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Isolation and Molecular Characterization of Infectious Bronchitis Virus from Broiler Chickens, Egypt during 2014 -2016

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Abstract

In this study, surveillance on infectious bronchitis virus strains in 75 Egyptian broiler chicken flocks was performed using real-time RT-PCR at eight Governorates during 2014-2016. The birds in the selected flocks were suffering from respiratory and renal manifestations. The results of the present study showed that 31 (41%) samples were positive for IBV. All isolates were classified as variants using sequencing analysis of the obtained data and can be divided into 2 groups. Group I (variant) Egyptian IBV included isolates, EG-F728-1-2015-SP1, EG-F728-7-2015-SP1, EG-F862-3-2015-SP1, EG-F862-4-2015-SP1, EG-F888-2015-SP1, EG-F777-2015-SP1 and EG-F183-1-2016-SP1 which were very close to IBV-IS-1494-06 and had amino acid sequence identities between 87.5% to 96.2%**.** Group II (variant) Egyptian IBV included isolates, EG-F183-2-2016-SP1 and EG-F183-3-2016-SP1. These two IBV isolates were very close to IBV-IS/885 (variant 2) and they shared amino acid sequence identities from 92.3% to 97.1% with each other**.** In conclusion, different variants of IBV are present in broiler farms in high prevalence rate even in vaccinated flocks.

Keywords: IBV, Broiler, Egypt, Sequencing, Variants

Introduction

Infectious bronchitis (IB) is an acute, highly contagious disease of chickens of all ages causing high morbidity. Infectious Bronchitis Virus (IBV) is a member in order Nidovirales, family Coronaviridae, subfamily Coronavirinae, genus *Gammacoronavirus* and species *Avian corona virus* [1]. The virus not only targets the respiratory tract but also the urogenital tract and it can spread to different organs [2]. Vaccination is an essential tool to increase protection of chicken against newly emerged variant IBV strains that challenge the success of vaccination strategies [3]. Despite the routine IBV vaccination, outbreaks of IB frequently occur in the field due to the presence of different serotypes as well as the emergence of multiple subtypes, generated by point mutations, insertions, deletions, or RNA recombination of the S1 genes [4]. Therefore, continuous genotyping of IBV field strains is very important for screening the emergence of new variants as well as evaluating the existing vaccination programs. Recently, other novel genotypes were reported in Egypt and classified as Egyptian variant I (e.g. CK/Eg/BSU-3/2011) and Egyptian variant II (e.g. CK/Eg/BSU-2/2011) [5]. In Egypt, commercial poultry industry suffered from heavy losses due to the emergence of new IBV strains that could compromise the immunity induced by most available vaccines [5]. The disease has been reported frequently in vaccinated and non-vaccinated flocks. The commonly used IBV attenuated vaccine is H120, while the Mass41 (M41) strain is commonly used in inactivated vaccines, but in many cases, renal damage was observed in IB-vaccinated flocks. Thus, suggesting that the currently used IB vaccination procedures may not be providing adequate protection [6]. The aim of the current study was the isolation and molecular identification of IBV in diseased broiler flocks in different Egyptian Governorates. Sequencing of some identified

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IBV and comparison with other obtained from GenBank were carried out.

Material and Methods

Examined field samples

The specimens included trachea, lung, kidney, cecal tonsil and air sac were collected from 75 broiler chicken flocks (5-10 birds per flock) with different age suffering from respiratory and renal troubles during 2014-2016 from 8 different Governorates in Egypt including Sharkia (n=13), Qalubia (n=15), Monofia (n=7), Bani-Sueif (n=5), Behara (n=10), Giza (n=8), Kafer elsheikh (n=9) and Gharbia (n=8) with age range 15 to 45 days.

