



# Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020

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## ABSTRACT

Poultry production has been affected by multiple respiratory diseases triggering serious economic losses in Egypt. The current study aimed to investigate the situation and genetic evolution of respiratory diseases in Egypt during 2020. A total of 53 samples were collected from infected flocks suffered from respiratory signs and variable mortality rates from nine governorates in Egypt during 2020. The collected samples were examined for the detection of respiratory disease viruses (Avian influenza virus (AIV (H5N8, H9N2), 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) + IBV and LPAIV (H9N2) +IBV were detected in 3.8% of nine governorates. The HA gene of HPAIV (H5N8) was cluster to clad 2.3.4.4.1b in a new branch with characteristic specific mutations especially in T140A in antigenic site A and R72S in the receptor-binding site, compared to A/duck/Egypt/F446/2017 with low A.A identity percent with vaccinal strains of H5N1 and H5N2 reaching to 91.9-94% and 84.6%, respectively. The HA gene of AIV (H9N2) belonged to A/quail/Hong Kong/G1/97-like virus clustered with group B with a specific mutation (212I) that may affect the human transmission of the virus. The HVRs of S1 gene of IBV cluster to GI23 (Egy Var I) clad with multiple mutations in HVR1 and HVR2, compared to IBV/CU/4/2014 and low identity percent (68.3-78.8%) with vaccine strains (H120, M41, 4/91). In conclusion, respiratory disease continues to circulate and rapidly evolved in Egypt during 2020.

**Keywords:** HPAIV (H5N8), IBV, Genetic characterization, LPAIV(H9N2), Respiratory disease

## 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, the poultry industry in Egypt has been affected mainly by viral respiratory diseases for several years (Haghighat-Jahromi et al., 2008; Sediek, 2013; Awad et al, 2016). The inability to control this disease results in significant economic losses in the poultry sector. Avian influenza (highly pathogenic, low pathogenic), Newcastle Disease Virus (NDV) and Infectious Bronchitis Virus (IBV) alone or mixed infection (with each other or other bacterial infection) are the main cause of respiratory disease affect poultry farm with a high mortality rate (Radwan et al., 2013; Hassan et al., 2016; 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 a wild bird (common coot) (Selim et al., 2017), then observed in domestic birds, including chickens and ducks, as single or co-infected with other respiratory viruses causing severe losses in poultry production (Salaheldin et al., 2018; Yehia et al., 2018; Shehata et al., 2018; Hassan et al., 2019). The low pathogenic avian influenza (LPAI) H9N2 was first recorded in 2010-2011 belonging to G1 lineage (El-Zoghby et al., 2012). It has immunosuppressive effects that lead to increased opportunism of the infection by other viral diseases as mainly observed in co-infection with H9N2 and I.B viruses (Hassan et al., 2017). In addition, the co-infection with other bacterial infections as *Escherichia coli* and *Haemophilus paragallinarum* contribute to increasing the virulence of H9N2 because it has an endoproteases enzyme that cleavage precursor HA proteins (Haghighat-Jahromi et al., 2008; Pan et al., 2012; Hassan et al., 2017).

Infectious bronchitis virus is one of the major significant respiratory infections (Hofstad, 1984). It is primarily recorded was in Egypt during the 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., 2006a). In 2011, the Egyptian virus was mutated and classified into variant I and variant II (Abdel-Moneim et al., 2012). Both types of IBV virus circulated in Egypt causing outbreaks despite vaccination programs, which all in all lead 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 was first recorded in 1948 (Daubney and Mansy, 1948) then spread rapidly in Egypt despite vaccination programs (Abdel-Moneim et al., 2006b; 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).

In the recent year, the respiratory viruses (HPAI (H5N8), H9N2 (LPAI)), IB, NDV has continuous evolution that may be affected on the pathogenicity of the virus and vaccine efficacy as previously recorded (Yehia et al., 2018; Hassan et al., 2017; Zanaty et al., 2016; Abdel-Moneim et al., 2006b). The aim of the current research was to explore the updated status of respiratory disease outbreaks in broiler Egyptian farms during 2020 using rRT-PCR and to study the genetic variation of the selected circulating respiratory viruses.

## MATERIALS AND METHODS

### Collection and preparation of specimens

In the present study, AIV (H5N8 and H9N2), NDV, and IBV were examined on 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, Figure 1). A total of 45 chicken flocks (including 36 broilers, 5 commercial layers, and 4 breeder farms), 2 flocks of turkeys, and 6 duck farms were examined. The samples from each flock were collected by 10-15 swabs were pooled in 2 ml of phosphate buffer saline (OIE, 2014; Naguib et al., 2017).

### RNA extraction, identification, and subtyping of viruses

The QIAamp Viral RNA Mini Kit (Qiagen) was used for the extraction of viral RNA from pooled oropharyngeal swabs following the manufacturer's guidelines, then eluted into 50 µl of nuclease-free water. All samples were tested for the 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). In the next step, subtyping using rRT-PCR targeted HA and NA genes as described by Hoffmann et al. (2016). In addition, all samples were tested for the 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 (velogenic strain) (Moharam et al., 2019). Furthermore, rRT-PCR was used for the detection of IBV RNA as described by (Naguib et al., 2017).

