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RESEARCH ARTICLE

Molecular and Histopathological Investigation of Avian Infectious Bronchitis Virus in the Delta of Egypt between 2016 and 2017

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Infectious bronchitis virus (IBV) is a highly contagious viral disease of Family Coronaviridae. The current study reports the molecular and histopathological investigation of IBV strains emerged in outbreaks in the Nile Delta of Egypt. Tissue specimens from thirty-six chicken flocks were subjected to virus isolation, histopathology and PCR. Twenty-three isolates were confirmed as IBVs based on the amplification of the highly conserved nucleocapsid (N) gene. Further characterization of five selected isolates was done by amplification of the (S1) glycoprotein gene. Phylogenetic analysis revealed that the isolated strains were clustered into three distinct groups within the variant II clade. Alignment of S1gene amino acid sequences showed significant amino acid substitutions in relation to the currently used vaccinal strains which displays that new IBV variants which are evolutionarily distant from vaccinal strains are still emerging within the Egyptian chicken flocks and are mostly associated with acute tubular nephrosis syndrome.

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INTRODUCTION

Poultry production in Egypt has become an industry, rather than an agricultural activity and is achieving an annual higher growth rate. However, it is faced with viral infections especially viruses affecting the respiratory tract which cause significant economic losses (Hassan *et al.*, 2016).

Infectious bronchitis virus (IBV) is considered one of the most prevalent respiratory viruses influencing the global poultry industry due to its highly contagious nature, continuous emergence of new variants and the evolution of specific tissue tropism (Liu *et al.,* 2017; Yang *et al.*, 2018). In the last two decades, Egyptian poultry industry has been greatly affected with IBV epidemics which are associated with high mortalities within the affected flocks. In addition, poor weight gains, condemnation at processing, decreased egg production and bad quality eggs increase the financial losses (Hasan *et al.*, 2002).

IBV infections are mainly characterized by respiratory manifestations and renal disorders with varying degrees (Liu *et al.*, 2015; Aslam *et al.*, 2016). Even in nephropathogenic strains, the virus replicates first in the tracheal cells, causing histological lesions similar to those induced by the respiratory strains. The nephritic form of IB is characterized by mild respiratory signs followed by depression, diarrhea and sever weight loss (Bayry *et al.*, 2005).

IBVs are enveloped viruses with positive sense, single stranded RNA genome of 27 kb length belonging to family *Coronaviridae* (Mahmood *et al.*, 2004). The virion consists of three major structural proteins; nucleocapsid (N), membrane (M) and the spike glycoproteins (S). The S1 subunit of the (S) glycoprotein is responsible for the serotype-specific antibodies and has more sequence variability than S2 subunit. Thus, genetic characterization of the IBV greatly depends on analysis of this unit (Abdel-Sabour *et al.*, 2017).

Evolution of new IBV variants is an ongoing process which is associated with frequent point mutations in the nucleotide sequences of the S1 subunit mainly without alteration of the remaining viral genome (Jackwood, 2012). This genetic variability represents a modified method of the virus to immune selective pressures due to the extensive use of IBV vaccines allowing new field strains to evolve (Jahantigh *et al.*, 2013). Although many

countries share some common antigenic types, IBV strains within a geographic region are usually unique and distinct (Bayry *et al.*, 2005).

IBVs have been diagnosed in Egypt since 1954 including Massachusetts, D274 and 4/91 strains (Abd El Rahman *et al.*, 2015). During 2003, a Mass-like strain (Egypt/F/ 03) was isolated from unvaccinated chicken flocks with severe renal disease (Abdel-Moneim *et al.*, 2012). Subsequently, IS585/98, Sul/01/09, IS/1494, and variants related to IS/885 have been detected in many provinces (Kiss *et al*., 2016). Despite continuous immunization, IBV has established an endemic status mainly in broiler chicken flocks (Abdel-Moneim *et al*., 2012). To study the existing IBV serotypes in a geographical region, several techniques such as haemagglutination inhibition, virus neutralization and antigen-capture ELISA have been shown to be effective. In addition, RT-PCR and S1 gene sequencing analysis are adopted for IBV serotyping (Poorbaghi *et al*., 2012). Improving vaccination potency and understanding IBV evolution requires continuous characterization of newly circulating IBV variants in a given region (Kiss *et al.*, 2016).

