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Molecular Characterization and Phylogenetic Analysis of Full-length S1 Gene of GI-16 and GI-23 Infectious Bronchitis Virus in Qena, Egypt

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ABSTRACT

Infectious Bronchitis Virus (IBV) is a highly evolving virus that affects respiratory, urinary and reproductive systems. This virus is recognized as an important pathogen due to the continuous genesis of new variants that threaten the poultry industry worldwide. The aim of this study was to characterize emerging IBV variants originated from field outbreaks in Qena province, Egypt, and to study their genetic relationships with global strains. From September 2017 to January 2019, 52 field samples were collected from broiler flocks suspected of being infected with IBV. The collected samples were inoculated into embryonated chicken eggs via allantoic route for virus isolation. The IBV presence was confirmed using real-time reverse transcriptase PCR (rRT-PCR) assay targeting nucleocapsid (N) gene and finally, nine samples were selected from 29 positive samples with rRT-PCR for further genetic characterization through full-length spike (S1) gene sequencing. Phylogenetic analyses indicated that one isolate (IBV/CK/EG/QENA-4/2017) clustered within genotype I lineage 16 (GI-16). On the other hand, the remaining eight isolates (2017-2018) belonged to genotype I lineage 23 (GI-23) and clustered separately in monophyletic clade. The isolates in this study were found to share only 74.6-82.1% amino acid identity with the commonly used vaccine strains in Egypt. In conclusion, findings of this study provide informative data on circulating IBVs in the study area and highlight the importance of adopting a convenient vaccination strategy that can be more efficient for controlling the emergence of new IBV variants.

Key words: Full-length spike gene, GI-16, GI-23, Infectious bronchitis virus, Phylogenetic, RT-PCR.

INTRODUCTION

Avian infectious bronchitis (IB) is a highly contagious acute viral disease of chickens that is of great economic importance in the poultry industry. This disease is caused by Infectious Bronchitis Virus (IBV) and the upper respiratory tract is considered the main site for virus replication (Raj and Jones, 1997; Jackwood and de Wit, 2013). However, some IBV strains have also a great affinity to replicate in the reproductive tract and the kidneys, as well as some other strains have been reported to replicate in other tissue such as proventriculus (Yu et al., 2001).

IBV is a member of genus *Gammacoronavirus* within family *Coronaviridae*. The virus has a positive sense, enveloped, single-stranded and non-segmented RNA genome (Boursnell et al., 1987; Cavanagh, 2007), consisting of regions that code for four structural proteins including the nucleocapsid protein (N), the membrane glycoprotein (M), the envelope protein (E), and the spike glycoprotein (S). It also includes regions 1a and 1ab expressing the replicase gene, in addition to that it comprises several accessory regions (Spaan et al., 1988; Masters, 2006; Jackwood and de Wit, 2013). The N gene is highly conserved even among IBV isolates of different serotypes, therefore, it is often chosen as the target gene for virus detection by real-time reverse transcriptase PCR (rRT-PCR) assay (Meir et al., 2010; Bande et al., 2016).

After translation, the S glycoprotein is cleaved into two subunits, S1 and S2 (Perlman and Netland, 2009). The S1 subunit encloses not only in the infectivity of the virus but also contains virus neutralization and serotype-specific epitopes which are located in three different Hyper Variable Regions (HVRs). These epitopes are responsible for the induction of neutralizing antibodies and immune responses (Cavanagh et al., 1988; Moore et al., 1997). The nucleotide sequence variation in the S1 gene may lead to lower cross-protection against serotypes and can modify the protection ability of a vaccine or immunity (Cavanagh and Gelb, 2008). Therefore, the nucleotide sequence of S1

gene is used to classify IBV strains and to identify new IBV variants that may challenge vaccination protocols (De Wit et al., 2011; Valastro et al., 2016). Recently, Valastro et al. (2016) proposed a unified IBV classification system based on S1 phylogeny that divided IBV strains into six main genotypes from GI to GVI comprising 32 viral lineages.