### Virus isolation

The positive samples were isolated into specific pathogen-free (SPF) embryonated chicken eggs (ECEs) aged 10 days old in allantoic fluid with daily observation. The mortalities were recorded and the allantoic sac was collected and tested by rapid slide haemagglutination (HA) test (OIE manual, 2008).

### Sequencing of the viral genome

The HA gene of AI (H5, H9) and HVRs of S1 gene of IBV amplification were examined by PCR using specific primers as described by Hoper et al. (2009), Selim et al. (2013) and Naguib et al. (2015), and high fidelity Phusion® DNA polymerase (Thermo Fisher Scientific, MA, USA). Using QIAquick Gel Extraction Kit, the PCR products were separated and purified from the gel (Qiagen).

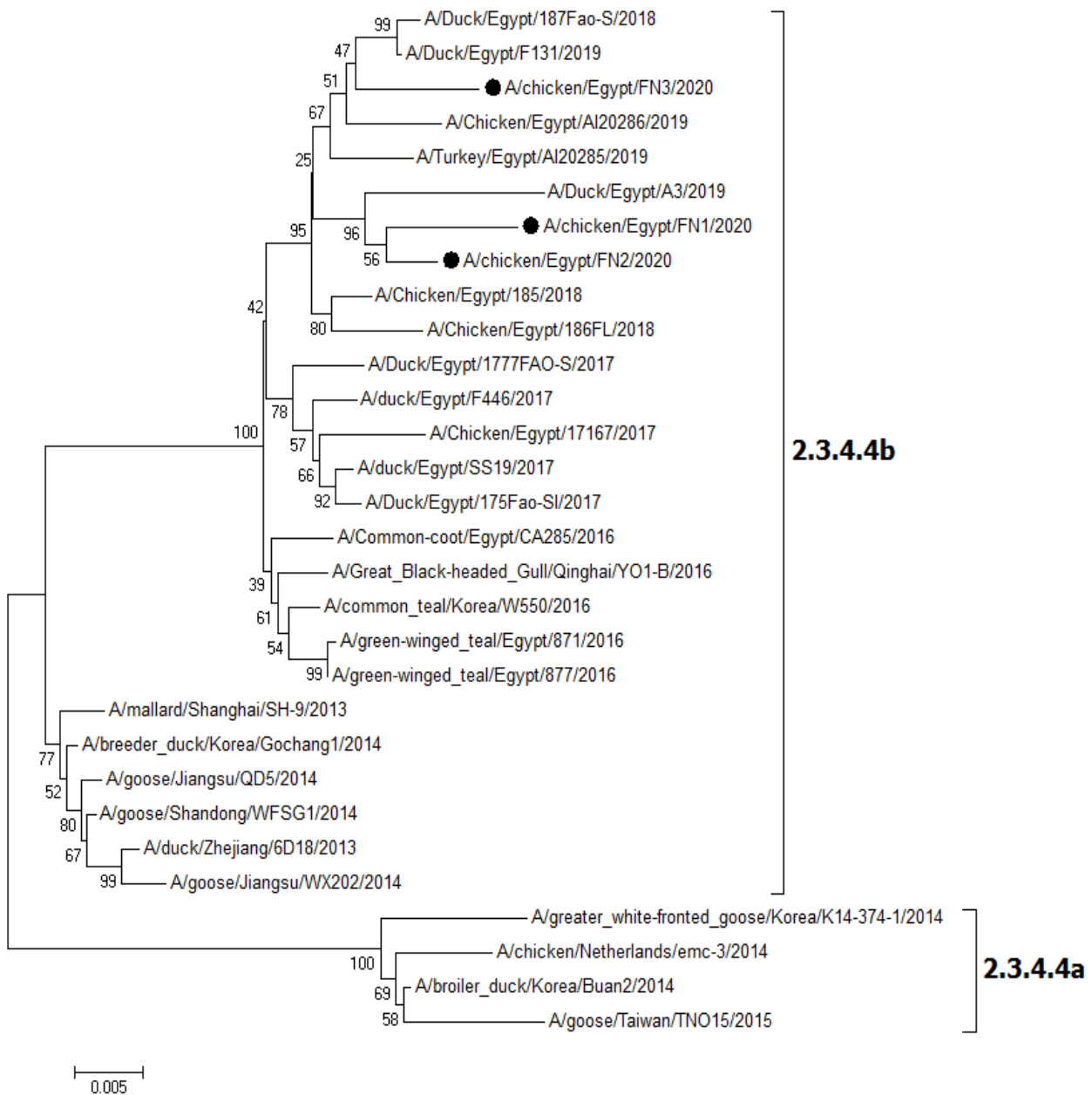
Cycle sequencing reactions have been performed on PCR products using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Using centri-sep columns (Macherey-Nagel GmbH & Co.) the sequence products were purified then sequenced on an ABI PRISM3130 Genetic Analyzer (Life Technologies). For 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 a bootstrap of 1000 trials of the Clustal W alignment algorithm (Tamura et al., 2013). The amino acid identity was analyzed using DNA star software (DNASStar, Madison, WI). Then, it was published by the national center for Biotechnology Information with the accession number provided in Table 2.