The data generated in this study aims to describe the genetic relationships between IBVs isolated from outbreaks in commercial chicken flocks located in the Egyptian Nile Delta during 2016-2017 and the previously isolated Egyptian strains, as well as strains from nearby countries along with vaccinal strains commonly used in Egypt. In addition, histopathological examination of suspected cases and infected embryos was carried out to study the pathological lesions associated with the disease outbreaks.

MATERIALS AND METHODS

Source and collection of samples: Fifty chicken farms were monitored in different provinces within the Nile Delta of Egypt during the period from January 2016 to September 2017. Thirty-six farms were suspected to be infected with IBV based on clinical signs and postmortem examination. The flocks were vaccinated at one day old with H120 or IB primer (H120+D274 strain) vaccines (Table 1). Four birds per flock were sampled by collecting either tracheal swabs or tissue specimens (kidney and trachea). Specimens were immediately preserved at -80°C, while other samples were trimmed and directly fixed in 10% neutral buffer formalin.

Histopathology: After fixation in formalin, the tracheal and kidney samples were dehydrated in ascending series of ethanol, cleared in xylene and embedded in paraffin wax. The samples were cut into 4-µm sections, stained with hematoxylin and eosin (H&E) and examined by light microscope.

Virus isolation: Tissue homogenates were pooled and centrifuged at 500 xg for 10 min. About 200 µl of each homogenate was inoculated into a number of five 10-days-old SPF ECEs (Koum Oshiem SPF farm, El-Fayoum, Egypt) via the allantoic cavity. The inoculated eggs were incubated at 37 ºC and daily candled. After 96 h post inoculation, the allantoic fluids were harvested and further passaged for up to three to five passages.

Table 1: Geographical region, vaccination history and mortalities of the Egyptian isolates

Isolates	Geographical region	Vaccine	Mortality %
IBV/KFS/2017/1	Dakahlya	H 120	07
IBV/KFS/2017/2	Gharbia	4/91	09
IBV/KFS/2017/3	Kafrelsheikh	*IB primer	12
IBV/KFS/2017/4	Sharqia	*IB primer	11
IBV/KFS/2017/6	Beheira	H120	18

*IB primer = H120 +D274 strain.

RNA extraction and PCR amplification: RNA was extracted from infected allantoic fluids using QIAamp Viral RNA Mini Kit (QIAGEN, Calif., USA). Then, a two-step RT-PCR (Koma Biotech, Korea) was conducted using oligonucleotide sequence primers for the highly conserved (N) gene with cycling conditions mentioned by (Rashid *et al*., 2009). Further subtyping of the positive samples using primers specific for the HVR of S1 gene was performed according to (Selim *et al*., 2013). Oligonucleotide primer sequences used in this study are shown in Table 2.

Purification and sequencing of PCR products: Amplicons of S1 gene of five selected isolates were purified using QIA quick PCR product extraction kit (Qiagen, Valencia) and sequenced using Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Fostercity, CA) and Applied Biosystems 3130 genetic analyzer (ABI, USA) using the same amplification primers.

Alignment and phylogenetic analysis: Blast analysis (BLASTn) of five isolates was performed to establish S1 gene identity to GenBank accessions. Comparative analysis of S1 sequences was performed using the CLUSTAL W Multiple Sequence Alignment tool of MEGA 6.0 software. Phylogenetic tree through a bootstrap of 1000 trials was constructed for the sequenced isolates along with other reference strains available in the GenBank database using MEGA version 6.0 software.

GenBank accession numbers: The amplified S1 gene sequences of five isolates were submitted to the GenBank database and assigned the accession numbers: IBV/KFS/2017/1 (KY613802), IBV/KFS/2017/2 (KY613803), IBV/KFS/2017/3 (KY613804), IBV/KFS/2017/4 (KY613805) and IBV/KFS/2017/6 (MG586045).

RESULTS

Isolation of IBV in ECE: The inoculated embryos showed stunted growth accompanied with characteristic curling appearance and uric acid deposition in the kidneys and ureter of some embryos. All isolates did not cause observable lesions in the embryos on the first egg passage, and some of them caused stunting and death of the embryos on the third to fifth passages.

