Abd El Rahman et al. 2015; Sultan et al. 2019). We need further study to evaluate vaccine efficacy and update the vaccine use to give highly protection against newly mutated strains.

The S1 subunit, which comprises three major hyperactive variable regions (HVRs) in the first 395 amino acid. The HVRs contain major antigenic sites between 38 to 67 amino acid (HVR-1), 97 to 141 amino acid (HVR2), and 274 to 387 amino acid (HVR-3) (Moore et al. 1997). In our study, we recorded multiple A.A. mutation in the HVR as previously recorded (Abozeid et al. 2017; Hassan et al. 2017; Zanaty et al. 2016) that may be due to widespread using of heterologous vaccine that led to vaccination pressure. It results in the increase the pathogenesis of the virus and change of tissue tropism (Cavanagh et al. 1992). In conclusion IBV and HPAIV (H5N8) are the main causes of respiratory disease in Egypt alone or co-

infected with other viruses with high mortality rate. Circulation of AI (H5N8) was detected with mutation in the antigenic site and receptor binding site, LPAI (H9N2) with specific mutation and IBV viruses were rapidly evolved in HVRs. the IBV and HPAI H5N8 viruses was distinct from vaccine strains so it is important to surveillance of respiratory disease viruses and study the genetic evolution and its effect on pathogenicity and vaccine efficacy.

Abbreviations

AIV: Avian influenza virus; IBV: Infectious bronchitis virus; NDV: Newcastle disease virus; RT-PCR: Reverse transcription polymerase chain reaction; AI: Avian influenza; HPAI: Highly pathogenic avian influenza; LPAI: Low pathogenic avian influenza; rRT-PCR: real-time reverse transcription polymerase chain reaction; PBS: Phosphate buffer saline; HVRs: hyperactive variable regions.

Declarations

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Authors' contribution

M.S. was carried out collection of samples from the affected flocks, F. A., A. S. were carried out detection of the DNA of respiratory diseases by RT-PCR. A. S. carried out isolation of samples in SPF ECE. N. Y., N. H. were carried out the sequencing, Genetic and phylogenetic Analysis and analysis of the data and writing the manuscript. W. M. supervised and final revision of the manuscript. S. M. A., M. M. S., M. T. E.S. provided technical help in writing the manuscript. The all authors read and approved final manuscript.

Conflict of interest

Authors declare that they have no conflict of interest.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participation

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.

The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Tables

Table (1) Epidemiological data of collected samples. FN: the table shows governorates, breeds, results of PCR of collected samples.

NO.	Governorates	Breed	Result
1	El-Dakhlia	Broiler	HPAIV(H5N8)
2	El-Dakhlia	Layer	HPAIV(H5N8)
3	El-Dakhlia	Layer	IBV
4	Giza	Breeder	IBV
5	Alexandria	Duck	IBV
6	Alexandria	Breeder	HPAIV(H5N8)
7	Giza	Broiler	HPAIV(H5N8)
8	Giza	Broiler	Negative
9	Giza	Layer	HPAIV(H5N8)
10	Giza	Broiler	NDV
11	El-sharqia	Broiler	IBV
12	El-Monofia	Broiler	NDV
13	El-sharqia	Breeder	LPAIV(H9N2)
14	El-Dakhlia	Broiler	IBV
15	Cairo	Broiler	IBV
16	Cairo	Layer	IBV
17	Domiat	Layer	HPAIV(H5N8)
18	El-Dakhlia	Broiler	IBV
19	El-Dakhlia	Broiler	IBV
20	El-Dakhlia	Breeder	HPAIV(H5N8)
21	El-Behira	Duck	Negative
22	Giza	Duck	IBV
23	Beni-suief	Duck	IBV
24	Beni-suief	Broiler	IBV
25	El-Dakhlia	Broiler	IBV
26	El-sharqia	Broiler	IBV
27	Domiat	Broiler	IBV
28	Domiat	Broiler	HPAIV(H5N8)