**Table 1.** Epidemiological data results of PCR of collected samples of collected samples

Number	Governorates	Species	Breeds	Age (day)	Results
1	El-Dakhliya	Chicken	Broiler	20	HPAIV(H5N8)
2	El-Dakhliya	Chicken	Layer	275	HPAIV(H5N8)
3	El-Dakhliya	Chicken	Broiler	25	IBV
4	Giza	Chicken	Breeder	90	IBV
5	Alexandria	Chicken	Broiler	30	IBV
6	Alexandria	Chicken	Layer	365	HPAIV(H5N8)
7	Giza	Chicken	Broiler	33	HPAIV(H5N8)
8	Giza	Chicken	Broiler	36	Negative
9	Giza	Turkey		65	HPAIV(H5N8)
10	Giza	Chicken	Broiler	40	NDV
11	El-sharqia	Chicken	Broiler	22	IBV
12	El-Monofia	Chicken	Layer	390	NDV
13	El-sharqia	Chicken	Broiler	15	LPAIV(H9N2)
14	El-Dakhliya	Chicken	Breeder	392	IBV
15	Cairo	Chicken	Broiler	23	IBV
16	Cairo	Chicken	Broiler	34	IBV
17	Domiya	Chicken	Broiler	42	HPAIV(H5N8)
18	El-Dakhliya	Chicken	Broiler	36	IBV
19	El-Dakhliya	Chicken	Layer	120	IBV
20	El-Dakhliya	Chicken	Broiler	22	HPAIV(H5N8)
21	El-Behira	Chicken	Breeder	196	Negative
22	Giza	Chicken	Broiler	20	IBV
23	Beni-suef	Turkey		60	IBV
24	Beni-suef	Chicken	Broiler	25	IBV
25	El-Dakhliya	Chicken	Broiler	15	IBV
26	El-sharqia	Chicken	Broiler	30	IBV
27	Domiya	Chicken	Broiler	15	IBV
28	Domiya	Chicken	Layer	120	HPAIV(H5N8)
29	Giza	Chicken	Broiler	33	IBV
30	Alexandria	Chicken	Broiler	33	HPAIV(H5N8)+IBV
31	Alexandria	Chicken	Broiler	34	IBV
32	Cairo	Chicken	Breeder	95	IBV
33	Cairo	Chicken	Broiler	22	HPAIV(H5N8)
34	El-Behira	Duck		12	HPAIV(H5N8)
35	Domiya	Chicken	Broiler	18	Negative
36	Giza	Duck		15	LPAIV(H9N2)
37	Giza	Chicken	Broiler	20	IBV
38	Beni-suef	Chicken	Broiler	15	HPAIV(H5N8)
39	El-Monofia	Duck		95	IBV
40	El-Dakhliya	Chicken	Broiler	20	HPAI (H5N8)
41	Cairo	Chicken	Broiler	28	LPAIV(H9N2)
42	El-sharqia	Duck		27	IBV+LPAIV(H9N2)
43	El-Monofia	Chicken	Broiler	18	NDV
44	Domiya	Chicken	Broiler	33	HPAIV(H5N8)
45	Domiya	Chicken	Broiler	23	IBV+LPAIV(H9N2)
46	El-sharqia	Duck		33	HPAI (H5N8)+IBV
47	El-Dakhliya	Chicken	Broiler	15	HPAIV(H5N8)
48	Beni-suef	Chicken	Broiler	35	Negative
49	Cairo	Chicken	Broiler	34	HPAIV(H5N8)
50	El-Behira	Chicken	Broiler	15	HPAIV(H5N8)
51	El-Dakhliya	Duck		17	LPAIV(H9N2)
52	El-Behira	Chicken	Broiler	28	LPAIV(H9N2)
53	El-sharqia	Chicken	Broiler	18	Negative

**Table 2.** Accession number of HA gene of HPAI (H5N8 and H9N2) and S1 gene of infectious bronchitis virus samples

Number	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	-	-



**Figure 1.** Phylogenetic tree of HA gene of highly pathogenic avian influenza (H5N8)

## RESULTS

### Result of real time-PCR

The findings indicated that 48 samples out of 53 tested samples were positive for RT-PCR for different respiratory diseases (20 samples were positive for IBV, 16 samples for HPAI (H5N8), and 5 samples for LPAI (H9N2), 3 samples for NDV (velogenic strain), 2 samples for HPAI (H5N8) +IBV, and 2 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 four selected AI (H5N8) isolates 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 belonged to clade 2.3.4.4b. They cluster with Egyptian viruses isolated in 2019 making a new subgroup with bootstrapping 95 as shown in Figure 1. They have five nucleotide mutations, compared to A/duck/Egypt/F446/2017, which is 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 was similar to A/Chicken/Egypt/185/2018 and A/Chicken/Egypt/186FL/2018. The S94R was also found only in the A/chicken/Egypt/FN3/2020. The A.A. identity of the current strains and H5N1 vectormune vaccine (A/mute\_swan/Hungary/4999/2006), B.E.S.T Vaccine (A/duck/China/E319-2/03) was within the range of 91.9-94% and H5N2 CEVac Flukem (A/chicken/Mexico/232/1994) was 84.6% (Figure 2).