IBV was first documented in Egypt by Ahmed (1954) and since then, several IBV strains with continuous diversity and recombination have been reported (Abdel-Moneim et al., 2006; Zanaty et al., 2016). The majority of IBV strains circulating in Egypt especially in the last years clustered into two distinct phylogenetic groups, the GI-1 lineage which contains classical strains and the GI-23 lineage that contains field IBV variants which was further sub-divided into Egy/var I and Egy/var II which are related to IS/1494 and IS/885 (Abdel-Moneim et al., 2002; Abdel-Moneim et al., 2012; Zanaty et al., 2016). In spite of this fact, other IBV lineages such as GI-12 (Abdel-Moneim et al., 2006; Valastro et al., 2016), GI-13 (Rohaim et al., 2019) and GI-16 (Q1 like strains) (Kiss et al., 2016; Abdel-Sabour et al., 2017) have been reported in Egypt.

Although chicken flocks are routinely vaccinated with live attenuated vaccines, outbreaks of IB in vaccinated flocks have been occurred, since there is little or no cross-protection among various serotypes of IBV (Reddy et al., 2015). The present study provided a monitoring data regarding the molecular characteristics, evolutionary relationship and genetic diversity of IBV strains isolated from chickens in Qena province as one of the southern provinces of Egypt in view of the fact that the majority of scientific research in Egypt focused on studying the IBV in the north and middle of Egypt.

MATERIAL AND METHODS

Ethical approval

This research did not involve the introduction of any intervention in/on birds, but the direct collection of tissue samples from freshly dead birds was conducted in full compliance with the recommendations of Faculty of Veterinary Medicine, South Valley University, Qena, Egypt for the care and use of laboratory animals.

Sampling and flocks' history

Fifty-two tissue samples (lung, trachea, and kidneys) were collected during the period from 2017 to 2019 from nine broiler chicken flocks in Qena province, South Egypt. These flocks were suspected of being infected with IBV and exhibited IB respiratory manifestations such as nasal discharge, sneezing, coughing, bronchial rales, gasping, tracheitis, airsacculitis, lung congestion, caseous materials in the trachea and/or nephropathogenic lesions such as pale enlarged kidneys with prominent tubular pattern. Most of these flocks had been previously vaccinated against IBV with one or more of vaccines of H120, H120 + D274, 1/96 and M41. The collected samples were labeled, stored on ice, transported to the Poultry Disease Department laboratory, Faculty of Veterinary Medicine, South Valley University, Egypt where kept frozen at -80 °C for further processing.

Egg inoculation and virus isolation

Collected tissue samples from each IBV-suspected flocks were homogenized in PBS (10% w/v) containing 5,000 IU /ml penicillin G, 5 µg/ml amphotericin B, and 5 mg/ml streptomycin (Sigma Chemical Co., USA). The homogenates were then centrifuged at 3000 rpm for 10 min after incubation at 4°C overnight and then 200 ul of the supernatant from each sample was inoculated into three 9-11-day-old embryonated chicken eggs. The allantoic fluid was harvested at 5-7 days post-inoculation and used for subsequent passages. The embryos were evaluated for gross lesions at each passage and this was performed as described by Guy (2008).

RNA extraction and real-time reverse transcriptase PCR

The genomic viral RNA of 52 samples were extracted from the harvested infected allantoic fluid using QIAmp® Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Presence of IBV in all samples was checked by rRT-PCR targeting the N gene of IBV using QuantiTect® probe RT-PCR kits (Qiagen, Hilden, Germany), and the reactions were carried out on Agilent Mx3005P thermocycler machine (Life Technologies, USA) using two specific primers, (AIBV-fr): 5'- ATG CTC AAC CTT GTC CCT AGC A -3' and (AIBV-as) 5'- TCA AAC TGC GGA TCA TCA CGT -3' and probe (AIBV-TM) 5'- [FAM] TTG GAA GTA GAG TGA CGC CCA AAC TTC A [TAMRA] -3' to amplify a 130 bp fragment of N gene as previously described by Meir et al. (2010) in the following conditions: 50 °C for 30 min, one cycle at 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 45 sec.

