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MOLECULAR ANALYSIS OF AVIAN INFECTIOUS BRONCHITIS VIRUS IN QALYOUBIA GOVERNORATE DURING 2017-2018

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ABSTRACT

The regular updates of molecular studies of Avian Infectious Bronchitis Virus in Egypt become imperative specially with the ongoing emergence of new strains of infectious bronchitis virus (IBV) that worsen the situation in poultry farms and causing extensive economic losses. In our study, we collect samples from twenty broiler flocks range from 18 to 36 days of age and manifested severe respiratory signs with mortality up to 30%. The collected flocks located in Qalyoubia governorate during period between January 2017 to December 2018. IBV Screening in collected samples was conducted by real time rRT-PCR targeting the nucleoprotein (N) gene. A total of 12 out of 20 examined farms were positive for IBV with percentage of (60%). Five positive IBV isolates were selected for amplification and sequencing of 400 –bp targeting the hypervariable region-3 (HVR 3) of S1 protein. Phylogenetic analysis confirmed that these viruses were closely related to each other (90-98% identity) and were clustered to variant II (GI 23) viruses within the Egy/Var-Ⅱ subgroup. The amino acids homology showed (75% to 80% identity) between our Egyptian/Qal variants and the common vaccine used for IBV infection in Egyptian poultry farms.

***Key words*:** Avian infectious bronchitis, Broiler, Molecular analysis, S1 gene (HVR3), Variant infectious bronchitis.

INTRODUCTION

Avian Infectious Bronchitis Virus (IBV) is a member of genus Gammacoronavirus, family Coronaviridae, order Nidovirales (De Groot *et al.,* 2011 and King *et al.,* 2011). Avian infectious bronchitis is one of highly contagious infectious diseases affect chicken with significant economic impact on poultry industry. Commonly, Respiratory manifestation, renal disorders, drop in egg production and both poor egg quality and quantity were seen and associated with IBV infection in chicken (Cavanagh, 2007). Some strains showing special tropism to proventriculus (Yu *et al.,* 2001) and this feature was recorded in strain called Egypt/Qal/014p isolated from 14-day-old broilers from Egypt (EL-Nahas *et al.,* 2017).

The IBV genome harbors of a single positive-stranded RNA of approximately 27.6 kb in size and encodes for four main structural proteins including, the phosphorylated nucleoprotien (N) protein, the membrane (M) glycoprotein, small envelope (E) protein and the spike (S) glycoprotein. The spike (S) glycoprotein, an integral membrane protein, is a major structural protein; it is cleaved post translationally into the S1 (N terminal part) and S2 fragments (Masters, 2006). The S1 subunit contains the receptor binding site and thus plays a pivotal role in tissue tropism and inducing protective immunity (Wickramasinghe *et al.,* 2011; Belouzard *et al.,* 2012). In the S1 gene, three HVRs located within amino acids 38-67 (HVR-1), 91-141 (HVR-2) and 274-387 (HVR-3) that contribute in induction of both neutralizing and serotype specific antibodies (Cavanagh *et al.,* 1988 and Moore *et al.,* 1997). Genotyping of IBV based on S1 gene sequencing, especially the HVRs, is the most reliable way to classify IBV isolates. Usually IBV serotypes have a wide range of genetic variations in the S1 gene ranged from 2% to 25% (Cavanagh *et al.,* 1992; Kingham *et al.,* 2000). In Egypt, many strains of live attenuated and inactivated vaccines were used to control IBV. However, the outbreaks of the disease have continued to cause severe infections (Abd El Rahman *et al.,* 2015). Different types of mutation including insertions, deletions, point mutations,

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substitutions and /or recombination of S1 gene are the main factors for emergence of new IBV variants (Alvarado *et al.,* 2005 and Carstens, 2009).