Real time reverse transcription PCR (rRT-PCR)

Viral RNA was extracted from the collected samples (trachea, kidney, air sac, cecal tonsil, lungs) using QIAamp Viral RNA Mini extraction kits (Qiagen Inc. Valencia CA) following the manufacturers' guidelines. One step rRT-PCR amplification of the coding regions of the SP1 gene was performed with gene-specific primer set using QuantiTect probe RT-PCR kits (Qiagen, Inc. Valencia CA). The sequences of the used primers are IBV5_GU391 Forward 5'-ACG TAT GAC TAC CCG CAG TAT TCA -'3, IBV5_GL533 Reverse 5'- AGA CCA GCC ACC ATG ATT GC-'3 target gene: N-gene [7]. IBV5-G probe 5′-FAM-CAC CAC CAG AAC CTG TCA CCT C-BHQ1-3′.

The reaction was performed in 25 µL using QantiTect probe RT-PCR kits (Qiagen). each reaction contained 12.5 µL of 2x QantiTect probe RT-PCR Master Mix, 0.1 units AmpErase (Qiagen), 5 µL DNA template. Nuclease free water was added to a final volume of 25 µL, the reaction conditions were 50°C for 2 min, 95°C for 15min, followed by 40 cycles at 94°C for 15 sec and 60°C for 60sec followed by plate read for fluorescence acquisition.

Virus isolation

Each IBV positive rRT-PCR sample (pooled samples) was inoculated into three, 9-11 day-old Specific Pathogen Free Embryonated Chicken Eggs (SPF-ECE) with 0.2mL via allantoic cavity [8]. Inoculated eggs were incubated at 37◦C for 96 h and candled daily for embryo viability. All allantoic fluids from inoculated eggs were harvested and tested for hemagglutination using 1% washed chicken red blood cells for the detection of presence of heamagglutination virus (s) in the collected samples [8].

Conventional RT-PCR

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for the amplification of hypervariable region of Spike 1 gene (HVR of Sp1 gene) sequence was carried out using Qiagen one step RT-PCR kit (Qiagen). The sequences of the used primers are IBV-S1-F Forward 5'-CAC TGG TAA TTT TTC AGA TGG- '3, IBV-S1-R Reverse 5'-C AGA TTG CTT ACA ACC ACC- '3 [9].

The final reaction volume was 25 µL containing; 12.5 µL RT-PCR Master mix, 5 µL template RNA, forward primer 1 µL, reverse primer 1 µL, RNase free water was added to reach 25 µL final volume. The reaction conditions were 95°C for 15 min, followed by 40 cycles at 95°C for 30 sec and 54°C for 45sec, 72°C for 2 min and final extension at 72°C for 10 min. PCR products were visualized by electrophoresis in 1.5% agarose in 1X TAE, ethidium bromide was added to a concentration of 0.5 µg/mL for nucleic acid visualization.

Sequencing and phylogenetic analysis

Amplicons of proper size were purified using QIAquick Gel Extraction Kit (Qiagen Inc. Valencia CA) according to manufactures' instructions. Sequencing was carried out in Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA) using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA). BLAST® analysis (Basic Local Alignment Search Tool) [10] was initially performed to establish sequence identity with other sequences on the GenBank.

Pairwise sequence alignment of the aligned sequences for the SP1 gene was performed with the MegAlign module of Lasergene DNAStar software (Madison, WI) (Lasergene version 7.5, DNASTAR, Madison, WI, USA) to determine nucleotide an amino acid sequence identities and divergences. A phylogenetic tree of the amino acid sequences

was constructed using MEGA6 software [11]. IBV sequences used for the alignments were obtained from the GenBank and EMBL database using multi sequence alignment (Table 1).

The tree topology was evaluated by 1000 boot strap analysis. The deduced amino acid sequences were determined to identify the pathotype of the isolated IBV strains. The Maximum Composite Likelihood method was used to compute the evolutionary distances (the units of the number of base substitutions per site). Evolutionary analyses were conducted in MEGA6.