### Genetic characterization of the H9N2 Virus HA gene

The phylogenetic analysis of the 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 belonged to the A/quail/Hong Kong/G1/97-like virus lineage clustered with group B (Figure 3). The viruses in the current study were closely related to other Egyptian strains with identities of 94.9- 98.2% (Figure 4). Considering mutation analysis, mutations in S16N, M58K, T121I/V, I134M, T145S, N179T as specific to all Egyptian viruses were recorded and N41G, I75V, V212I, T413N resemble Egyptian viruses in 2018-2019, compared to the A/quail/Hong Kong/G1/97. In addition, R180K/Q was specific for viruses in the present study. The amino acid sequences at the cleavage site of HA contained a low pathogenic RSSR/GLF motif.

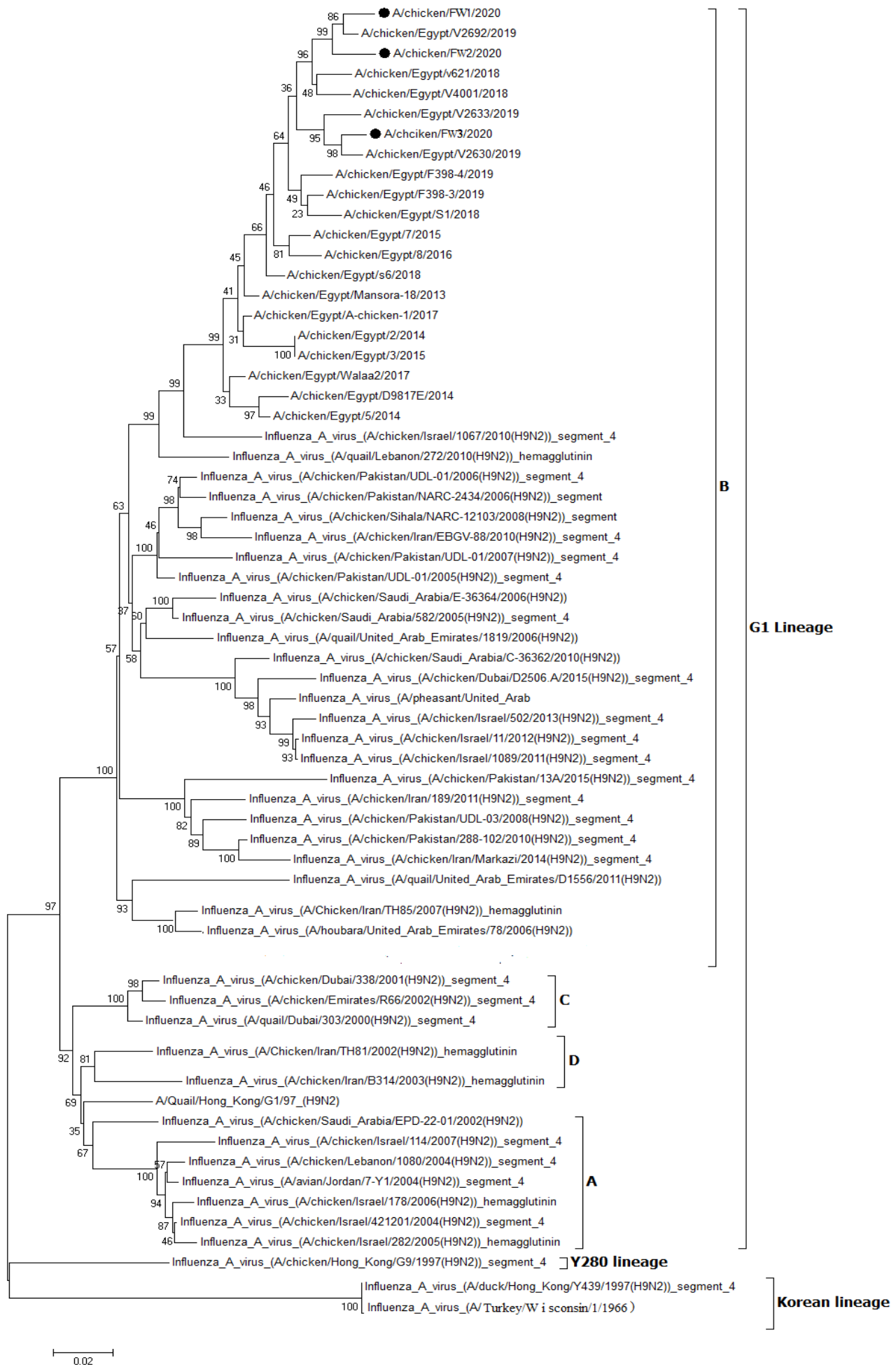
### Genetic characterization of S gene of infectious bronchitis virus

The phylogenetic analyses 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) (Figure 5).