IBV strains have been reported in chickens worldwide (Jackwood, 2012). Since the first report of isolation of IBV in Egypt in 1950 (Ahmed, (1954), ongoing scientific attention was directed in several publications for isolation and/ or detection of IBV strains from poultry farms in Egypt. Earlier IBV genotypes related to Massachusetts, D3128, D274, D08880 and 4/91 were circulating in poultry farms with nephropathy (Abdel-Moneim *et al.,* 2006 and Jackwood *et al.,* 2012) and with the continuing detection of IBV strains, different variants closely related to the Israeli variant strain were identified including Egyptian variant I (Egypt/Beni-Seuf/01) in 2001 (Abdel-Moneim *et al.,* 2002) and Egyptian variant II (CK/Eg/BSU-2,3/2011) in 2012 (Abdel-Moneim *et al.,* 2012). Moreover, IS/885/00 and IS/1494/06 were dominant variants circulating in different Egyptian poultry farms causing respiratory and renal disorders (Meir *et al.,* 2004; El-Mahdy *et al.,* 2012; Selim *et al.,* 2013). The continous circulation of different variants of IBV in majority of farms all over Egypt even in vaccinated ones with different strains (H120, MA5 and 4/91) was dramatically complicated the situation in poultry sector and unfortunately, allow to emerge new mutants with risk of IBV vaccination failure (Zanaty *et al.,* 2016). Our study was a contribution in IBV surveillance in Qalyoubia which represent one of the provinces that characterized by the intensity of poultry production in Egypt for updating and monitoring the most important molecular changes in currently circulating IBV isolated form poultry farms through the partial sequence of S1 gene during a period between 2017-2018.

MATERIALS AND METHODS

Samples collection

In the present study, samples were collected from broiler flocks range from 18 to 36-day-old and suffering from severe respiratory signs from twenty different farms located in AL-Qalyoubia Governorate during the period from January 2017 till December 2018. The chickens were vaccinated with H120 vaccine against IBV at one day of age. Chickens showed respiratory signs such as gasping, coughing, sneezing and tracheal rales with white diarrhea in some cases. Necropsy showed mild to severe tracheitis with congested lung. In addition, birds were suffering from kidney lesions such as enlargement and congestion. The samples were collected as pooled homogenate from trachea and kidneys representing 20 farms were collected from diseased birds and freshly dead birds, stored in sterile Falcon tubes containing transport media with antibiotics. The samples were transported to the Reference Laboratory for veterinary Quality control on Poultry production (RLQP), Animal Health Research Institute, Dokki, Giza, Egypt., in ice box and stored at -80 °C until use.

Virus detection and isolation

Viral RNA was extracted directly from the samples by using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), following the kit manufacturer's instructions. The virus identification was confirmed by real-time reverse transcription-polymerase chain reaction (rRT-PCR) for the presence of the neucloprotein (NP) gene of the IBV using Quantitect probe RT-PCR kit (Qiagen, Hilden, Germany), with specific primers and probe targeting the NP gene (Callison *et al.,* 2001).

For Viral isolation, samples from trachea and kidneys were prepared as 10% tissue suspensions in transport medium (phosphate-buffered saline) (pH 7.4), after three cycles of freezing and thawing the suspension was clarified by centrifugation at 3000 rpm for 10 min and filtered through 0.45 micron membrane filters (Millipore) before inoculation in 10 day-old embryonated SPF eggs via allantoic sac route. Three passages were performed and characteristic embryo changes such as dwarfing, stunting, curling of the embryos, subcutaneous hemorrhage and ureate deposition in the mesonephros were observed between post-inoculation days 2 and 7 (OIE, 2013). The harvested allantoic fluids from each sample were screened using rRT-PCR for further confirmation.

Sequencing of the S1 gene

Screening was further tested using a specific primer set for the amplifications of the HVR-3 of the S1 gene using Qiagen one-step RT-PCR (Qiagen, Hilden, Germany), according to (Adzhar *et al.,* 1997). Amplificates of 400 bp were excised and purified from gels using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The purified RT-PCR products were sequenced using Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA) and Applied Biosystems 3130 genetic analyzer (ABI, United States).

Phylogenetic analysis

Sequences similarities and relationships of the HVR-3 of the S1 gene of positive samples in the present study were compared with previously published IBV vaccine and reference strains available in the public database (NCBI, United States). Amino acids phylogenetic tree was drawn for the sequenced isolates along with other vaccine and reference strains available in the GenBank database using MEGA program version 6 (Tamura *et al.,* 2013). A comparative analysis of deduced

amino acids and nucleotide sequences of the HVR-3 was created using the CLUSTAL W Multiple Sequence Alignment Program, version 1.83 of MegAlign module of Lasergene DNAStar software (Ziegler *et al.*, 2002). Sequences generated in the frame of this study were submitted to the GenBank database with accession numbers showed in Table 1.