Table 1: Reference and vaccinal IBV strains with GenBank accession numbers used in this study

IBV strain	Tropism	GenBank accession number
UK /4/91	Vaccine	AF093794
Connecticut 46	Respiratory	L18990
H120	Vaccine	M21970
Ma5	Vaccine	AY561713
M41	Respiratory	AY561711
IBV-CK-CH-Guangdong-Lezhu2-0905-S1	Respiratory	GU938398
IBV-strain-NGA-310-2006	Respiratory	FN182278
IBV-IS-1494-06-S1	Proventriculus/Nephropathogenic	EU780077
IBV-isolate-IS-885-S1	Nephropathogenic	AY279533
CR88121	Vaccine	JN542567
J2	Proventriculus	AF286303
Variant 2	Respiratory/Nephropathogenic	AF093796
QXIBV	Proventriculus	AF193423
Ck/Eg/BSU-1/2011	Nephropathogenic	JX174184
Ck/Eg/BSU-3/2011	Nephropathogenic	JX174186
Ck/Eg/BSU-2/2011	Nephropathogenic	JX174185
Egypt/F/03	Respiratory	DQ487085
D274	Proventriculus/Nephropathogenic	X15832
JX	Respiratory	HQ018891
IBV/ck/CH/LNM/091017	Respiratory	HM194682
IBV-strain-JF24-S1	Respiratory	AF218851
D41	Respiratory	AF036937
Variant 1	Respiratory/Nephropathogenic	AF093795
Sul/01/09	Respiratory/Nephropathogenic	GQ281656
EG/101	Proventriculus/Nephropathogenic	HM851180
Ck/Eg/BSU-4/2011	Nephropathogenic	JX174187

Results

Clinical and postmortem findings

The examined birds in the 75 broiler flocks showed clinical signs of depression, decrease in feed consumption and huddling together under heat source. Most of the birds were suffering from variable respiratory troubles in the form of gasping, lacrimation, nasal discharge and head swelling. Some of the examined chicks showed also renal signs in the form of chalky droppings, increased water intake and unsteady gait.

Postmortem examination of both freshly dead and sacrificed affected chickens revealed gross lesions in the form of serous, catarrhal, or caseous exudate in the trachea. Also, caseated plugs at tracheal bifurcation were observed in some examined flocks. In addition, of pneumonia**,** cloudiness of air sacs with or without yellow caseous exudates were observed. The kidneys of some birds were swollen and pale with tubules and ureters distended with ureats**.**

Virus identification using rRT-PCR

The results showed that out of 75 broiler farms 31 (41%) pooled samples were positive for IBV. The highest prevalence rate of IBV virus was recorded in Bani-Sueif Governorate (80%), while the lowest was recorded in Giza Governorate 12.5%.

Table 2: Result of RRT-PCR from different governorates:

Locality	Total number examined	Positive number	%
Sharkia	13	7	54%
Monofia	7	5	71%
Qalubia	15	2	13%
Bani-Sueif	5	4	80%
Behara	10	2	20%
Giza	8	1	12.5%
Kafer-Elshikh	9	4	44%
Gharbia	8	6	75%
Total	75	31	41%

Isolation in ECE

Positive rRT-PCR samples were prepared to be inoculated in SPF Embryonated chicken eggs via allantoic route for virus isolation. Three passages of each isolate were performed and infected embryos showed dwarfism, curling, subcutaneous congestion and hemorrhage after the third passage. Examination of the allantoic fluid revealed negative HA test.

