

## Molecular Studies on Infectious Bronchitis Virus Isolated from Broiler Chickens in Damietta Governorate, Egypt

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### Abstract

Fifty-two samples of tracheal swabs and organs were collected from broiler chickens at different districts from Damietta Governorate during December 2012 to August 2014. The samples were collected from chicken farms suffered from respiratory signs, diarrhea and high mortality. Thirty-six samples tested positive for IBV infection when screened by real time quantitative polymerase chain reaction (RT-qPCR). Seven of them were subjected for further isolation and characterization of the virus. The seven selected IBV isolates were propagated in embryonated chicken eggs (ECE) and were confirmed using PCR amplification of the partial sequence of the S1 spike gene. The amplified products were sequenced and the phylogenetic analysis was performed. The seven IBV viruses were closely related to each other with 94-100% nucleotide identity and clustered within the same group with IBV/Eg/CLEVB-2/IBV/012 and Eg/12120s/2012 strains (variant 2-like strain), while their identity with the vaccinal strains used in Egypt was ranged from 67-91%, particularly D274 (89%-91%). These results indicated the complexity of IBV control in relation to the current vaccination programs in Damietta.

**Keywords:** Infectious Bronchitis Virus, RT-PCR, Sp1 gene, Phylogenetic Analysis

### Introduction

Infectious bronchitis (IB) is an extremely contagious viral infection that induces serious economic losses of the poultry industry around the world. The respiratory manifestation of the disease is the most common among clinical cases and it is characterized by tracheal rales, coughing, and sneezing [1]. The infection may spread to the reproductive and renal systems, leading to oviduct and kidney lesions in layers. Infectious bronchitis virus (IBV) is a Coronavirus belonging to Family Coronaviridae (Genus Coronavirus, group III) [2]. The virus is single stranded, non-segmented, positive sense RNA virus with an envelope of approximately 120 nm in diameter [3-5].

The genome of IBV is 27 kB length and encodes for four structural proteins; the spike (S) glycoprotein, the membrane (M) glycoprotein, the nucleocapsid (N) phosphoprotein, and the envelope (E) protein

[4,6]. The S protein is the structural protein of the spikes, which are essential for viral attachment to the cells, fusion of the viral envelop with the host cell membrane, and neutralizing antibodies induction [7]. The S1 and S2 portions of the S protein result from the cleavage of the spike precursor polypeptide [8]. In the S1 subunit, three hypervariable regions (HVRs) have been identified [9,10]. Haemagglutination-inhibiting and most of the virus-neutralizing antibodies are induced by S1 [11]. Therefore, S1 gene analysis is mainly used for molecular characterization of IBV.

In different Egyptian poultry farms, Massachusetts D3128, D274, D-08880 and 4/91 genotypes of IBV strains have been detected [12]. In 2001, the Egyptian variant, Egypt/Beni-Suef/01 which was closely related to the Israeli variant strain was isolated from different poultry farms [13]. Selim et al. [14] reported the isolation of the Egyptian variants

Eg/12120s/2012 and Eg/1265B/2012 from broiler and layer chicken farms in Egypt during 2013. Accordingly, molecular epidemiological studies are very important for genotyping of IBV field strains.

The main objective of the study was to determine the prevalence and diversity of IBV genotypes at broiler chicken farms in Damietta Governorate.

## **Material and Methods**

### ***Sample collection***

In this study, 52 pooled samples were collected from 52 broiler chicken farms located at different districts of Damietta Governorate, Egypt. The investigated broiler farms showed respiratory signs, diarrhea and high mortality during surveillance from December 2012 to August 2014. Five tracheal swabs/each case and organs (trachea, lungs and kidneys) were collected from dead birds. Out of 52 broiler farms 18 were vaccinated against IBV with commercial live attenuated H120 strain vaccines.

### ***Detection of IBV by real time RT-PCR***

Real-time reverse transcription-polymerase chain reaction (RT-qPCR) was carried out for the direct detection of IBV in the clinical samples. The viral RNA was extracted using QiaAmp viral RNA mini kit (Qiagen, Germany) following the manufacturer's guidelines. The reaction was performed using Qiagen one step RT-PCR Kit (Qiagen, GmbH, Hilden, Germany). Amplification of the specific target was conducted using the forward primer IBV5\_GU391, 5'-ACGTATGACTACCCGCAGTATTCA-3', reverse primer IBV5\_GL533, 5'-AGACCAGCCACCATGATTGC-3' and probe IBV5\_G, 5'-FAMCACCACCAGAACCTGTCACC TC-BHQ1-3' [15]. Seven positive samples were then selected for further virus isolation and characterization based on the district locality. The selected isolates were not vaccinated with live attenuated vaccine.