29	Giza	Turkey	IBV
30	Alexandria	Turkey	HPAIV(H5N8)+IBV
31	Alexandria	Broiler	IBV
32	Cairo	Broiler	IBV
33	Cairo	Broiler	HPAIV(H5N8)
34	El-Behira	Broiler	HPAIV(H5N8)
35	Domiat	Broiler	Negative
36	Giza	Broiler	LPAIV(H9N2)
37	Giza	Broiler	IBV
38	Beni-suief	Broiler	HPAIV(H5N8)
39	El-Monofia	Duck	IBV
40	El-Dakhlia	Duck	HPAI (H5N8)
41	Cairo	Broiler	LPAIV(H9N2)
42	El-sharqia	Broiler	IBV+LPAIV(H9N2)
43	El-Monofia	Broiler	NDV
44	Domiat	Broiler	HPAIV(H5N8)
45	Domiat	Broiler	IBV+LPAIV(H9N2)
46	El-sharqia	Broiler	HPAI (H5N8)+IBV
47	El-Dakhlia	Broiler	HPAIV(H5N8)
48	Beni-suief	Broiler	Negative
49	Cairo	Broiler	HPAIV(H5N8)
50	El-Behira	Broiler	HPAIV(H5N8)
51	El-Dakhlia	Broiler	LPAIV(H9N2)
52	El-Behira	Broiler	LPAIV(H9N2)
53	El-sharqia	Broiler	Negative

Table (2) Accession number of sequenced samples. FN: the table shows accession numbers of HA gene of HPA I (H5N8 and H9N2) and S1 gene of IBV samples.

NO.	Code	GenBank Accession number		
		HA(H5)	HA(H9)	S1
45	IBV/EGY/CH/AY1/2020, A/chicken/FW1/2020	-	MW227513	MW240842
30	IBV/EGY/CH/AY2/2020 A/chicken/Egypt/FN1/2020	MW227501	-	MW240843
3	IBV/EGY/CH/AY3/2020	-	-	MW240844
11	IBV/EGY/CH/AY4/2020	-	-	MW240845
15	IBV/EGY/CH/AY5/2020	-	-	MW240846
39	IBV/EGY/CH/AY6/2020	-	-	MW240847
13	A/chicken/FW2/2020	-	MW227514	-
36	A/chicken/FW3/2020	-	MW227515	-
28	A/chicken/Egypt/FN2/2020	MW227502	-	-
40	A/chicken/Egypt/FN3/2020	MW227503	-	-

Figures

Figure 1

Phylogenetic tree of HA gene of H5N8. FN: The figure shows the phylogenetic analysis of HA gene of AIV H5N8 revealing that all Egyptian strains cluster were related 2.3.3.4b and begin to make new branch with H5N8 Egyptian viruses in 2019. The AI H5N8 viruses in our study are indicated with a black dot.

Percent Identity																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16			
Divergence	1		97.2	98.5	98.5	98.7	99.4	99.6	99.4	98.9	99.6	99.4	99.4	99.2	91.9	94.0	84.6	1	A-duck-Egypt-F446-2017
	2	2.9		97.6	97.6	96.8	97.4	97.6	97.6	97.4	97.6	97.7	97.7	97.4	91.7	93.8	84.9	2	A-broiler-duck-Korea-Buan2-2014
	3	1.5	2.5		100.0	98.1	98.7	98.9	98.7	98.1	98.9	98.7	98.7	98.5	91.7	94.0	84.6	3	A-goose-Jiangsu-QD5-2014
	4	1.5	2.5	0.0		98.1	98.7	98.9	98.7	98.1	98.9	98.7	98.7	98.5	91.7	94.0	84.6	4	A-goose-Shandong-WFSG1-2014
	5	1.3	3.3	1.9	1.9		98.7	99.1	98.9	98.1	98.9	98.7	98.7	98.5	91.5	93.4	84.4	5	A-Chicken-Egypt-17167-2017
	6	0.6	2.7	1.3	1.3	1.3		99.4	99.2	99.1	99.8	99.6	99.6	99.4	91.7	93.8	84.4	6	A-Common-coot-Egypt-CA285-2016
	7	0.4	2.5	1.1	1.1	0.9	0.6		99.8	98.9	99.6	99.4	99.4	99.2	91.7	94.0	84.6	7	A-duck-Egypt-SS19-2017
	8	0.6	2.5	1.3	1.3	1.1	0.8	0.2		98.7	99.4	99.2	99.2	99.1	91.7	94.0	84.6	8	A-Duck-Egypt-175Fao-SI-2017
	9	1.1	2.7	1.9	1.9	1.9	0.9	1.1	1.3		99.2	99.2	99.2	99.2	91.9	94.0	84.2	9	A-Chicken-Egypt-185-2018
	10	0.4	2.5	1.1	1.1	1.1	0.2	0.4	0.6	0.8		99.8	99.8	99.6	91.9	94.0	84.6	10	A-Turkey-Egypt-AI20285-2019
	11	0.6	2.3	1.3	1.3	1.3	0.4	0.6	0.8	0.8	0.2		100.0	99.4	91.9	94.0	84.6	11	A-chicken-Egypt-FN1-2020
	12	0.6	2.3	1.3	1.3	1.3	0.4	0.6	0.8	0.8	0.2	0.0		99.4	91.9	94.0	84.6	12	A-chicken-Egypt-FN2-2020
	13	0.8	2.7	1.5	1.5	1.5	0.6	0.8	0.9	0.8	0.4	0.6	0.6		91.9	94.0	84.6	13	A-chicken-Egypt-FN3-2020
	14	8.2	8.4	8.4	8.4	8.6	8.4	8.4	8.4	8.2	8.2	8.2	8.2	8.2		97.0	87.4	14	A-mute-swan-Hungary-4999-H5N1-vectoremu
	15	6.1	6.3	6.1	6.1	6.7	6.3	6.1	6.1	6.1	6.1	6.1	6.1	6.1	2.9		87.6	15	A-duck-China-E319-2-03-H5N1-B.E.S.T-Vac
	16	16.4	15.9	16.4	16.4	16.6	16.6	16.4	16.4	16.8	16.4	16.4	16.4	16.4	12.9	12.9		16	A-chicken-Mexico-232-1994-H5N2
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16			