By mutation analysis of HVRs, the recorded mutation was compared with the reference strain of IBV/CU/4/2014. The HVR1 of three Egyptian viruses (IBV-EGY-CH-AY1-2020, IBV-EGY-CH-AY2-2020, IBV-EGY-CH-AY3-2020) had three A.A. and the other two viruses (IBV-EGY-CH-AY4-2020, IBV-EGY-CH-AY6-2020) had four A.A. However, the IBV-EGY-CH-AY5-2020 had two A.A. The HVRII had 7A.A. mutation in all viruses except IBV-EGY-CH-AY2-2020, which had six amino acid mutations, and IBV-EGY-CH-AY1-2020, IBV-EGY-CH-AY5-2020 which had four A.A. (Figure 6). The S1 gene identity of amino acids revealed that viruses in the current study related to vaccine seeds used commonly in Egypt (M41, H120, 4/91) within the range of 68.3-78.8% (Figure 7).

		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	16.4	16.4	16.4	16.8	16.4	16.4	16.4	16.4	16.4	12.9	12.9	█	16
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		

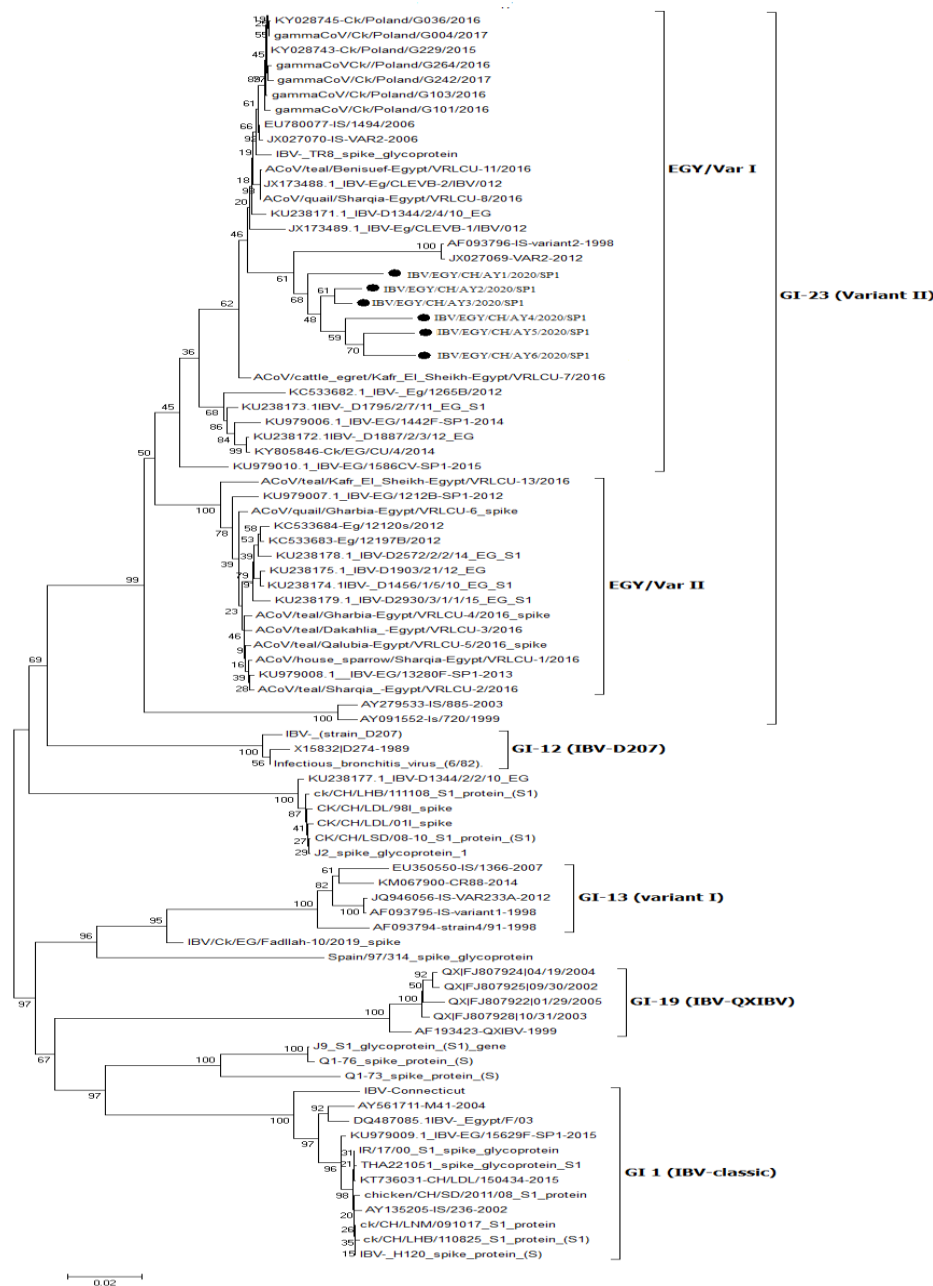
**Figure 2.** Amino acid identities and divergence of HA gene of H5N8 viruses compared to other selected strains and vaccinal strains