Reverse transcriptase PCR and full S1 sequencing

RT-PCR was performed to amplify the full S1 gene using Qiagen one step RT-PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, using two specific primer pair sets (Table 1) in two overlapping PCR fragments. The RT- PCR using the first primer pair was performed under the following conditions: 50°C for 30 min, then 95°C for 15 min, 35 cycles (94°C for 1 min, 54°C for 45 s, 72°C for 1 min), 72°C for 10 min, while the same conditions were used with the second primer pair except the number of cycles were 40 cycles and the extension time changed to be 72°C for 1.30 min. The PCR product was analyzed by electrophoresis on 1.2% agarose gel stained with SYBR Green and visualized using an ultraviolet transilluminator. After gel electrophoresis confirmation, the PCR products were sent for sequencing by a commercial service provider (Macrogen, Inc., South

Korea) where the PCR products were purified and sequenced in both forward and reverse directions using the same primer pair sets.

Table 1. Primers used in this study to amplify the full-length spike glycoprotein (S1) gene

Name	Sequence (5'-3')	Band size (base pair)	Reference
SF 1-1	GCCAGTTGTTAATTTGAAAAC	980	(Pohuang et al., 2011; Yousefi et al., 2019)
SR 1-1	TAATAACCACTCTGAGCTGT		
SF 1-2	ACTGGCAATTTTTCAGATGG	1065	
SR 1-2	AACTGTTAGGTATGAGCACA		

Sequence analysis and phylogenetic analysis

All chromatograms were analyzed, assembled using DNA Baser Assembler v5.15.0 software. The sequences obtained in the current study together with the other representative IBV sequences obtained from NCBI GenBank (n=106) were aligned based on the nucleotide sequences with MUSCLE criterion (Edgar, 2004), implemented in MEGA6 software (Tamura et al., 2013). Prior to phylogenetic analysis, Gblocks software (Talavera and Castresana, 2007) was used to remove all potentially poorly aligned regions. For this analysis, a less stringent procedure, allowing for gap positions within final blocks, was applied. The phylogenetic analysis based on the full S1 gene was carried out with MEGA6 software using Maximum likelihood method with the general time reversible model as implemented in MEGA6 and a discrete gamma distribution with 1000 bootstrap replicates (Felsenstein, 1985) was used in the data analysis to assess the robustness of the branches. Evolutionary distances between the studied sequences and the reference and vaccine strains were inferred using the full S1 dataset, with pairwise comparisons of nucleotide sequences performed using BioEdit software v7.0.5.3 (Hall, 1999). Multiple alignment of deduced amino acid sequences with the amino acid sequences of the most currently used vaccine strains in Egypt was carried out using BioEdit software v7.0.5.3 (Hall, 1999). The nucleotide sequences obtained in this study were submitted to the NCBI GenBank to assign accession numbers (Table 2).

RESULTS AND DISCUSSION

Virus isolation and identification

Similar to other RNA viruses, the IBV virus is constantly evolving and mutating (Jackwood, 2012). Studies of genetic diversity and the relationships among other viruses circulating globally are very important for tracking the circulation of viruses and for better understanding of how isolates evolve to give rise to new variants of IBV. In this study, inoculation of collected tissue samples in embryonated chicken eggs revealed the ability of many isolated samples to induce typical IBV lesions such as stunting, curling, dwarfing, abnormal feathering and subcutaneous congestion (De Wit, 2000; Guy, 2008) in some inoculated embryos. Testing of harvested allantoic fluids for IBV by using rRT-PCR assay revealed that 29 out of 52 samples were positive for IBV and 9 out of 29 positive samples were selected to be amplified with one-step RT- PCR assay for full S1 sequencing and genetic characterization (Table 2).