### ***Virus isolation and propagation***

The virus was isolated from seven RT-PCR positive samples. The supernatant was

inoculated into 10-day-old embryonated specific pathogen free (SPF) chicken eggs (Komoshim, Fayuom). Procedures were performed according to OIE terrestrial manual [16]. Embryos were examined for curling and dwarfism after harvesting of the allantoic fluids [17]. Each sample was subjected to three serial blind passages to induce the IBV characteristic lesions in the chicken embryos.

### ***Genetic characterization of hypervariable regions (HVRs) of Spike 1 (S1) gene***

The HVRs of S1 gene were amplified by conventional PCR using Qiagen one step RT-PCR Kit (Qiagen, GmbH, Hilden, Germany). The used primers were IBV-S1-F 5'-CACTGGTAATTTTCAGATGG-3' and IBV-S1-R 5'-CAGATTGCTTACAACCACC-3' [18]. The reaction conditions were reverse transcription at 50°C for 30 min, primary denaturation at 95°C for 15 min followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 54°C for 45 sec and extension at 72°C for 2 min, and finally a final extension at 72°C for 10 min. RT-PCR product was visualized by Electrophoresis in 1.5% agarose in IX TAE, ethidium bromide was added to a concentration of 0.5 µg /mL for nucleic acid visualization. A positive control that was obtained from known positive reference sample from National Laboratory for Veterinary Quality Control on Poultry Production (NLQP) deposits was also run in the reaction.

### ***Sequencing***

Amplicons of the proper molecular size were purified using QIAquick PCR Product extraction kit (Qiagen Inc. Valencia CA) following the manufacturer guidelines. Sequencing was then carried out using Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA, USA, cat. no. 4336817) in an Applied Biosystems 3130 genetic analyzer (Hitachi, Japan), with 3130 Genetic Analyzer Capillary Array, 3130 Running Buffer, BigDye® Terminator v3.1 5X Sequencing Buffer, Hi-Di™ Formamide. Submission of the nucleotide sequences to GenBank for getting accession numbers was carried out.

**Table 1: The IBV reference strains published in Gene Bank used in this study**

IBV strain	Accession number	IBV strain	Accession number
UK /4/91	AF093794	Egypt/F/03	DQ487085
D274	X15832	IBV/Egypt/Zag/07-01 spike	EU368592
H120	M21970	Sul-01-09	GQ281656
Ma5	AY561713	IS-885	AY279533
M41	AY561711	IS-1494-06	EU780077
IBV-strain-NGA-310-2006	FN182278	Mans-5 sp1	KF856876
IBV/Eg/CLEVB-2/IBV/012	JX173488	Mans-2 sp1	KF856873
Eg/12120s/2012	KC533684	IBV/Eg/CLEVB-1/IBV/012	JX173489
CR88121	JN542567	Variant 2	AF093796
Eg/1265B/2012	KC533682	strain NGA/310/2006	FN182278
QXIBV	AF193423	Connecticut 46	DQ487085

### *Sequence and phylogenetic analysis*

Alignment of the sequences was carried out by the Clustal W method using MegAlign module of DNASTar software (Lasergene version 7.2, DNASTAR, Madison, WI, USA). The nucleotide sequences were compared with IBV sequences available in GenBank and commonly used vaccinal strains (Table 1). Sequence identities were calculated using DNASTar software [19]. Construction of the phylogenetic tree of the aligned sequences by

neighbor-joining method in MEGA5 ([www.megasoftware.net](http://www.megasoftware.net)) was performed. The tree topology was evaluated by 500 bootstrap analysis [20]. 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 MEGA5.

**Table 2: The result of real-time RT-PCR in vaccinated and non-vaccinated flocks**

Serial	district	Number of flocks	Total no. of positive flocks	Results in relation to vaccination against IB	
				Positive/Total vaccinated (%)	Positive /Total not vaccinated
1	<b>Damietta</b>	13	10	3/4	7/9
2	<b>Fariskour</b>	18	12	2/6	10/12
3	<b>Kafr saad</b>	11	8	2/4	6/7
4	<b>Elzarka</b>	10	6	2/4	4/6
	<b>Total</b>	52	36	9/18 (50%)	27/34 (79.4%)