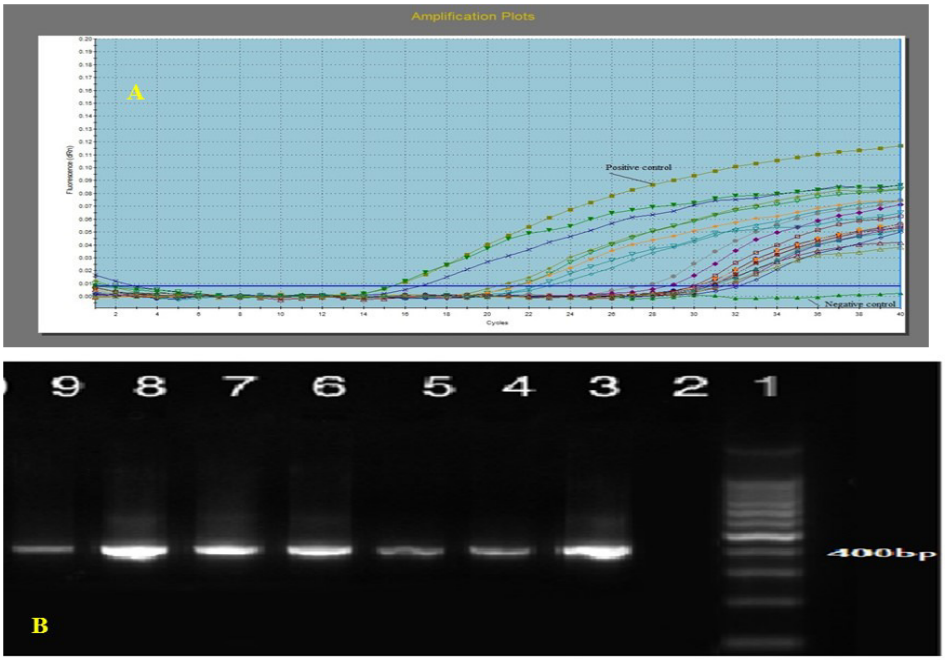

Figure 1: (A) Amplification curve for IBV samples using rRT-PCR. (B): Conventional PCR results for 6 isolates for IBV gene (Lane 1: 100 bp ladder, Lane 2: negative control, Lane 3: positive control obtained from poultry production reference lab., Dokki, Giza and Lanes 4-9: positive samples.

Sequencing and phylogenetic analysis

Nine samples were chosen based on the high CT values and lesion in embryos for SP1 gene characterization by sequencing. The analysis for the sequence data obtained from the nucleotide and amino acids sequences of HVR 3 of S1 gene was done to determine the differences among the 9 isolates. Table (1) shows the similarity percent for HVR 3 of S1 protein of IBV strains in comparison with other reference and vaccinal strains previously published in the GenBank database. Sequence analysis revealed that all the isolates were variants and divided into 2 groups. Group I (variant) Egyptian IBV included isolates, EG-F728-1-2015-SP1, EG-F728-7-2015-SP1, EG-

F862-3-2015-SP1, EG-F862-4-2015-SP1, EG-F888-2015-SP1, EG-F777-2015-SP1 and EG-F183-1-2016-SP1 which were very close to IBV-IS-1494-06 and had amino acid sequence identities between 87.5% to 96.2%**.** Group II (variant) Egyptian IBV included isolates, EG-F183-2-2016-SP1 and EG-F183-3-2016-SP1. These two IBV isolates were very close to IBV-IS/885 (variant 2) and they share amino acid sequence identities from 92.3% to 97.1% with each other (Figure 2).

In relation to vaccinal strains, the strains isolated in this study shared different amino acids identity. For the variant strains included in group I IBV-IS-1494-06 (Variant 2) including strain number EG-F728-1-2015-SP1 , EG-F728-7-2015-SP1 , EG-F862-3-2015-SP1, EG-F862-4-2015-SP1 , EG-F888-2015-SP1 , EG-F777-2015-SP1 and EG-F183-1-2016-SP1,They shared 76.6% to 88.5% with H120 and Ma5 and with 76% and 88.5% 4/91 and CR88, and, 80% with D274, respectively (Figures 2, 3).The other subgroup of the variant strains resembling IBV-isolate-IS-885,They shared amino acid similarity of 79%-83.7%, 79%-83.7%, 82.7-85.6%, 77.9-82.7 and 81.7-84.6% with H120, Ma5, 4/91, CR88 and D274, respectively (Figures 2, 3).