Figure 2

Amino acid identities and divergence of HA gene of H5N8 viruses compared to other selected strains and vaccinal strains. FN: Amino acid identity of HA gene of H5N8 viruses showed that A.A. identity percent of Egyptian H5N8 strain in this study ranged from 91.9-94% and 84.6% with H5N1 and H5N2 vaccine respectively.

Figure 3

Phylogenetic tree of HA gene of H9N2. FN: The figure shows the phylogenetic analysis of HA gene of AIV H9N2 revealing that all Egyptian strains cluster were was belonged to the A/quail/Hong Kong/G1/97-like virus lineage clustered with group B. The AI H9N2 viruses in our study are indicated with a black dot.

Figure 4

Amino acid identities and divergence of HA gene of H9N2 viruses compared to other selected strains and vaccinal strains. FN: Amino acid identity of HA gene of H9N2 viruses showed that closely related to other Egyptian strains with identities of 94.9- 98.2%.

Figure 5

Phylogenetic tree of partial S1 gene of IBV. FN: The figure shows the phylogenetic analysis of HA gene of AIV H9N2 revealing that all Egyptian strains cluster were cluster to clad GI23 (Egy Var I). The IBV viruses in our study are indicated with a black dot.

	HVR1 (60-88)	HVR2 (115-149)
cu-4	SG~QCTAGSIYWSKNFSASSVAMTAPDT	YKNGQGSCPLTGLIPQNHIRISAMKNSSL
F859	I	R
Eg-CLEVB-2-IBV-012	T	R
IBV-Eg-CLEVB-1-IBV-012	..V...A.....	R
IBV-Eg-CLEVB-2-012	T	R
IBV-EG-1586CV-SP1-2015	..H...A.....A.....QN	H
IBV-EG-13280F-SP1-2013	..Q...A.....A.....QN	..SSS...M...HY...R.N.
IBV-EG-1212B-SP1-2012	..Q...A.....A.....QN	..SSS...M...YY...R.N.
IBV-EG-1442F-SP1-2014	D	R
IBV-D2930-3-1-1-15	..Q...A.....A.....QN	..SSS...M...Y...R.N.
IBV-D2572-2-2-14 EG	..Q...A.....A.....QN	..S.S...M...Y...R.N.
IBV-D1903-21-12	..Q...A.....A.....QN	..SSS...M...Y...R.N.
IBV-D1456-1-5-10	..Q...A.....A.....QN	..SSS...M...YY...R.N.
IBV-D1795-2-7-11		
IBV-D1887-2-3-12		
IBV-D1344-2-4-10	I	
IBV-EGY-CH-AY1-2020	..S...T.....V	..S...L...M.....R
IBV-EGY-CH-AY2-2020	..S...H.....V	..S...SKDLF.....
IBV-EGY-CH-AY3-2020	..G...H.....V	..S...SKDLF.....H
IBV-EGY-CH-AY4-2020	..S...T.H.....A	..H...I.M...HL.V.....H
IBV-EGY-CH-AY5-2020	H.....V	..S...L...KD.....H
IBV-EGY-CH-AY6-2020	..S...T.H.....V	..S...SKDL.....T.H

Figure 6

Amino acid mutation in HVRS in S1 gene of IBV. FN: The HVR1 had 3,4,2 A.A. mutations in three, two and one samples in this study respectively. The HVR2 had 7A.A. mutation in all viruses except two viruses had 6 and 4 mutations.