Histopathological findings: Regarding to histological report, the trachea of normal birds showed pseudostratified ciliated columnar epithelium (Fig. 1A). Kidney of normal birds revealed normal renal glomeruli and tubules (Fig. 1B). Trachea of birds infected with IBV/KFS/2017/1 showed marked tracheitis features (Fig. 1C). The kidneys showed multifocal presence of interstitial nephritis associated with mononuclear cell

Table 2: Oligonucleotide primers used in the study

Primer	Sequence	Target **gene**	Amplified product	Reference
IBV-F	GCTTTTGAGCCTAGCGTT	N gene	149 bp	(Rashid *et al*., 2009)
IBV-R	GCCATGTTGTCACTGTCTATT	N gene		
IB-S1-F	CACTGGTAATTTTTCAGATGG	S1 gene	403 bp	(Selim *et al*., 2013)
IB-S1-R	CAGATTGCTTACAACCACC	S1 gene		

F= forward, R= reverse primer, N=nucleocapsid, S1=spike glycoprotein.

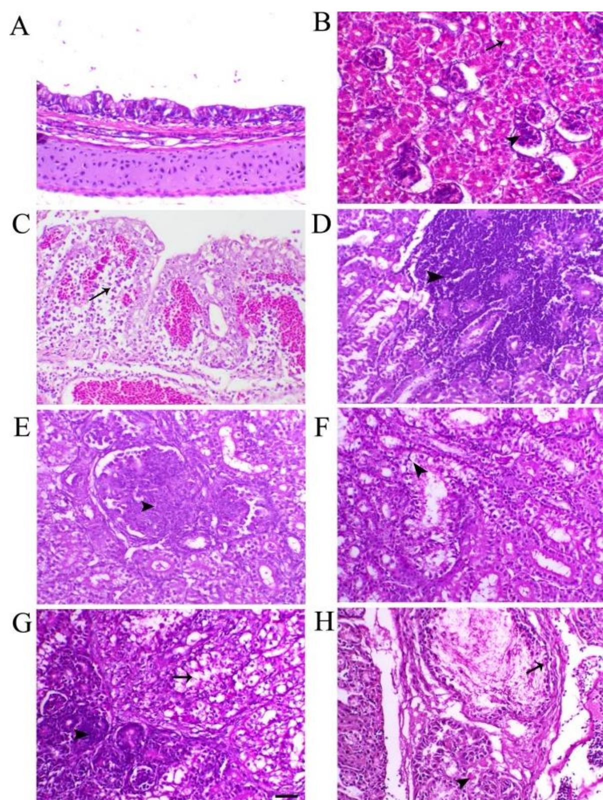

Fig. 1: (A). Trachea of normal chicken showing normal pseudostratified ciliated lining epithelium (arrow); (B). Kidney of normal chicken showing normal renal glomeruli (arrowhead) and tubules (arrow); (C) Trachea of chicken infected with IBV/KFS/2017/1 showing tracheitis with inflammatory cell infiltration (arrow); (D) Kidney of birds infected with IBV/KFS/2017/1 showing interstitial nephritis (arrowhead indicates marked lymphocytic cells infiltration); (E) Kidney of chicken infected with IBV/KFS/2017/4 showing proliferative glomerulonephritis (arrowhead); (F) Renal medulla of chicken infected with IBV/KFS/2017/6 showing tubular degeneration (arrowhead); (G) Renal medulla of bird infected with IBV/KFS/2017/6 showing tubular degeneration (arrow) associated with focal regenerative tubular basophilia (arrowhead); (H) Renal medulla of embryo infected with isolate IBV/KFS/2017/6 showing marked degeneration of some renal tubular epithelium (arrow) with casts formation within some renal tubules (arrowhead). H&E, bar= 50 µm.

infiltration (Fig. 1D). The trachea of birds infected with IBV/KFS/2017/2, IBV/KFS/2017/3 showed tracheitis associated with congestion of the blood capillaries within the lamina propria, hyperplasia of the lining epithelium and mononuclear inflammatory cells infiltration. Kidneys of these birds demonstrated mild proliferation of the mesangial cells with hyalinized matrix (Fig. 1E). Birds infected with IBV/KFS/2017/4 and IBV/KFS/2017/6 showed mild to moderate tracheitis and severe renal tubular epithelial lining degeneration (Fig. 1F and G). The kidney of infected embryos with different IBV isolates showed medullary nephrotic features characterized by tubular degeneration and necrosis within distal renal tubules and collecting ducts. While the cortical portion revealed congestion of glomerular tufts and peritubular blood capillaries and vacuolation of renal tubules lining epithelium consistent with fatty changes (Fig. 1H).