Distance and phylogenetic analysis of full-length S1 gene

Recently, genetic characterization based on the full S1 gene of IBV has become the primary method for classifying IBV strains because of its functional significance and heterogenicity (Valastro et al., 2016). The phylogenetic analysis based on the full S1 sequences representing different IBV strains (n=106) demonstrated that all nine isolates in this current study were variants and none of them were classic or of vaccine origin. One of those nine isolates named (IBV/CK/EG/QENA-4/2017) clustered with other isolates from Italy, China, South Korea, Taiwan, Vietnam, and Peru in GI-16 (Valastro et al., 2016) (Figure 1). Interestingly, this isolate, which was isolated from a unvaccinated broiler flock, was very closely related to other strains isolated in China (GU938413.1, HM363027.1, AF286302.1 and AF286303.1), Italy (KP780179.1) and Vietnam (KY992863.1) (Yu et al., 2001; Zou et al., 2010; Ji et al., 2011; Franzo et al., 2015; Le et al., 2019) and showed high levels of nucleotide (99.7-99.9%) and deduced amino acid (99.2-99.8%) identities. These very high identities based on the nucleotide and amino acid sequences with other viruses indicate a common origin among these viruses.

The GI-16 lineage previously identified as Q1-like IBV or CK/CH/LDL/97I-type IBV was first identified in China in 1995 from layer flock with proventriculitis (Yu et al., 2001). The strains of this lineage were isolated regularly from both vaccinated and non-vaccinated chicken flocks (Yu et al., 2001; Luo et al., 2012). Also, in 2011, the GI-16 lineage was isolated from three different Middle Eastern countries (Jordan, Saudi Arabia, and Iraq) from chicken flocks suffered from respiratory manifestations, kidney affections and decrease in egg production (Ababneh et al., 2012), and in Egypt, the strains of this lineage were isolated from broiler flocks suffered from respiratory symptoms associated with renal lesions and increased mortality (Kiss et al., 2016; Abdel-Sabour et al., 2017).

The remaining eight isolates were clustered together in a separate monophyletic branch within GI-23 (Valastro et al., 2016) (Figure 1), suggesting that IBV circulating in this area is undergoing evolution. These eight variant isolates were found to be highly related among themselves with 95.4-99.9% and 94.1-99.8% nucleotide and amino acid sequence identities, respectively (Table 3). The percentage of the nucleotide and amino acid sequence identities (87.49-93.9% and 81.2-92.2%, respectively) among these eight isolates and the rest of the GI-23 viruses used in the construction of the phylogenetic tree showed higher nucleotide and amino acid diversity within the lineage.

GI-23 lineage of IBV was first recognized in Israel in 1998 from chickens suffering from respiratory and kidney lesions (Meir et al., 2004; Valastro et al., 2016) then spread rapidly to Egypt and other Middle East countries. For nearly 20 years, the strains of GI-23 have been geographically limited to Middle East countries, but have recently spread to some European countries (Valastro et al., 2016; Lisowska et al., 2017; Fischer et al., 2019). In Egypt, GI-23 lineage has become the most prevalent lineage in the field as the majority of circulating IBV variant strains reported in chickens belong to this lineage as stated in many studies (Awad et al., 2014; Valastro et al., 2016; Zanaty et al., 2016; Abdel-Sabour et al., 2017; Abozeid et al., 2017; Naguib et al., 2017), Which is consistent with the results obtained in this study.

Compared to the commonly used vaccine strains in Egypt, the nine isolates in this study showed different levels of nucleotide (77.4-82.3%) and amino acid (74.2-81.8%) identities to the H120, Ma5, Mass41, 4/91, CR88, D274 and Israel variant 1(1/96) strains. The vaccine strain D274 shared the highest nucleotide amino acid identities among the vaccine strains with isolates obtained in this study. In addition to the pairwise identity, the phylogenetic analysis revealed that these nine variant isolates had a far distant relation to these vaccine strains. The high sequence differences between our isolates and the commonly used vaccine strains in Egypt may explain the reason for the failure of the vaccines to protect against challenge with these field strains.

On the other hand, there are other factors should be taken in consideration such as immunocompromised chicken flocks with other pathogens (Cheng et al., 2018), lack of bio-security (Jackwood and Lee, 2017) as well as the improper application of vaccines (Magouz et al., 2018). However, the frequent evolution of novel IBV variants is still the main cause of vaccination failure (Reddy et al., 2015; Khataby et al., 2016).