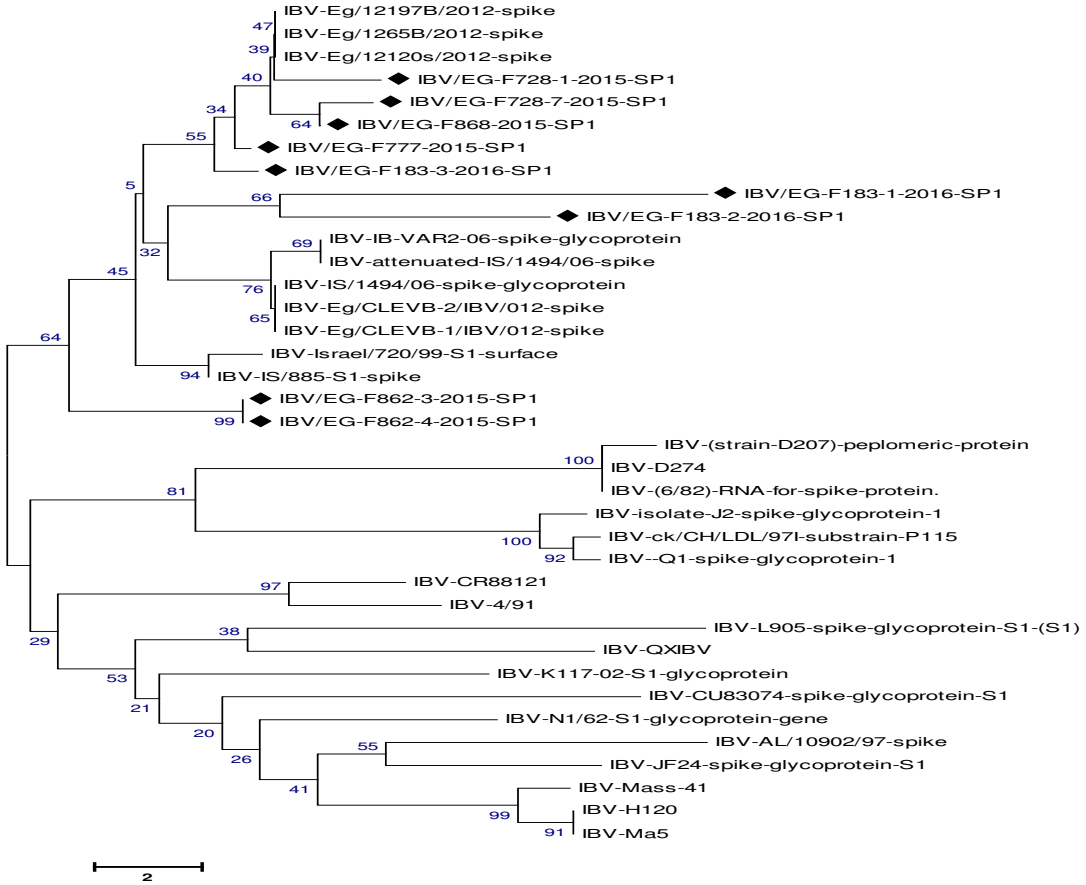

Figure 2: Phylogenetic tree of the partial S1 amino acids sequence of the nine Egyptian IBV isolates in this study, vaccinal strains present in Egypt and other reference IBV strains. The phylogenetic tree analysis was conducted by neighbor-joining method using bootstrap analysis (1000 replications) using Mega 6 software.

Discussion

Increased incidence of respiratory and nephritis symptoms related to infection with (IBV) in vaccinated and non-vaccinated Egyptian flocks have been reported and resulted in severe economic losses [12]. Different serotypes have been reported worldwide and new variant serotypes continue to be recognized [5,13]. Concerning the prevalence of IB in Egyptian chicken broiler farms, examination of 75 chicken flocks

distributed in 8 Governorates from 2014 to 2016, revealed that 31 (41%) were positive for IBV. Thus, indicating that IBV is widely prevalent in Egypt, since the initial description and isolation of the virus [14-16].

Infection of vaccinated flocks could be attributed to virus mutation, while, in non-vaccinated flocks is due to highly contagious nature of the virus and emergence of new IBV variants with nephropathogenic property [4,5,17,18].