RESULTS

Clinical reports of examined farms:

The main complaints of the majority of examined farms were summarized in the following signs, high mortalities up to 30%, ruffled feathers, depression, loss of appetite, mild to severe respiratory signs in form of difficult breathing, tracheal rales, gasping, sneezing and coughing. White diarrhea in some cases was also observed. The most prominent findings at necropsy were mild to severe tracheitis with congested lung, in addition to swollen and pale kidneys and in some cases the tubules and ureters filled with ureate.

Virus detection and isolation

IBV was detected in examined samples in 12 out of 20 farms with percentage of (60%) using rRT-PCR **(Fig. 1).** Virus isolation was obtained from the homogenate pool of the trachea and kidneys from each flock. The allantoic fluid from the 3rd passage of each sample was further confirmed positive using rRT-PCR.

Genetic and phylogenetic characterization

The 400 bp amplified PCR products of the HVR-3 of S1 gene were obtained from the 5 positive selected samples (Fig. 2), and then the partial sequencing of HVR-3 and sequence analysis was conducted. Phylogenetic tree was constructed from the amino acid sequences of HVR-3 of the S1 protein. The results indicated that Egyptian IBV viruses in our study found to be closely related to the variant II isolates and were clustered within the Egy/Var-Ⅱ subgroup (IBV-Eg-12120s-2012 and IBV/IS/885-00) and other Egyptian related strains in the GenBank (Fig. 3).

Alignment report of the S1-HVR3 for both nucleotide and deduced amino acid were performed and compared with the previously published reference and vaccine IBV strains commonly used in the field (H120, Ma5, D274, 4/91,1/96 and CR88121) as well as original Egyptian viruses of Egy/Var-Ⅰ and Egy/Var-Ⅱ. In comparison to vaccine strains used in Egypt, Egy/ Var-Ⅱ strains isolated in this study showed identity from 75% to 80% for vaccinal strains H120, Ma5, 4/91, CR88 and D274 (Table, 2). In the meantime, strains shared 90% to 98% amino acid with each other according to sequence of HVR3 (Table, 2)

Table 1: Data including time of collection, ages of birds and accession no. of Infectious bronchits virus in the present study.

Isolate name	Date of collection	Age of birds/d	GeneBank accession number
IBV- EGY/QAL-1-2017	2017	35	MK492508
IBV- EGY/QAL-2-2017	2017	24	MK492509
IBV- EGY/QAL-3-2018	2018	32	MK492510
IBV- EGY/QAL-4-2018	2018	36	MK492511
IBV- EGY/QAL-5-2018	2018	25	MK492512