PCR for detection and genotyping of IBV: Out of 36 examined field samples, the detection of IBV in the collected allantoic fluids was confirmed in 23 (63.8%) cases through amplification of the (N) gene giving the specific size bands at (149 bp). By further examination of the positive samples by PCR, specific bands of 403 bp size of the HVR of S1 gene were amplified.

Sequencing and genetic analysis: Blast results and phylogenetic analysis revealed that the isolates under study belong to variant II like strains and they are further separated into three subclusters. The three isolates designed as IBV/KFS/2017/1-2-3 were clustered together with the Egyptian variants IB/chicken/Egypt/BSU-FA-KB27/2013, IB/chicken/Egypt/BSU-MNKB44/2013, EGY/Qalyobia/121, IBV/Egypt/VRLCU02/2012 with nucleotide sequence identity (97-100%). They shared 98% nucleotide homology with the Libyan strains IBV-Chicken-Libya-03-2012 and IBV-Chicken-Libya-12-2012. Their nucleotide homology with the Israel strains IS/1494/06 and IS/885 S1 was (95%) and (96%) respectively. Isolate IBV/KFS/2017/4 was grouped into the same cluster with the Egyptian IBV/Ck/EG/CU/4/ 2014 variant strain with 99% nucleotide identity. While isolate IBV/KFS/2017/6 shared a common ancestor with IB/chicken/Egypt/BSU-FA-KB23/2013 with similarity (96%) (Fig. 2). Compared to the vaccinal strains, the isolates shared 82-83% nucleotide identity with H120, 4/91, MA5, D274, and Connecticut. The nucleotide and amino acid sequence similarities were compared among each other and with other IBV strains published in the GenBank database (Table 3). Amino acid alignment analysis of the five isolates with the commonly used vaccinal strains showed multiple amino acid substitutions (Fig. 3).

DISCUSSION

Avian IBVs are pervasive all over the world including Egypt and have been inducting significant losses to the poultry sector. In Egypt, the poultry industry is mainly centralized in the Nile Delta where poultry farming is intensively practiced (Hosny, 2006).

Detection and identification of the circulating IBV strains is fairly a challenging task. Many systems are used, but virus isolation and molecular assays cooperatively should be used for the detection and characterization of IBVs (Kiss *et al*., 2016).

In this study, thirty-six different clinically affected poultry farms located in the Nile Delta provinces were investigated for the presence of IBV infection. Virus isolation was done by intra-allantoic passage of tissue homogenate into ECE. After 3-5 blind passages in embryonated eggs, embryo mortalities, dwarfing, curling and uric acid deposition in the kidneys and ureters were observed.