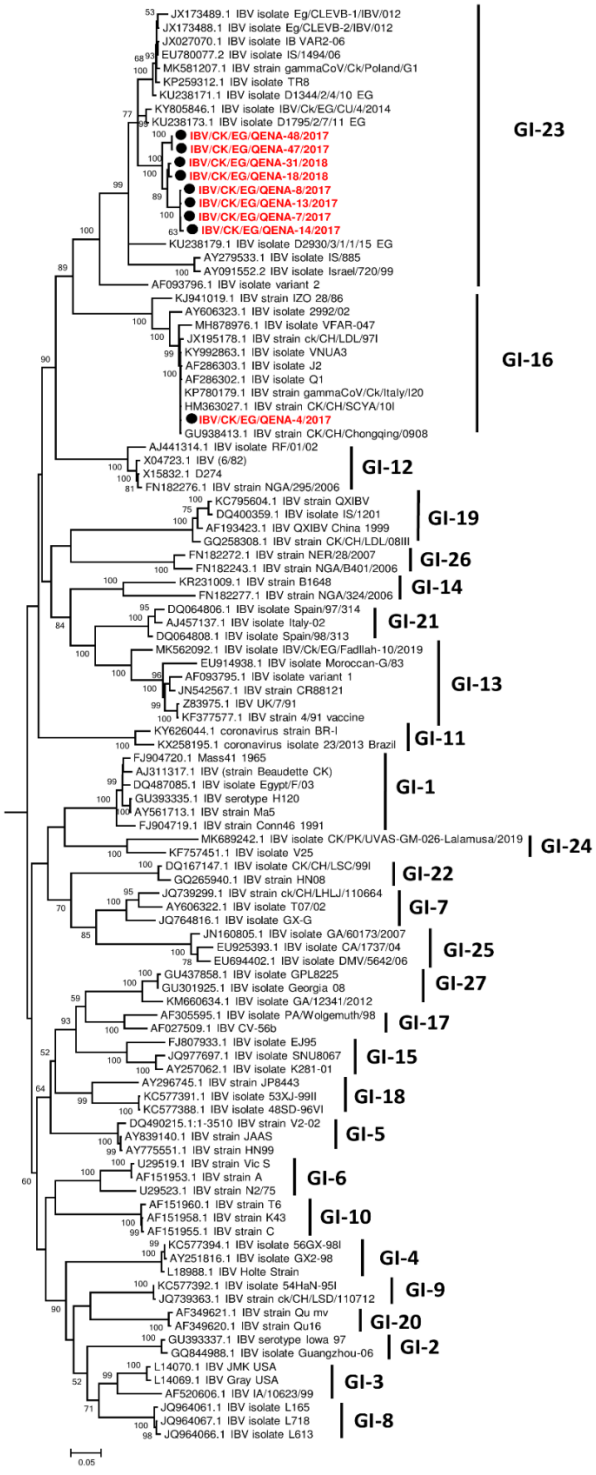

Figure 1. Phylogenetic analysis based on a full-length nucleotide sequence of the S1 gene, showing the relationship among the isolates in this study and other infectious bronchitis virus strains retrieved from Genbank. The tree was constructed using the maximum-likelihood method with (GTR+G+I) model and 1000 bootstrap replicates using MEGA 6 software. There were a total of 1570 positions in the final dataset. The isolates sequenced in this study are highlighted in bold font with red color.

Table 2. Flocks data sampled for infectious bronchitis virus isolates used for full-length spike glycoprotein (S1) gene characterization