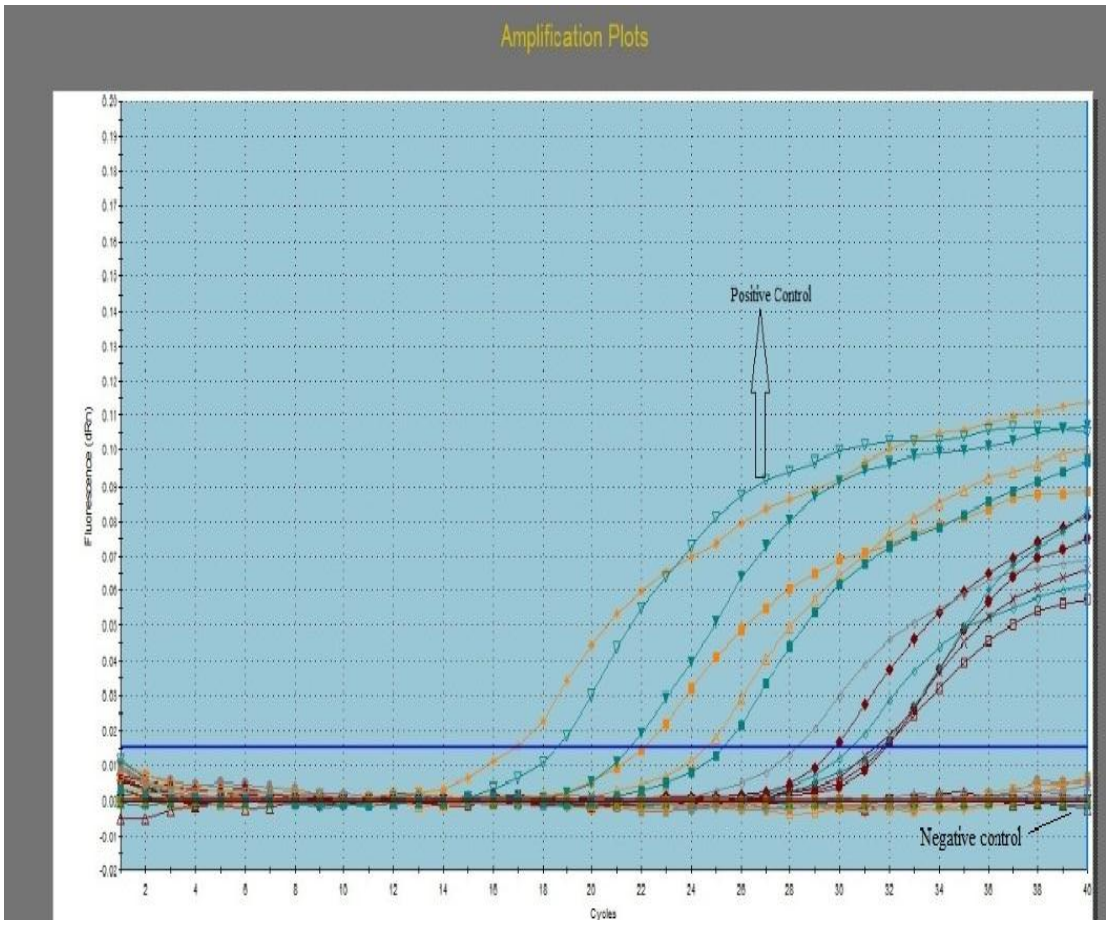

Fig. 1: Amplification curve for IBV (N) gene rRT- PCR.

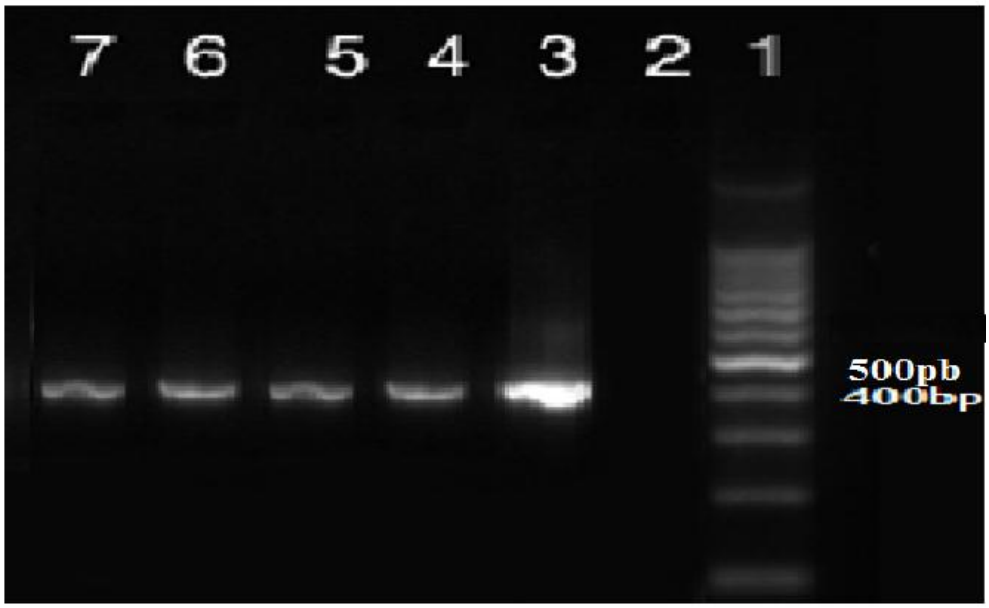

Fig. 2: Gel electrophoresis shows the result of PCR products of five selected isolates.