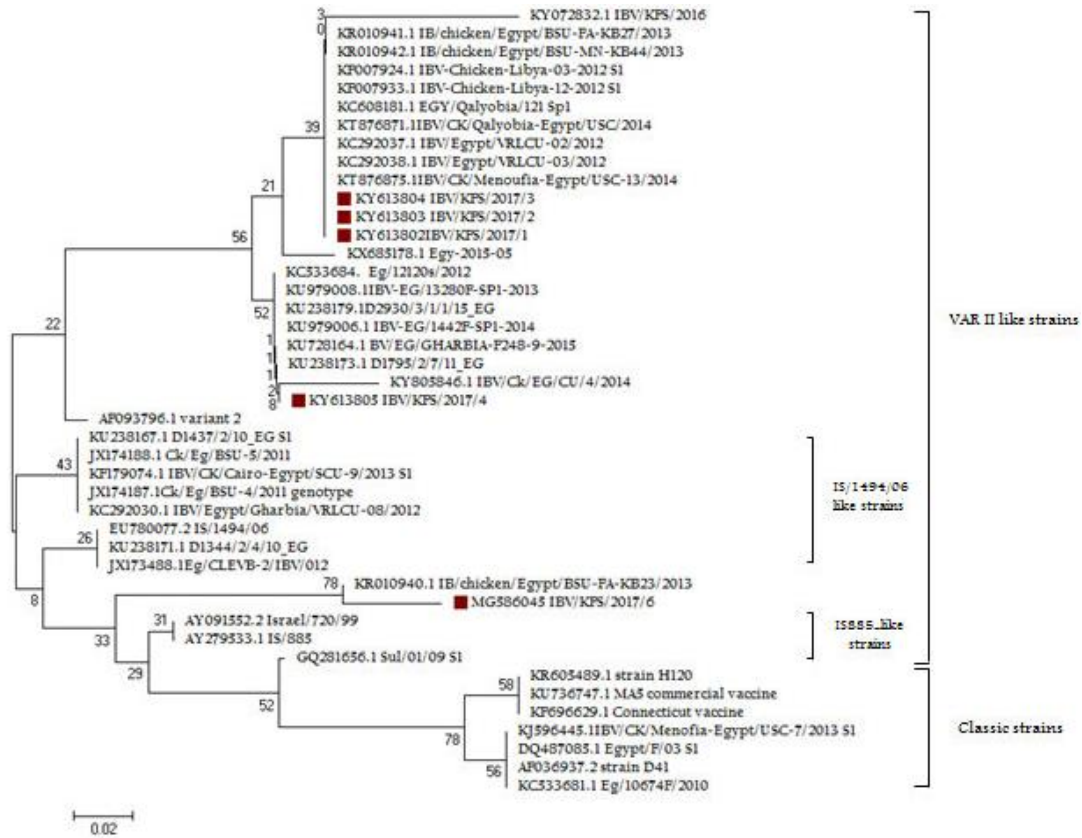

Fig. 2: Phylogenetic tree based on partial S1 gene sequencing of five IBV isolates compared with other Genbank reference and vaccinal strains.

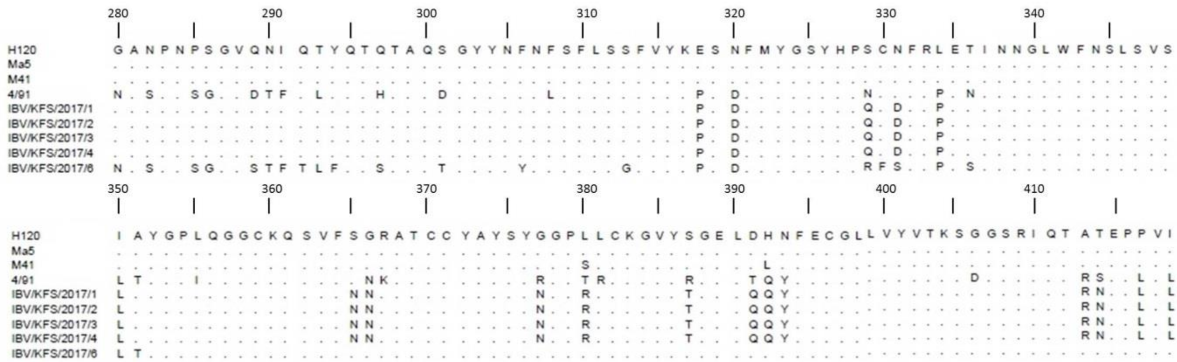

Fig. 3: Alignment of the amino acid residues of the five isolates compared with the currently used vaccinal strains. Sequence identities are indicated by dots.