No.	Isolate identification	Flock age (day)	Vaccines used in flock against infectious bronchitis	Collection date	Flock location	Signs and postmortem lesions	Accession No.
1	IBV/CK/EG/QENA-4/2017	29	Non vaccinated	November 2017	Qena- Dishna	Sever respiratory manifestations	MN890126
2	IBV/CK/EG/QENA-7/2017	29	M41	September 2017	Qena- Abu Tesht	Kidney damage with high morbidity and mortality rates	MN890127
3	IBV/CK/EG/QENA-8/2017	29	M41	September 2017	Qena- Abu Tesht	Kidney damage with high morbidity and mortality rates	MN890128
4	IBV/CK/EG/QENA-13/2017	29	M41	September 2017	Qena- Abu Tesht	Kidney damage with high morbidity and mortality rates	MN890129
5	IBV/CK/EG/QENA-14/2017	29	M41	September 2017	Qena- Abu Tesht	Kidney damage with high morbidity and mortality rates	MN890130
6	IBV/CK/EG/QENA-18/2018	17	H120	March 2018	Qena- Dishna	Sever respiratory manifestations	MN890131
7	IBV/CK/EG/QENA-31/2018	32	H120 & 1/96	December 2018	Qena- Qus	Sever respiratory manifestations	MN890132
8	IBV/CK/EG/QENA-47/2017	32	H120 + D274	December 2017	Qena- Abu Tesht	Kidney damage with high morbidity and mortality rates	MN890133
9	IBV/CK/EG/QENA-48/2017	32	H120 + D274	December 2017	Qena- Abu Tesht	Kidney damage with high morbidity and mortality rates	MN890134

Table 3. Nucleotide and amino acid identities of full-length spike glycoprotein (S1) gene sequence of the nine infectious bronchitis virus isolates in this study with other Egyptian strains, reference strains and vaccine strains.

		Amino acid identity (%)																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	
1	GU393335.1_H120		99.8	96.4	78.6	75.4	75.0	75.2	75.9	77.5	76.3	75.8	75.8	75.8	75.9	75.6	75.0	75.4	75.8	75.4	75.6	**1**
2	AY561713.1_Ma5	99.8		96.2	78.4	75.2	74.8	75.0	75.9	77.5	76.3	75.6	75.6	75.8	75.9	75.6	75.0	75.4	75.8	75.4	75.6	**2**
3	FJ904720.1_Mass41_1965	97.6	97.7		77.8	74.8	74.2	74.6	74.8	76.7	75.6	75.4	75.4	75.0	74.8	74.8	74.2	74.6	75.0	74.6	74.8	**3**
4	X15832.1_D274	80.9	80.9	80.6		80.1	79.0	78.6	79.3	82.9	83.5	81.4	81.6	81.8	81.6	81.4	81.0	81.2	81.4	81.4	81.6	**4**