1 - Ladder
2 - Negative control
3 - Sample 1
4 - Sample 2
5 - Sample 3
6 - Sample 4
7 - Sample 5

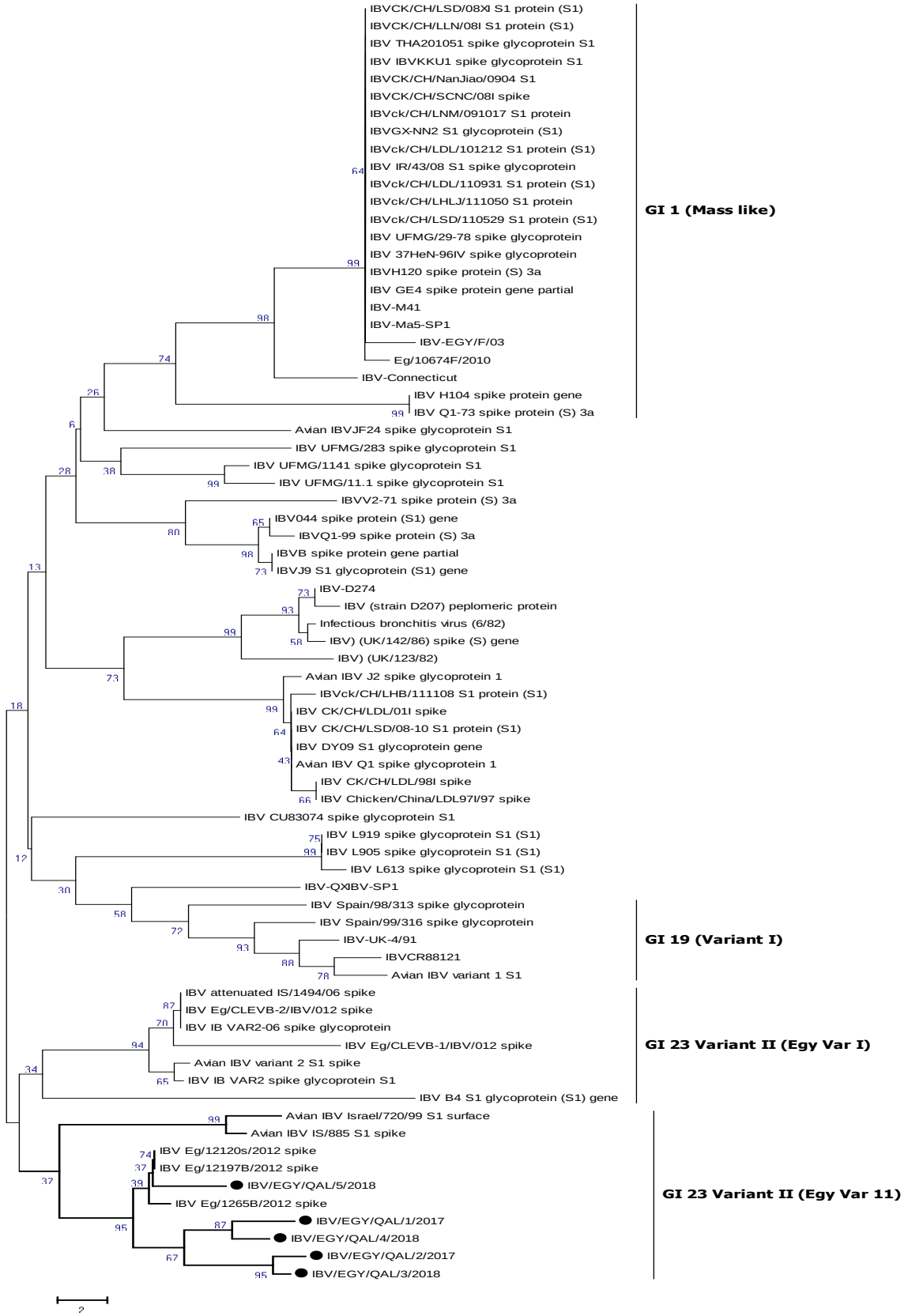

Fig. 3: Phylogenetic tree representing the partial amino acid sequences of the S1 gene for 5 avian infectious bronchitis virus isolates (marked with black circle) with other related infectious bronchitis virus and vaccine strains**.**