The observed clinical signs and gross lesions resembled those reported by several studies including wet eyes, swollen sinuses, reduced feed consumption and body weight, varying mortality, wet droppings, respiratory lesions and renal lesions [19-22].

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
1		95.2	95.2	96.2	84.6	84.6	84.6	87.5	83.7	86.5	98.1	98.1	93.3	93.3	99.0	99.0	88.5	89.4	98.1	1	IBV-Eg/12120s/2012-spike
2	5.0		100.0	96.2	84.6	84.6	84.6	88.5	84.6	87.5	93.3	95.2	92.3	92.3	96.2	94.2	87.5	90.4	95.2	2	IBV-IS/1494/06-spike
3	5.0	0.0		96.2	84.6	84.6	84.6	88.5	84.6	87.5	93.3	95.2	92.3	92.3	96.2	94.2	87.5	90.4	95.2	3	IBV-Eg/CLEVB-2/IBV/012
4	4.0	4.0	4.0		82.7	82.7	82.7	86.5	84.6	85.6	94.2	94.2	95.2	95.2	95.2	97.1	87.5	92.3	97.1	4	IBV-IS/885-S1-spike
5	17.3	17.3	17.3	19.7		98.1	98.1	81.7	80.8	80.8	84.6	85.6	80.8	80.8	85.6	83.7	76.9	79.8	83.7	5	IBV-Mass-41
6	17.3	17.3	17.3	19.7	1.9		100.0	80.8	79.8	80.8	84.6	85.6	80.8	80.8	85.6	83.7	76.9	79.8	83.7	6	IBV-H120
7	17.3	17.3	17.3	19.7	1.9	0.0		80.8	79.8	80.8	84.6	85.6	80.8	80.8	85.6	83.7	76.9	79.8	83.7	7	IBV-Ma5
8	13.7	12.6	12.6	14.9	21.0	22.3	22.3		85.6	95.2	87.5	88.5	86.5	86.5	88.5	86.5	76.9	82.7	85.6	8	IBV-CR88121
9	18.5	17.3	17.3	17.3	22.3	23.6	23.6	16.1		82.7	81.7	83.7	83.7	83.7	83.7	82.7	75.0	77.9	82.7	9	IBV-D274
10	14.9	13.7	13.7	16.1	22.3	22.3	22.3	5.0	19.7		86.5	87.5	87.5	87.5	87.5	85.6	76.0	81.7	84.6	10	IBV-4/91
11	1.9	7.1	7.1	6.0	17.3	17.3	17.3	13.7	21.0	14.9		96.2	91.3	91.3	97.1	97.1	86.5	88.5	96.2	11	EG-F728-1-2015-SP1
12	1.9	5.0	5.0	6.0	16.1	16.1	16.1	12.6	18.5	13.7	4.0		91.3	91.3	99.0	97.1	86.5	87.5	96.2	12	EG-F728-7-2015-SP1
13	7.1	8.1	8.1	5.0	22.3	22.3	22.3	14.9	18.5	13.7	9.2	9.2		100.0	92.3	94.2	83.7	90.4	93.3	13	EG-F862-3-2015-SP1
14	7.1	8.1	8.1	5.0	22.3	22.3	22.3	14.9	18.5	13.7	9.2	9.2	0.0		92.3	94.2	83.7	90.4	93.3	14	EG-F862-4-2015-SP1
15	1.0	4.0	4.0	5.0	16.1	16.1	16.1	12.6	18.5	13.7	2.9	1.0	8.1	8.1		98.1	87.5	88.5	97.1	15	EG-F868-2015-SP1
16	1.0	6.0	6.0	2.9	18.5	18.5	18.5	14.9	19.7	16.1	2.9	2.9	6.0	6.0	1.9		87.5	90.4	99.0	16	EG-F777-2015-SP1
17	12.6	13.7	13.7	13.7	27.6	27.6	27.6	27.6	30.4	29.0	14.9	14.9	18.5	18.5	13.7	13.7		87.5	88.5	17	EG-F183-1-2016-SP1
18	11.4	10.3	10.3	8.1	23.6	23.6	23.6	19.7	26.3	21.0	12.6	13.7	10.3	10.3	12.6	10.3	13.7		91.3	18	EG-F183-2-2016-SP1
19	1.9	5.0	5.0	2.9	18.5	18.5	18.5	16.1	19.7	17.3	4.0	4.0	7.1	7.1	2.9	1.0	12.6	9.2		19	EG-F183-3-2016-SP1
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		

Figure 3: Amino acid identities of Egyptian isolates with other selected infectious bronchitis virus sequence.