Percent Identity																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	100	90.7	71.7	75.3	71.7	72.1	73.3	71.7	72.5	72.5	80.0	68.3	75.0	72.1	76.7	74.2	75.0	78.8	76.7	73.3
2	10.1	100	76.3	75.3	73.2	69.1	69.1	70.0	68.0	69.1	81.7	70.1	76.7	69.1	78.3	72.2	76.7	76.3	78.3	70.1
3	34.4	25.2	100	67.0	68.8	77.1	77.9	75.0	77.1	77.5	80.0	86.2	78.3	77.1	80.0	75.4	78.3	77.1	80.0	76.7
4	30.2	30.4	40.0	100	67.0	64.9	64.9	66.7	67.0	67.0	70.0	69.1	70.0	66.0	71.7	70.1	71.7	72.2	71.7	68.0
5	36.2	33.9	39.6	43.7	100	74.1	74.3	68.3	71.1	73.8	70.0	69.3	71.7	71.1	70.0	71.8	68.3	75.1	70.0	71.5
6	33.7	36.7	27.2	43.6	32.2	100	94.0	81.7	96.8	98.0	95.0	83.8	98.3	97.5	96.7	92.8	95.0	91.7	96.7	94.2
7	31.1	35.2	26.6	42.2	32.4	5.8	100	80.0	90.6	93.2	88.3	81.6	91.7	91.3	90.0	88.1	88.3	87.7	90.0	89.2
8	35.7	39.2	30.5	44.1	41.1	21.2	23.6	100	80.0	81.7	83.3	83.3	81.7	81.7	83.3	85.0	85.0	83.3	83.3	85.0
9	32.9	38.4	27.1	39.7	34.2	2.6	8.1	23.4	100	98.2	91.7	83.8	96.7	98.6	96.7	93.5	95.0	93.5	96.7	94.9
10	33.0	37.0	26.6	39.9	33.5	2.6	7.2	21.1	1.7	100	93.3	84.5	98.3	98.9	98.3	93.1	96.7	93.1	98.3	94.6
11	23.3	21.1	23.3	38.4	38.4	5.2	12.8	19.1	8.9	7.0	100	95.0	93.3	93.3	95.0	93.3	93.3	95.0	95.0	93.3
12	40.3	36.2	14.5	36.4	38.0	19.3	22.9	18.9	18.7	18.1	5.2	100	98.3	84.1	100.0	82.3	98.3	83.8	100.0	83.8
13	30.8	28.7	25.9	38.3	35.7	1.7	8.9	21.2	3.4	1.7	7.1	1.7	100	98.3	98.3	96.7	96.7	98.3	98.3	96.7
14	33.6	36.6	27.1	41.6	34.9	2.1	7.7	21.1	1.3	1.3	7.0	18.7	1.7	100	98.3	93.5	96.7	93.1	98.3	94.9
15	28.2	26.1	23.4	35.6	38.5	3.4	10.8	18.9	3.4	1.7	5.2	0.0	1.7	1.7	100	98.3	98.3	100.0	100.0	98.3
16	31.8	35.5	28.9	38.3	33.7	5.7	10.5	16.8	5.3	6.2	7.1	18.7	3.4	5.7	1.7	100	100.0	94.6	98.3	97.1
17	30.8	28.7	25.9	35.6	41.6	5.3	12.9	16.8	5.2	3.4	7.1	1.7	3.4	3.4	1.7	0.0	100	98.3	98.3	100.0
18	25.0	29.0	26.4	35.0	29.2	7.1	11.5	18.9	5.7	6.2	5.2	17.6	1.7	6.2	0.0	4.7	1.7	100	93.9	98.3
19	28.2	26.1	23.4	35.6	38.5	3.4	10.8	18.9	3.4	1.7	5.2	0.0	1.7	1.7	0.0	1.7	1.7	0.0	100	98.3
20	31.7	34.9	27.7	38.0	33.5	5.3	9.6	16.8	4.8	5.7	7.1	18.1	3.4	5.3	1.7	1.3	0.0	4.4	1.7	100
1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Figure 7

Amino acid identities and divergence of partial S1 gene of IBV sequenced viruses compared to other selected strains and vaccinal strains. FN: Amino acid identity of partial S1 gene of IBV viruses showed

that A.A. identity percent of Egyptian IBV strain in this study ranged from 68.3%-78.8% with H120, M41, 4/91 vaccine strains.