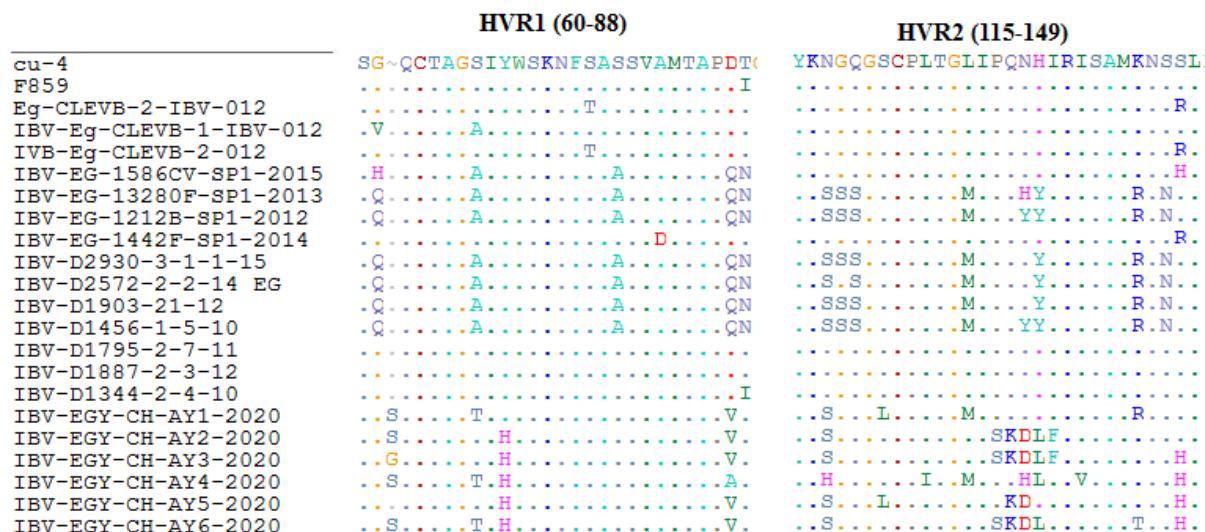
**Figure 3.** Phylogenetic tree of HA gene of H9N2

		Percent Identity																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
Divergence	1	85.3	88.7	92.5	92.1	92.7	93.3	91.3	90.5	87.9	88.1	87.1	89.3	88.9	88.3	87.9	87.5	87.5	88.5	1	A-Quail-HongKong-G1-97
	2	14.8	87.5	84.2	83.6	84.6	84.2	83.6	84.0	83.6	82.4	82.2	83.4	83.2	82.6	82.8	82.4	82.4	82.8	2	A-duck-HongKong-Y439-1997
	3	10.8	12.3	89.5	89.3	89.1	89.5	87.7	89.3	87.1	87.1	86.3	87.5	87.5	87.3	87.1	86.5	86.5	87.9	3	A-chicken-HongKong-G9-1997
	4	6.4	16.5	10.1	98.2	93.3	93.3	91.3	90.7	87.9	88.1	87.9	89.3	88.9	88.3	87.9	87.7	87.7	88.9	4	A-chicken-Israel-421201-2004
	5	6.9	17.2	10.3	0.8	92.9	93.1	91.1	90.5	87.7	88.1	87.7	89.1	88.7	88.1	87.7	87.5	87.5	88.7	5	A-chicken-Israel-178-2006
	6	6.0	15.5	10.4	5.5	6.0	93.1	90.9	91.3	88.3	87.9	87.1	89.3	88.7	88.1	87.9	87.3	87.3	88.7	6	A-Chicken-Iran-TH81-2002
	7	5.5	16.3	10.1	5.8	6.0	5.8	92.7	92.7	89.3	89.1	89.3	90.9	90.5	89.9	89.5	89.5	89.5	90.3	7	A-chicken-Dubai-338-2001
	8	7.8	17.2	12.2	8.0	8.2	8.3	6.4	95.8	92.5	92.3	91.7	94.3	93.7	92.5	92.5	91.7	91.7	92.7	8	A-chicken-Saudi
	9	8.7	16.9	10.5	8.9	9.1	7.8	6.4	3.3	93.7	93.3	93.1	95.8	95.2	93.9	93.7	93.1	93.1	93.7	9	A-chicken-Israel-1067-2010
	10	11.8	17.5	13.2	12.2	12.5	11.3	10.3	6.9	5.9	98.4	95.2	96.2	96.8	97.4	97.8	94.9	94.9	97.6	10	A-chicken-FW1-2020
	11	11.5	19.0	13.2	12.0	12.0	11.8	10.6	7.1	6.4	1.4	95.4	96.0	96.8	97.4	98.2	94.9	94.9	97.6	11	A-chicken-FW2-2020
	12	12.8	19.3	14.2	12.2	12.5	12.8	10.3	7.8	6.6	4.6	4.4	95.2	95.8	95.6	95.6	97.6	97.6	95.8	12	A-chicken-FW3-2020
	13	10.1	17.7	12.5	10.5	10.8	10.1	8.5	4.9	3.6	3.1	3.3	4.2	97.8	96.2	96.0	94.9	94.9	96.2	13	A-chicken-Egypt-2-2014
	14	10.6	18.0	12.7	11.0	11.3	10.8	8.9	5.5	4.4	2.7	2.7	3.8	1.4	97.2	97.0	95.8	95.8	97.0	14	A-chicken-Egypt-7-2015
	15	11.3	18.8	12.9	11.7	12.0	11.5	9.6	6.9	5.7	2.3	2.3	4.2	3.1	2.3	97.8	95.6	95.6	97.8	15	A-chicken-Egypt-v621-2018
	16	11.8	18.5	13.2	12.2	12.5	11.8	10.1	6.9	5.9	2.1	1.6	4.2	3.3	2.5	1.9	95.6	95.6	97.2	16	A-chicken-Egypt-V4001-2018
	17	12.3	19.0	13.9	12.5	12.7	12.5	10.1	7.8	6.6	4.8	4.8	2.1	4.4	3.8	4.2	4.2	99.6	95.8	17	A-chicken-Egypt-V2630-2019
	18	12.3	19.0	13.9	12.5	12.7	12.5	10.1	7.8	6.6	4.8	4.8	2.1	4.4	3.8	4.2	4.2	0.0	95.8	18	A-chicken-Egypt-V2633-2019
	19	11.1	18.5	12.2	11.0	11.3	10.8	9.2	6.6	5.9	2.1	2.1	4.0	3.1	2.5	1.9	2.5	4.0	4.0	19	A-chicken-Egypt-V2692-2019
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		