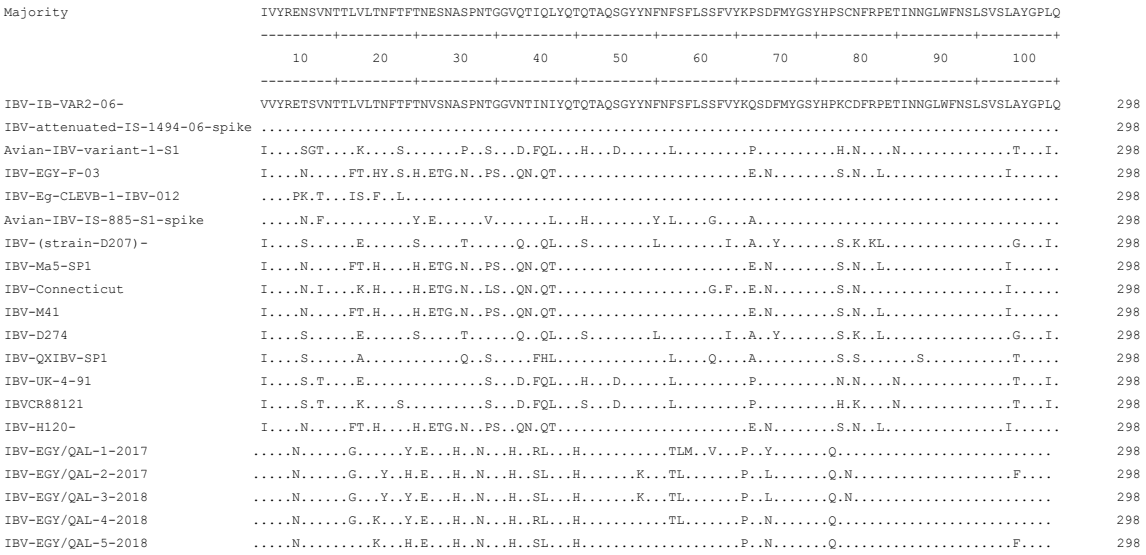

Fig. 4: Sequence alignment for the amino acids of S1 gene for the 5 isolates of the study; vaccine strains and the reference strains.

Table 2: Nucleotide and deduced amino acid identities of infectious bronchitis virus Egyptian isolates with other selected references and vaccine strains from different serotypes.

Sequence name	IBV-IB-VAR2-06-	IB-IS1494-06-	IBV-Eg-12120s-2012	IBV-Eg-12197B-2012	IBV-Ma5-SP1	IBV-Connecticut	IBV-M41	IBV-D274	IBV-QXIBV-SP1	IBV-UK-4-91	IBVCR88121	Avian-IBV-variant-1-S1	IBV-Eg-1265B-2012	IBV-Eg-CLEVB-2-IBV-012-	Avian-IBV-IS-885-S1-	IBV-EGY-QAL-1-2017	IBV-EGY-QAL-2-2017	IBV-EGY-QAL-3-2018	IBV-EGY-QAL-4-2018	IBV-EGY-QAL-5-2018
NUCLEOTIDE IDENTITY %																				
IBV-IB-VAR2-06	ID	100%	89%	89%	81%	80%	81%	82%	82%	82%	80%	81%	90%	100%	90%	88%	87%	87%	88%	89%
IBV-IS-1494-06-	100%	ID	89%	89%	81%	80%	81%	82%	82%	82%	80%	81%	90%	100%	90%	88%	87%	87%	88%	89%
IBV-Eg-12120s-2012	90%	90%	ID	100%	80%	79%	80%	80%	81%	80%	79%	80%	99%	89%	90%	97%	96%	96%	96%	97%
IBV-Eg-12197B-2012	90%	90%	100%	ID	80%	79%	80%	80%	81%	80%	79%	80%	99%	89%	90%	97%	96%	96%	96%	97%
IBV-Ma5-SP1	79%	79%	81%	81%	ID	97%	99%	82%	79%	78%	76%	77%	80%	81%	80%	79%	79%	78%	78%	79%
IBV-Connecticut	78%	78%	80%	80%	93%	ID	98%	81%	79%	78%	77%	78%	79%	80%	82%	78%	78%	78%	78%	79%
IBV-M41	79%	79%	81%	81%	100%	93%	ID	82%	79%	79%	77%	78%	80%	81%	81%	79%	79%	79%	79%	80%
IBV-D274	83%	83%	82%	82%	78%	76%	78%	ID	81%	83%	83%	82%	81%	82%	79%	80%	79%	79%	79%	79%
IBV-QXIBV-SP1	86%	86%	83%	83%	76%	76%	76%	83%	ID	83%	81%	82%	82%	82%	82%	80%	79%	79%	79%	80%
IBV-UK-4-91	83%	83%	83%	83%	74%	74%	74%	83%	87%	ID	98%	98%	81%	82%	81%	80%	79%	79%	80%	80%
IBVCR88121	82%	82%	81%	81%	72%	73%	72%	83%	86%	95%	ID	98%	79%	80%	79%	79%	77%	78%	79%	79%
Avian-IBV-variant-1-S1	80%	80%	81%	81%	72%	73%	72%	80%	85%	95%	96%	ID	81%	81%	80%	80%	79%	79%	80%	80%
IBV-Eg-1265B-2012-	90%	90%	99%	99%	80%	79%	80%	82%	83%	83%	81%	81%	ID	90%	90%	97%	96%	96%	97%	97%
IBV-Eg-CLEVB-2-IBV--	100%	100%	90%	90%	79%	78%	79%	83%	86%	83%	82%	80%	90%	ID	90%	88%	87%	87%	88%	89%
Avian-IBV-IS-885-S1	90%	90%	90%	90%	76%	78%	76%	80%	84%	82%	80%	79%	90%	90%	ID	88%	87%	87%	88%	88%
IBV-EGY-QAL-1-2017	84%	84%	92%	92%	75%	74%	75%	79%	78%	78%	76%	76%	93%	84%	85%	ID	96%	97%	98%	95%
IBV-EGY-QAL-2-2017	83%	83%	91%	91%	75%	74%	75%	77%	77%	78%	75%	76%	90%	83%	81%	90%	ID	99%	95%	95%
IBV-EGY-QAL-3-2018	84%	84%	91%	91%	75%	74%	75%	78%	78%	79%	76%	77%	92%	84%	83%	92%	98%	ID	96%	94%
IBV-EGY-QAL-4-2018	85%	85%	93%	93%	76%	75%	76%	79%	79%	79%	77%	77%	94%	85%	86%	96%	91%	93%	ID	97%
IBV-EGY-QAL-5-2018	87%	87%	97%	97%	78%	77%	78%	80%	80%	80%	78%	78%	96%	87%	87%	90%	91%	90%	94%	ID
AMINO ACIDS IDENTITY %																				