## Results

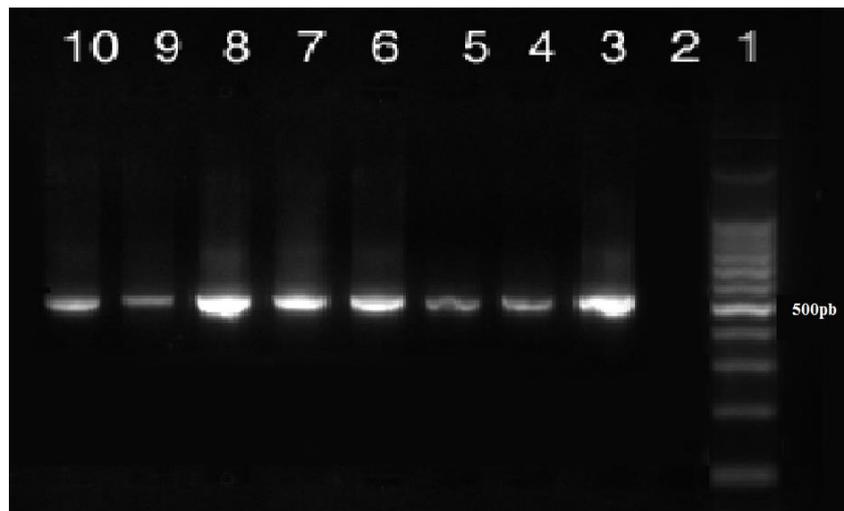
Out of the examined 52 farms, 36 were positive for IBV by RT-qPCR using specific primers and probes (Table 2). According to the locality of the positive districts, seven isolates were selected for further molecular characterization.

After three blind passages of the seven IBV isolates, the inoculated embryos showed obvious curling and dwarfing with subcutaneous hemorrhages. These lesions were characteristic for the seven tested samples.

The selected seven isolates were positive for hypervariable regions of S1 gene by the amplification of 500 bp amplicon (Figure 1).

Sequencing and alignment analysis of such region in the seven strains showed 94-100% nucleotide identity when they were compared together. In addition, when the seven strains were compared with other vaccine strains used in Egypt (M41, H120, Ma5, 4/91, CR88 and D274), the nucleotide identity ranged from 67-91% (Table 3).

Figure 2 shows the analysis of the phylogenetic tree of the seven selected strains. All of the sequences are grouped together with Eg/1265B/2012 and Eg/12120s/2012 Egyptian strains of Variant 2-like lineage. The vaccine strain (D274) was shown to be the nearest strain to the studied strains with nucleotide identity ranged from 89-91% (Table 3).



**Figure 1: Gel electrophoresis of the 500 bp RT-PCR products of the selected seven isolates, Lane 1: 100 bp ladder, Lane 2: negative control, Lane 3: positive control (known positive reference sample from NLQP deposits), Lanes 4-10: the seven isolates under investigation.**

## Discussion

The current study is considered the first molecular record for IBV in Damietta Governorate. Fifty-two broiler chicken farms were examined by real time PCR to highlight the distribution of infection in the districts of Damietta Governorate. Out of 52 examined clinical samples, 36 were positive by real time PCR. Based on their origin, seven samples were selected for further isolation. The selected isolates were not vaccinated with live

attenuated vaccine to avoid isolation of the vaccine stain.

Inoculated SPF-ECE showed typical IBV lesions as curling and dwarfing of the embryo and this agreed with the results reported before [21]. The isolates were confirmed positive by real time PCR.

Genotyping of the seven isolates was carried out using partial sequencing of S1 gene [22]. Relation between recent isolates and other IBV isolates circulating in the Middle

East and with vaccine strains was conducted with phylogenetic analysis.

The sequences of the seven IBV field strains in this study (IBV-EG-Damietta1, IBV-EG-Damietta2, IBV-EG-Elzarka1, IBV-EG-Fariskur1, IBV-EG-Fariskur2, IBV-EG-Kafrsaad1, IBV-EG-Kafrsaad2) present in the same group with Eg/1265B/2012, Eg/12120s/2012 (Figure 2). In accordance with Selim *et al.* [14], the vaccinal strain (D274) is found to be the nearest vaccine strain to these seven isolates.

Kafrsaad1, IBV-EG-Kafrsaad2) present in the same group with Eg/1265B/2012, Eg/12120s/2012 (Figure 2). In accordance with Selim *et al.* [14], the vaccinal strain (D274) is found to be the nearest vaccine strain to these seven isolates.