**Figure 4.** Amino acid identities and divergence of HA gene of H9N2 viruses compared to other selected strains and vaccinal strains



**Figure 5.** Phylogenetic tree of partial S1 gene of infectious bronchitis virus



**Figure 6.** Amino acid mutation in HVRS in S1 gene of infectious bronchitis virus

		Percent Identity																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
Divergence	1	█	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	1	IBV-H120
	2	10.1	█	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	2	IBV-M41
	3	34.4	25.2	█	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	3	IBV-D207
	4	30.2	30.4	40.0	█	67.0	64.9	64.9	66.7	67.0	67.0	69.1	70.0	66.0	71.7	70.1	71.7	72.2	71.7	72.2	68.0	4	IBV-QXIF
	5	36.2	33.9	39.6	43.7	█	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	5	IBV-UK-4-91
	6	33.7	36.7	27.2	43.6	32.2	█	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	6	IBV-IS-1494-06
	7	31.1	35.2	26.6	42.2	32.4	5.8	█	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	7	IBV-VAR2
	8	35.7	39.2	30.5	44.1	41.1	21.2	23.6	█	80.0	81.7	83.3	83.3	81.7	81.7	83.3	85.0	85.0	83.3	83.3	85.0	8	IBV-IS-885
	9	32.9	38.4	27.1	39.7	34.2	2.6	8.1	23.4	█	98.2	91.7	83.8	96.7	98.6	96.7	93.5	95.0	93.5	96.7	94.9	9	IBV-cu-4
	10	33.0	37.0	26.6	39.9	33.5	2.6	7.2	21.1	1.7	█	93.3	84.5	98.3	98.9	98.3	93.1	96.7	93.1	98.3	94.6	10	IBV-EG-CLEVB-1
	11	23.3	21.1	23.3	38.4	38.4	5.2	12.8	19.1	8.9	7.0	█	95.0	93.3	93.3	95.0	93.3	93.3	95.0	95.0	93.3	11	IBV-EG-13280F
	12	40.3	36.2	14.5	36.4	38.0	19.3	22.9	18.9	18.7	18.1	5.2	█	98.3	84.1	100.0	82.3	98.3	83.8	100.0	83.8	12	IBV-EG-1212B
	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	█	98.3	98.3	96.7	96.7	98.3	98.3	96.7	13	IBV-EG-1442F
	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	█	98.3	93.5	96.7	93.1	98.3	94.9	14	IBV-D1344-2-4-10
	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	█	98.3	98.3	100.0	100.0	98.3	15	IBV-EGY-AY1-2020
	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	█	100.0	94.6	98.3	97.1	16	IBV-EGY-AY2-2020
	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	█	98.3	98.3	100.0	17	IBV-EGY-AY3-2020
	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	█	100.0	93.9	18	IBV-EGY-AY4-2020
	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	0.0	█	98.3	19	IBV-EGY-AY5-2020	
	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	█	20	IBV-EGY-AY6-2020
	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 infectious bronchitis virus sequenced viruses compared to other selected strains and vaccinal strains

## DISCUSSION

In Egypt during the last years, the outbreaks due to respiratory disease with high mortality rates have increased and affect poultry production. 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 chickens (Hassan et al., 2016). The current study aimed to identify the incidence of viral respiratory disease in broiler chickens, especially AIV (HP, LP), IBV, and NDV in Egypt during 2020, and study the molecular characterization and evolution of detected viruses.