5	JN542567.1_CR88121	78.1	78.0	78.4	79.6		93.1	95.4	76.7	79.0	80.3	77.8	77.8	80.3	79.9	79.7	79.3	79.0	79.2	79.0	79.2	**5**
6	KF377577.1_4/91	78.6	78.6	78.9	79.1	96.6		92.2	77.5	77.6	79.2	77.3	77.3	79.5	79.5	79.3	78.4	78.0	78.6	78.0	78.2	**6**
7	AF093795.1/IS variant1 (1/96)	78.0	78.0	78.3	79.4	97.4	95.5		76.9	78.8	79.3	77.6	77.6	79.7	79.7	79.5	78.6	78.6	78.8	78.6	78.8	**7**
8	AY279533.1_IS/885_S1	78.6	78.6	78.6	78.9	77.9	78.6	77.7		87.9	87.9	79.7	79.7	87.1	87.1	86.7	86.2	86.0	86.7	86.3	86.5	**8**
9	KY805846.1 EG/CU/4/2014	80.3	80.1	80.3	82.8	78.8	78.5	79.0	88.4		94.5	81.6	81.8	91.6	91.3	91.1	90.7	92.4	92.0	92.4	92.6	**9**
10	EU780077.2_IS/1494/06	80.1	79.9	79.9	83.1	79.2	79.2	79.2	88.1	95.4		82.0	82.2	91.4	91.1	90.9	90.5	91.8	91.8	91.8	92.0	**10**
11	AF286302.1_Q1	77.5	77.4	78.1	80.7	78.6	78.8	78.9	80.1	82.1	81.6		99.6	82.4	82.4	82.0	81.8	80.9	81.2	82.4	82.6	**11**
12	IBV/CK/EG/QENA-4/2017	77.5	77.4	78.1	80.6	78.6	78.8	78.9	80.2	82.2	81.6	99.8		82.4	82.4	82.0	81.8	81.0	81.4	82.6	82.7	**12**
13	IBV/CK/EG/QENA-7/2017	79.6	79.4	79.6	82.3	79.1	79.4	79.0	87.4	92.3	92.6	81.5	81.5		99.6	99.4	98.8	96.5	96.9	95.2	95.4	**13**
14	IBV/CK/EG/QENA-8/2017	79.7	79.5	79.6	82.1	79.0	79.4	79.0	87.4	92.2	92.5	81.4	81.4	99.8		99.4	98.4	96.7	97.1	94.8	95.0	**14**
15	IBV/CK/EG/QENA-13/2017	79.5	79.3	79.5	82.1	78.9	79.3	78.9	87.3	92.1	92.5	81.3	81.3	99.8	99.8		98.2	96.4	96.7	94.7	94.8	**15**
16	IBV/CK/EG/QENA-14/2017	79.2	79.0	79.2	82.0	78.8	79.1	78.7	87.0	91.9	92.2	81.1	81.1	99.5	99.4	99.3		95.4	95.8	94.1	94.3	**16**
17	IBV/CK/EG/QENA-18/2018	79.6	79.4	79.6	82.0	79.0	78.9	78.9	87.4	93.6	93.4	81.4	81.4	97.1	97.2	97.1	96.7		99.2	96.2	96.4	**17**
18	IBV/CK/EG/QENA-31/2018	79.7	79.5	79.7	81.9	78.8	78.9	78.7	87.6	93.6	93.5	81.3	81.3	97.2	97.3	97.2	96.8	99.3		96.2	96.4	**18**
19	IBV/CK/EG/QENA-47/2017	79.4	79.3	79.4	82.2	78.9	78.9	78.8	87.2	93.7	93.0	82.0	82.1	95.8	95.7	95.6	95.4	97.4	97.2		99.8	**19**
20	IBV/CK/EG/QENA-48/2017	79.4	79.4	79.5	82.3	79.0	78.9	78.9	87.2	93.7	93.0	82.1	82.1	95.9	95.7	95.7	95.4	97.4	97.3	99.9		**20**
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	
Nucleotide identity (%)																						