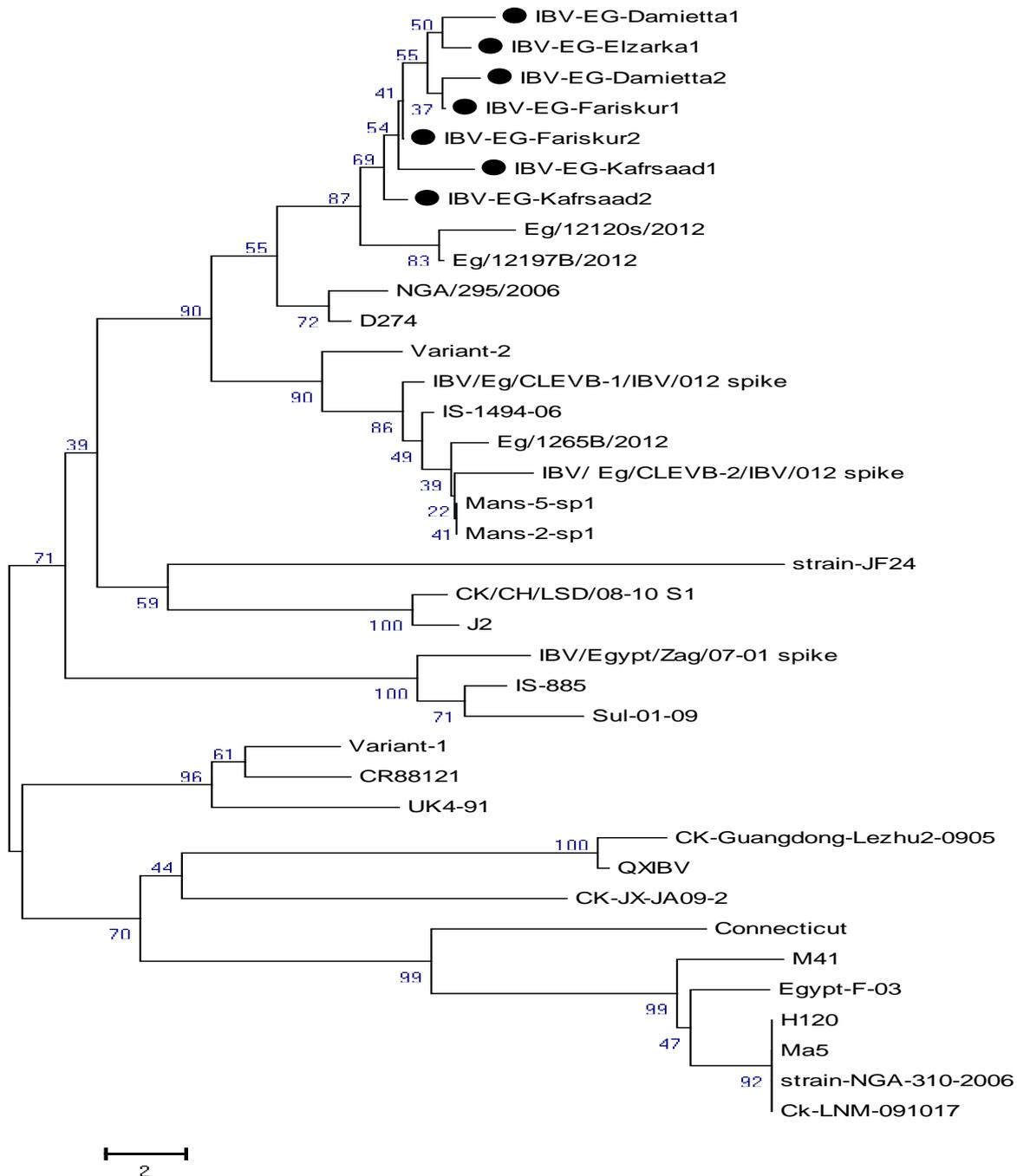


Figure 2: Phylogenetic tree of the 318 b fragment of the S1 nucleotide sequence of the seven Egyptian IBV isolates in this study, vaccinal strain present in Egypt and other reference IBV strains. The phylogenetic tree analysis was conducted by neighbor-joining method using bootstrap analysis (500 replications) using Mega 5 software.