In the present study, multiple respiratory viruses as single or mixed infections were detected. The AIV subtypes and IBV were recorded in 48 out of 53 farms. Single infection represented 90.6% (37.7% I.B, 30.2% H5N8, 9.4% I.B and 5.7% NDV). Furthermore, co-presence of HPAI (H5N8) and IBV, unique detection of these co-infected flocks, and LPAI (H9N2) and IBV, were detected in 3.8% as previously recorded by El-Shall et al. (2019). The occurrence of IBV and HPAI (H5N8) in Egyptian poultry with a high mortality rate in different vaccinated poultry flocks has previously been reported by a number of researchers (Abdel-Moneim et al., 2006a, Abd El Rahman et al., 2015; Yehia et al., 2020). It was indicated the high incidence of respiratory disease in broiler chicken during 2020 in single infection more than co-infected with other viruses.

In recent years, the avian influenza situation in Egypt has been more complicated due to the detection and circulation of many serotypes, including HPAI (H5N1), HPAI (H5N8), and LPAI (H9N2) (Shehata et al., 2019). The



phylogenetic analysis of four selected samples of the HA gene of HPAI H5N8 revealed that the four samples were cluster to clad 2.3.4.4.1b as previously recorded by Shehata et al. (2018) and El-Shall et al. (2019) in a new subgroup with characteristic specific mutations due to multiple mutations that may affect the pathogenicity and vaccine efficacy.

Considering mutation analysis, a change was detected in the (T140A) antigenic site A in two samples of A/chicken/Egypt/FN1/2020 and A/chicken/Egypt/FN2/2020 as previously reported by Yehia et al. (2020) that may be due to excessive use 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 mentioned by Nabil et al. (2020).

The A.A. identity was very low in H5N1 and H5N2 vaccine reaching 91.9-94% and 84.6%, respectively, similar to previous studies (Kandeil et al., 2018; Yehia et al., 2020). It explained where the outbreaks occurred in the vaccinated flocks and causing high mortality.

The phylogenetic analysis of three LPAI (H9N2) selected strains revealed that all viruses in the current study belonged to A/quail/Hong Kong/G1/97-like virus lineage clustered with group B as previously recorded (Kareem et al., 2015; El-Shall et al., 2019). Regarding mutation analysis, the LPAI (H9N2) Egyptian viruses had six A.A. mutation-specific. In addition, I75V, N41G, and T413N mutation specific to 2018-2019 Egyptian viruses and 212I mutation that recorded previously had an important effect on the increased pathogenicity of the H9N2 in mice as previously recorded (Yang et al., 2014) that may affect the human transmission of the virus. So, there is a need to conduct further studies to detect the effect of these mutations on the pathogenicity of the virus and vaccine efficacy in poultry.

In the current study, a high number of infected vaccinated flocks with IBV viruses by RT-PCR was recorded. Therefore, partial S1 gene sequencing is important to identify the IBV strains. Furthermore, the partial IBV S1 gene was sequenced, and the analysis revealed that six selected viruses were related to clad GI23 (Egy Var I) as previously indicated (Hassan et al., 2016; Zanaty et al., 2016; Abozeid et al., 2017). The Egyptian strains in this study had low identity percent with vaccine strains H120, M41, 4/91 ranging 68.3-78.8% that lead to the appearance of infection in vaccinated farms as previously recorded (Abd El Rahman et al., 2015; Sultan et al., 2019). Accordingly, future studies should be conducted to evaluate vaccine efficacy and update the vaccine used to give high protection against newly mutated strains.

The S1 subunit, which comprises three major hypervariable regions (HVRs) in the first 395 amino acids. The HVRs contain major antigenic sites between 38 and 67 amino acids for HVR-1, 97 and 141 amino acids for HVR2, and 274 and 387 amino acids for HVR-3 (Moore et al., 1997). The present study indicated multiple A.A. mutations in the HVR as previously recorded (Hassan et al., 2016; Zanaty et al., 2016; Abozeid et al., 2017) that may be due to widespread use of heterologous vaccine leading to vaccination pressure, which increases the pathogenicity of the virus and changes in tissue tropism (Cavanagh et al., 1992).

## CONCLUSION

Infectious bronchitis virus and highly pathogenic avian influenza (H5N8) are the main causes of respiratory disease in Egyptian flocks alone or co-infected with other viruses with a high mortality rate. Circulation of avian influenza (H5N8) was detected with a mutation in the antigenic site and receptor binding site, low pathogenic avian influenza (H9N2) with a specific mutation, and infectious bronchitis viruses were rapidly evolved in hypervariable regions. The infectious bronchitis virus and highly pathogenic avian influenza H5N8 viruses were 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.

## DECLARATIONS

### Authors' contribution

Nahed Yehia contributed to molecular characterization and data analysis and the write-up of the manuscript and Fatma Amer contributed to sample collection and diagnosis.

### Competing interests

The authors have not declared any conflict of interest.

### Consent to publish

All the authors approved and agreed to publish the manuscript.

### Ethical consideration

All authors approved the final draft of the manuscript for publication. Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

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