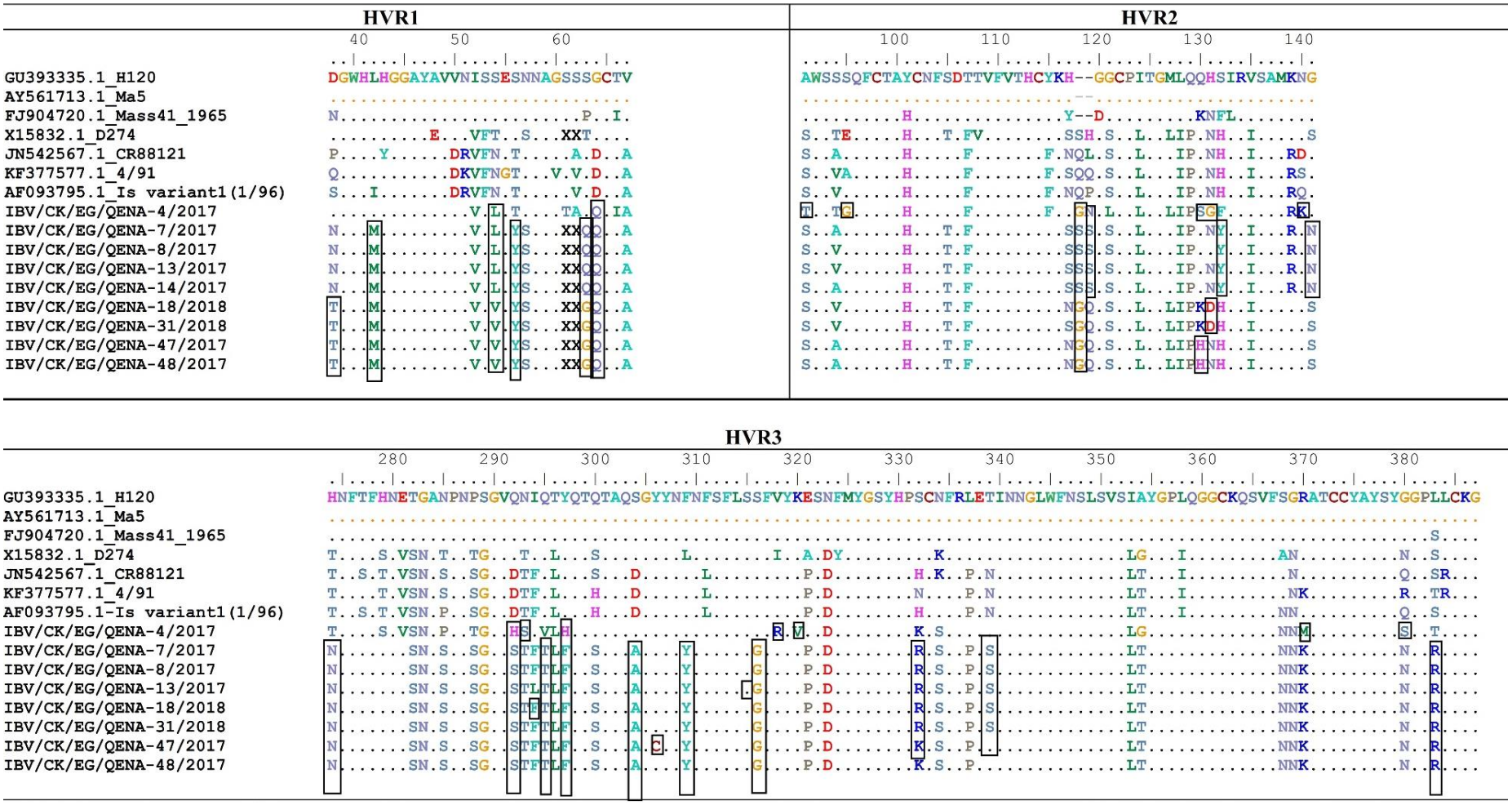

Figure 2. Multiple alignment of deduced amino acid sequences of hypervariable regions (HVR) from the infectious bronchitis virus (IBV) isolates in this study and some IBV vaccine strains. A dot indicates an identical amino acid with H120 strain. A dash indicates an amino acid deletion, "X" means any gap within the codon not translated to a valid amino acid.

Alignment analysis of deduced amino acid

The S1 protein contains three HVRs associated with serotype specificity and virus-neutralizing epitopes and located within the amino acid residues 38–67, 91–141, and 274–387 (Moore et al., 1997; Wang and Huang, 2000). In this study, full-length S1 gene sequences of obtained isolates were translated and aligned with the amino acid sequences of the most currently used vaccine strains in Egypt. Based on the alignment in three HVRs of S1, the nine isolates showed unique amino acid differences in comparison to the commonly used vaccine strains (Figure 2). It is well known that even small changes in the amino acid sequence of the spike protein can contribute to the generation of new antigenic types that can alter the protective ability of a vaccine (Adzhar et al., 1997; Casais et al., 2003).

CONCLUSION

The present study provided a robust depiction of genetic characteristics of IBVs isolated from chickens in Qena province, Egypt as well as the evidence for the emergence of IBV variants in vaccinated and unvaccinated broiler flocks. This study demonstrated the circulation of two IBV variants lineages (GI-16 and GI-23). The genetic variability among studied isolates and commonly used vaccine strains can explain the poor vaccination performance and disease outbreak in this region. The continuous disease monitoring and surveillance are required not only to elucidate sequence characteristics of prevailing strains but also to revise appropriate vaccine strategies. These data will be essential as a step for selecting appropriate vaccine strains as well as planning for future vaccine strategies.

DECLARATIONS

Authors' contributions

All authors contributed equally to this work.

Competing interests

The authors declare that there is no competing of interests.

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