In this study, the prevalence of IBV infection in 75 examined farms by rRT-PCR indicated the widespread distribution of the virus all over Egypt. Higher isolation rates were observed in Bani-Sueif (80%), Gharbia (75%) and Monofia (71%) Governorates compared to the other localities.

The primary isolation of IBV from positive rRT-PCR samples, via inoculation of 9-11 day- old SPF egg was adopted. After three blind passages of rRT-PCR pooled samples in ECE, marked curling and dwarfing as well as high embryonic mortality were observed. The recorded results were considered suggestive to IBV infection as previously recorded [8].

Identification and genotyping of IBV was performed by the sequencing of hyper variable region (HVR3) of the SP1 gene [9]. Sequence analysis showed that these isolates were subdivided into 2 groups. The first subgroup (7 isolates) resembling the variant 1 strains including IBV-IS-1494-06 that match with them 87.5% to 96.2% amino acid identity. This finding agreed with previously recorded studies [23]. The second subgroup resembling the new variant 2 strains including isolates IS/885/00 isolated in Israel and Egypt, respectively revealed (92.3%-97.1%) amino acid identities). This finding agreed with a previously reported study [5].

Conclusion

It could be concluded that different variants of IBV are present in high rate in broiler farms even in vaccinated farms and cause severe economic losses. Therefore, the variant strains vaccine may play a role in spreading IBV in broiler farms. IBV has high mutation rate, consequently, continuous surveillance of new IBV strains is important for understanding the molecular evolution of different genotypes and for selecting candidate virus strains for vaccination regimes.

Conflict of interest

The authors have no conflict of interest to declare.

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الملخص العربي

عزل وتصنيف فيروس التهاب الشعب المعدى فى بدارى دجاج التسمين من بعض محافظات مصر

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في هذه الدراسة تم اجراء مسح عن فيروسات التهاب الشعب المعدى فى عدد (٧٥) قطيع من بدارى دجاج التسمين المصرية في ثمان محافظات خلال الفترة ٢٠١٤-٢٠١٦ وذلك بإستخدام تفاعل البلمرة المتسلسل الكمي. وكانت الطيور تعانى من اعراض تنفسيه وكلوية متفاوته. وأوضحت النتائج وجود ٣١ (٤١%) عينة إيجابية لفيروس الإلتهاب الشعبي المعدي. والتي تم تصنيفها جميعاً على انها من المجموعة الأولي (Variant 1) للعزلات المصرية والتي تضمنت العترات(EG-F728-1-2015-SP1, EG-F728-7-2015-SP1, EG-F862-3-2015-SP1, EG-F862-4-2015-SP1, EG-F888-2015-IBV-IS-1494-06 EG-F183-1-2016-SP1,SP1, EG-F777-2015-SP1). كما اتضح أن هذه العترات تشبه الي حد كبير بنسبة تتراوح بين ٨٧.٥%-٩٦.٢%. بينما عترتان من الفيروسات المعزولة (EG-F183-3-2016, EG-F183-2-2016) تشابهت كثيرا مع المجموعة الثانية Variant II (IS/885) بنسبة ٩٢.٣%-٩٧.١% تشابه مع تتابع الأحماض الأمينية. وعليه يمكن إستنتاج أن فيروسات متحورة مختلفة من فيروس التهاب الشعب المعدي متواجدة في مزارع بداري التسمين بنسبة عالية حتى ولو كانت محصنة.