DISCUSSION

IBV, one of the important viruses that infect poultry flocks worldwide and recently the new variants topped the scene in Egypt and putting poultry policy makers in the face of a catastrophic crisis that represented in huge economic losses and failure of vaccination. Our study was a contribution to follow up and identify the IBV genotypes circulated in the poultry flocks in Qalyoubia locality. Herein, we examined twenty broilers farms distributed in different centers followed the Qalyoubia governorate, the majority of complaints of examined farms were high mortalities up to 30%, with the inspection of affected birds the most common signs were seen in form of respiratory manifestation including, difficult breathing, tracheal rales, sneezing and coughing. On necropsy, the frequently PM findings were seen in form of mild to severe tracheitis with caseous exudate in some cases, air-sacculitis and swollen, congested and pale kidney with distended ureter with urates. Similar signs and PM findings were observed in previous study isolated IBV from poultry flocks in Delta region in Egypt during 2014-2015 (Sultan *et al.,* 2017).

The confirm and on-time diagnosis was urgent need specially after increasing incidence of mixed infection in commercial Egyptian poultry farms nowadays. RRT-PCR was rapid, sensitive and specific molecular diagnostic tool for detection and screening of IBV suspect samples (Naguib *et al.,* 2017). In our study the screening conducted on 20 different farms for the presence of the neucloprotein (N) gene sequence of the IBV using rRT-PCR confirmed IBV in 12 farms with percentage of 60%.

Recently IBV variants are widely distributed in many countries all over the world with more than 20 different serotype (Sjaak de Wit *et al.,* 2011). In Egypt, from the available molecular data reported during the few years ago, the IBVs were evolved into two groups classic (GI-1) and variant (GI-23), the last one were subdivided into two subgroups (Egy/Var-Ⅰand Egy/Var-Ⅱ) according to the sequence of the HVR-3 (Abdel-Moneim *et al.,* 2012 and *Ganapathy et al.,* 2015). The viruses clustered to the subgroup Egy/Var-Ⅱ were shown to be widely spread in Egypt in last years (Abo-Elkhair *et al.,* 2012 and Valastro *et al.,* 2016).