**Table 3: Nucleotide identity between the seven selected isolates and Sequences of IBV reference strains published in Gene Bank**

Isolates	Accession number	country	Year	Identity to No.1	Identity to No.2	Identity to No.3	Identity to No.4	Identity to No.5	Identity to No.6	Identity to No.7
IBV/Eg/CLEVB-2	JX173488	Egypt	2012	65%	65%	66%	66%	66%	66%	67%
IS-1494-06	EU780077	Israel	2006	88%	88%	89%	89%	88%	89%	89%
IS-885	AY279533	Israel	2003	82%	82%	82%	82%	81%	81%	82%
Eg/12120s/2012	KC533684	Egypt	2012	69%	68%	69%	69%	69%	69%	69%
Eg/1265B/2012	KC533682	Egypt	2012	65%	64%	65%	66%	65%	65%	66%
QXIBV	AF193423	China	1999	76%	77%	78%	77%	77%	77%	78%
Egypt-F-03	DQ487085	Egypt	2003	72%	72%	73%	73%	72%	73%	73%
CR88121	JN542567	France	2011	81%	82%	83%	82%	82%	82%	83%
H120	M21970	Netherlands	1989	71%	71%	72%	72%	71%	72%	73%
UK4-91	AF093794	United Kingdom	1998	80%	80%	81%	81%	80%	81%	81%
Ma5	AY561713	USA	1990	72%	72%	73%	73%	72%	73%	73%
M41	AY561711	USA	2004	70%	69%	70%	71%	70%	70%	71%
D274	X15832	Netherlands	1989	90%	90%	91%	91%	89%	91%	91%

As a result of few changes in the amino acid structure of the S1 protein, new IBV genotypes have usually emerged [23]. Such changes could be attributed to the immune pressure caused by the misuse of multiple types of vaccines or emergence of new strains as a result of mutation and recombination [24].

The nucleotide identity between the seven Damietta Governorate IBV field isolates was ranged from 94% to 100% with each other, while, the similarity with the vaccine strains used in Egypt (M41, H120, Ma5, 4/91, CR88 and D274) ranged from 67% to 71%, 69% to 73%, 69% to 73%, 77% to 81% ,78% to 83% ,87% to 91%, respectively. Mutation, recombination and strong positive selection *in vivo* could explain the diversity in S1 [25]. In addition, changes in few amino acids in the spike (S) glycoprotein might result in the generation of genetic variants of IBV.

Circulation of Egyptian IBV variants among vaccinated and non-vaccinated flocks results in severe economic problems [26]. Thus, indicating partial protection after vaccination with a live attenuated heterologous strain [27].

### Conclusion

In conclusion, IBV still circulating in Egyptian flocks causing high economic losses in broilers, Continuous surveillance of IBV strains in Egypt is essential in defining ideal control procedures, including the choice of the appropriate vaccine strains and vaccination programs.

### Conflict of interest

None of the authors have any conflict of interest to declare.

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### الملخص العربي

#### دراسات جزيئية على فيروس التهاب الشعب الهوائية المعدي في بدارى التسمين في محافظة دمياط

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في الفترة من ديسمبر ٢٠١٢ الى اغسطس ٢٠١٤ تم تجميع ٥٢ عينة مسحات وأنسجه من مزارع دواجن التسمين من مناطق مختلفة من محافظة دمياط. تم جمع هذه العينات من مزارع دواجن تعاني من اعراض تنفسيه واسهالات مائيه وزياده في معدل النفوق. ومن خلال فحص هذه العينات عن طريق تفاعل البلمرة العكسي الحقيقي وجد ان هناك ٣٦ عينة ثبت اصابتها بفيروس التهاب الشعبى الهوائى المعدي. تم اختيار ٧ معزولات ممثله عن مناطق المحافظه للعزل والتوصيف الجزيئى . تم تمرير هذه السبع معزولات على بيض خالى من المسببات المرضيه وتم فحص السائل الالنتيوسى لدراسة النتائج النيوكليتيدي لهذه العينات ومقارنتها بمعزولات اخرى لدراسة التغيرات على مستوى النيوكليتيديات والاحماض الامينيه ودراسة مدى اختلافها عن السلالات المعزوله في مصر والدول المحيطة. ومن ثم وجد ان هذه العينات تقع في مجموعه واحده وتتشابه فيما بينها بنسبة ٩٤%-١٠٠% وتقع في نفس المجموعه مع المعزوله IBV/Eg/CLEVB-2/IBV/012 والمعزوله Eg/12120s/2012 strains (variant 2-likestrain) وتراوح معدل تشابه المعزولات مع العترات من اللقاحات المتواجده في مصر ٦٧%-٩١% خصوصاً D274 والذي بلغت ٨٩%-٩١% نسبة تشابه مع المعزولات. من هذه الدراسة نخلص الي ان هناك تعقيدات للتحكم في مرض التهاب الشعب المعدي في ظل اللقاحات المستخدمة حالياً ضد المرض بمحافظة دمياط.