Table 3: Nucleotide and amino acid sequence identities of the five Egyptian IBV isolates compared with reference strains based on the partial S1-gene sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1-Eg/12120s/2012	-	99	100	95	95	84	95	99	99	96	95	99	96	96	96	96	81	82	82	82
2-IBV/Ck/EG/CU/4/2014	94	-	99	95	95	84	99	98	99	95	95	99	98	98	98	98	81	82	82	82
3-IBV-EG/13280F-SP1-2013	98	93	-	95	95	82	95	99	99	96	95	99	96	98	96	96	81	82	82	82
4-Eg/CLEVB-2/IBV/012	89	94	90	-	99	84	95	95	95	95	99	95	95	94	95	95	83	83	83	82
5-IS/1494/06	90	94	91	98	-	84	95	95	95	95	99	95	95	94	95	95	83	83	83	82
6-*IBV/KFS/2017/6	82	80	81	79	79	-	84	83	82	84	84	84	84	84	84	84	84	83	83	85
7-Israel/720/99	90	94	91	98	100	79	-	95	95	99	95	95	95	95	95	95	82	81	81	82
8-D2930/3/1/1/15_EG	98	93	98	90	90	80	90	-	98	96	95	99	98	98	98	98	81	82	82	82
9-IBV-EG/1442F-SP1-2014	93	97	94	94	93	81	93	93	-	95	95	99	98	98	98	98	81	82	82	82
10-IS/885	87	87	88	87	87	82	87	87	87	-	95	96	96	95	96	96	82	82	82	82
11-D1344/2/4/10_EG	90	94	90	98	98	79	98	90	93	87	-	95	95	94	95	95	83	83	83	82
12-D1795/2/7/11_EG	94	98	94	95	94	82	94	94	97	87	95	-	98	98	98	98	81	82	82	82
13-IBV/KFS/2017/2	97	97	98	95	95	87	95	98	98	95	95	96	-	98	100	100	81	82	82	82
14-IBV/KFS/2017/3	97	98	99	95	95	87	95	98	98	95	95	96	100	-	98	99	80	81	81	81
15-IBV/KFS/2017/4	98	97	99	95	95	87	95	98	98	95	95	96	100	99	-	100	81	82	82	82
16-IBV/KFS/2017/1	98	98	99	95	95	87	95	98	98	95	95	96	100	100	100	-	81	82	82	82
17-Connecticut vaccine	74	83	75	75	75	75	75	75	75	75	75	76	83	82	83	83	-	95	95	81
18-strain H120	77	77	76	75	75	72	75	76	76	75	76	77	82	82	82	82	91	-	100	80
19-MA5 commercial vaccine	77	77	76	76	76	72	76	76	76	75	76	77	82	82	82	82	91	99	-	80
20-4/91 attenuated vaccine	77	77	78	78	78	82	78	77	77	77	78	78	86	86	86	86	74	74	74	-

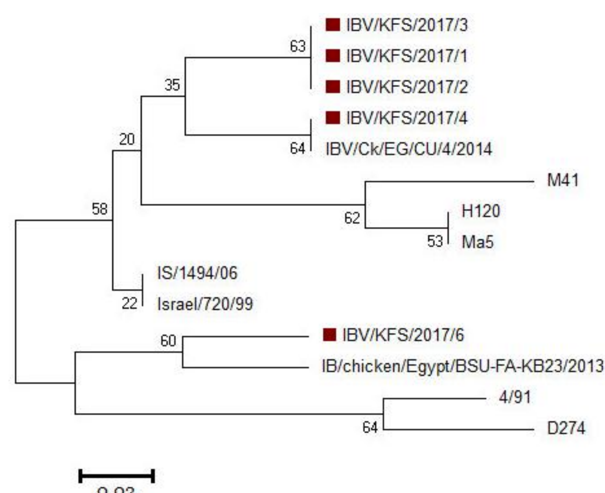

Fig. 4: Phylogenetic tree of the partial S1 gene sequences of IBV isolates and the Genbank reference and vaccinal strains on amino acid level.

Microscopic picture of infected tracheas showed degenerative changes of the tracheal mucosa, mononuclear cell infiltration and epithelial desquamation in the tracheal lumen (Cavanagh *et al*., 1992). Kidney damage was the prominent lesion related to the isolated variants and was evidenced more readily on histological study rather than by gross examination. Parallel to previous studies, the general findings in the renal tubules matched with that recorded by Chousalkar *et al.* (2007) where they showed mild proliferation of the mesangial cells with hyalinized matrix and severe degree of renal tubular lining epithelial degeneration and proliferative glomerulonephritis. In accordance, a previous investigation showed that the virus replicates more frequently in the epithelial cells of the collecting tubules and the distal convoluted tubules (Cavanagh *et al*., 1992).