The phylogenetic analysis conducted on the selected five IBVs showed that these viruses were related to each other (90-98% identity) according to sequence of HVR-3 and found to be closely related to the variant isolates and were clustered within the Egy/Var-Ⅱsubgroup (IBV-Eg-12120s-2012 and IBV/IS/885-00) and other Egyptian related strains deposited in the GenBank database. These findings referred to the circulation of strains belongs to Egy/Var-Ⅱ in broiler flocks in Qalyoubia during 2017-2018, also our findings were agree with Abdel-Moneim *et al.,* 2012; Sultan *et al.,* 2015; Tatar-Kis *et al.,* 2015; Zanaty *et al.,* 2016 and EL Samadony *et al.,* 2017) who reported that Egyptian variant II strains were the most dominant strains circulated in Egyptian poultry flocks recently.

One of our study aims is to compare between the isolated variants circulated in poultry flocks located in Qalyoubia governorate and the vaccine strains currently used for IBV vaccination (H120, Ma5, 4/91, CR88, and D274). Herein, the comparison showed that there was 75% to 80% identity between our isolates and the mentioned vaccine strains which indicate to the wide gap of difference between the isolated viruses and the existing vaccines types which resulted in emerging of new antigenic types that increase the risk of vaccine failure. Similar observation were recorded in previous studies (Samir *et al.,* 2014 and bdel-ELGhany *et al.,* 2015). The small changes detected along the S1 protein lead to genetic alteration and recombination and subsequently evolve new field strains (liu and Kong, 2004), these changes arises from immunological pressure caused by the massive use of live vaccines in presence of infection with field strain (Zanaty *et al.,* 2016), so it is necessary to evaluate the protective capabilities of the commonly used vaccine in Egypt as well as preparation of vaccine from indigenous isolates for achieving the successful control of IBV in Egypt (EL-Nahas *et al.,* 2017).

CONCLUSION

The present study confirmed the detection and isolation of IBVs from broilers flocks in Qalyoubia governorate during 2017-2018, the Egypt/Qal strains were closely related to Egy/Var-Ⅱthat dominant circulated in last years in commercial poultry farms in Egypt. Also the phylogenetic analysis showed the dissimilarities between our isolates and the currently used vaccines for IBV in Egypt. The continuous follow up through surveillance activities and preparation of vaccines from indigenous isolates were necessary issues for successful control of IBV in Egypt.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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التحليل الجزيئي لفيروس التهاب الشعب الهوائية المعدي للدواجن في محافظة القليوبية خلال ٢٠١٧ ـ٢٠١٨

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التحديث الدوري للدراسات الجزيئية لفيروس التهاب الشعب الهوائية المعدي للدواجن في مصر أصبح ضروري وخاصة مع الظهور المستمر للعترات الجديدة من فيروس IBV والتي تزيد من سوء الوضع في مزارع الدواجن وتتسبب في خسائر اقتصادية واسعة النطاق. في دراستنا ، قمنا بتجميع عينات من قطعان دجاج التسمين من عشرين مزرعة تقع في محافظة القليوبية خلال الفترة من يناير ٢٠١٧ حتى ديسمبر ٢٠١٨. تم إجراء فحص IBV في العينات بواسطة اختبار تفاعل البلمرة المتسلسل حقيقي الزمن rRT-PCR والذي يستهدف جين nucleoprotein (N). حوالى ١٢ مزرعة من أصل ٢٠ مزرعة تم فحصها كانت إيجابية لـفيروس IBV بنسبة ٦٠٪. تم اختيار خمس معزولات إيجابية لـفيروس IBV لتضخيم وتسلسل ٤٠٠ bp تستهدف منطقة the hyper variable region-3-من بروتين S1. وقد أكد التحليل التطوري أن هذه الفيروسات تراوحت في ارتباطها ببعضها البعض ما بين (90-98% identity) وتم تجميعها لفيروسات مختلفة variant II (GI 23) ضمن مجموعة فرعية Egy/Var-II. أظهر تقرير محاذاة الأحماض الأمينية االتفاوت (75% to 80% identity) بين معزولاتنا المصرية Egyptian /Qal variants واللقاحات الشائعة الاستخدام لعدوى الـ IBV في مزارع الدواجن المصرية.