Molecular confirmation revealed 63.8% IBV positive samples based on amplification of the N gene which contains highly conserved amino acid and nucleotide sequences and is closely associated with the genomic RNA showing insignificant variations thus facilitating the detection of various strains of IBV (Yan *et al*., 2013). The remaining IBV negative samples may possibly contain other pathogens with similar respiratory signs (Siddique *et al*., 2012). In contrast to the (N) gene, the S1 protein highly diverse with reference to both nucleotide sequences and deduced amino acids (Cavanagh *et al*., 1992). For further characterization of IBV positive samples, a PCR assay was conducted using primers for the HVR of S1 gene. Five selected IBV isolates were genetically characterized based on sequence analysis of the partial S1 gene. The five Egyptian isolates had 87–100% nucleotide sequence homology with each other. They exhibited similarity with other strains from neighboring countries like Israel and Libya ranging between 82-96% and 98% respectively, which is mainly due to illegal movement of poultry and poultry by-products through country borders. Both IS/1494/06 and IS/885 strains have been recorded as the main variants associated with recent IBV outbreaks in the Middle East (Mahmood *et al*., 2011). Phylogenetic analysis showed that the isolates fall into the variant 2-like group and had an apart relation to the vaccinal strains commonly used in Egypt including H120, Ma5, D274 and 4/91 vaccines showing nucleotide and amino acid identity ranging from 82-84% and 72-86% respectively. Hence, flocks infected with these variants were not protected by the currently used IBV vaccination program. With reference to the deduced amino acids; multiple substitutions were detected in the five IBV isolates between amino acid residues 280-419 of S1 gene when compared with the H120 vaccinal strain (Fig. 3). The regions between amino acid residues 251-347 have previously been identified as a HVR which is highly associated with serotype-specific epitopes (Abdel-Sabour *et al*., 2017). Isolates IBV/KFS/2017/1-2-3-4 had 17 amino acid substitutions while IBV/KFS/2017/6 showed higher genetic diversity by 23 amino acid substitutions in comparison to H120 vaccinal strain (Fig. 3). By phylogenetic analysis of the partial S1 sequences on amino acids level, the four Egyptian isolates IBV/KFS/ 2017/1-2-3-4 form a distinct cluster from the vaccinal strains with 82-83% identity to H120 vaccinal strain; while isolate IBV/KFS/2017/6 was clustered with the 4/91 vaccinal strain with 82% amino acid identity and 72-75% identity to H120 and D274 strains respectively (Fig. 4). Isolate IBV/KFS/2017/4 was closely related to the Egyptian strain IBV/Ck/EG/ CU/4/2014 which has shown evidence for recombination from the Italian strain (90254/2005), 4/91 and H120 vaccinal strains (Abozeid *et al*., 2017).

These results indicate that IBV viruses in vaccinated chicken populations exhibit higher mutation rates and the extensive use of live attenuated vaccines plays a significant role in the ongoing evolution of new variants (Jackwood and Lee, 2017). Conversely, it has been proposed that vaccination with two antigenically distinct live-attenuated vaccines can result in effectual cross-protection against different IBV serotypes (De Wit *et al*., 2011).

In Egypt, the more commonly applicable live attenuated vaccine in day–old broiler chicks are H120 vaccine (Abdelmoneim *et al*., 2002). The low sequence homology between these isolates and the H120 vaccinal strain may explains the failure of the vaccine to protect against challenge with these strains. Although this is surely an important consideration, but before blaming the vaccine, there are other factors that must be evaluated such as shortage of bio-security (Jackwood and Lee, 2017), immunosuppressed flocks (Cheng *et al*., 2018) inadequate distribution of live vaccines administered by drinking water or spraying or inactivation of live vaccines due to incorrect handling and administration. However, the continuous evolution of new IBV variant strains is still the main cause of poor vaccine immunization (Khataby *et al*., 2016). Even though S1 gene sequencing is considered a sensitive and specific tool providing rapid genotyping of the circulating IBV strains, yet it is strongly recommended to standardize the type and length of the target gene which is used for genotyping in order to ensure common understanding of genotype distributions (Bande *et al*., 2016).

Conclusions: Our data document the broad circulation of emerging IBV variants among chicken flocks in the Egyptian Nile Delta provinces despite of the vaccination programs. The significant amino acid substitutions in

these isolates in relation to the currently used vaccinal strains may provide evidence for continuous emergence of new IBV strains which raises the concern to develop different vaccination programs to confer complete protection to the chicken flocks and to prevent future IBV outbreaks.

Authors contribution: AM designed the study, performed virus isolation, PCR and phylogenetic analysis; WA designed the study and performed histopathological examination. AA designed the study, collected the tissue samples and performed virus isolation. AE designed the study, collected the tissue samples and performed PCR. AD designed the study and analyzed the results. All authors revised the manuscript and approved the final version to be published.

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