

Evolution of New Variant Strains of Infectious Bronchitis Virus Isolated from Broiler Chickens

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Abstract: *In order to investigate the prevalence and track the genetic evolutions of the circulated infectious bronchitis virus (IBV) strains and their prevalence in Assiut, Egypt, partial S1 gene amplification and sequencing were done during 2013-2014. Three infectious bronchitis virus (IBV) strains with the following accession numbers (KR003438, KR003439 and KR003440 respectively) were detected from 21 tracheal samples originated from suspected broiler flock with a percentage of infection 14.28%. Nucleotide sequencing and analyses of the hypervariable region of the S1 subunit of the virus spike gene showed that the three isolates, shared from 94.3% to 99.3% homologies between each other. 46.5 to 84.6% similarities were observed with the global reference strains which reflected the variant nature of the three isolated viruses. In their comparison with the commonly used vaccine strains (793/B and Massachusetts types) the isolates revealed low nucleotide homologies ranged from 66.3 to 85.7% which the lower values 67% to 69.8% were mentioned with the most used vaccinal strain HI20 that explain the farness of isolated viruses from vaccines used in Egypt. The pathogenicity testing of the 3 IBV isolates in 30-day-old chickens revealed the respiratory nature of these strains with mortality percentages 20-30%.*

These results demonstrates for the first time in Assiut province, the variant nature of the circulated IBV serotypes associated with disease cases and its farness from the used vaccinal strains that emphasize the importance of continued monitoring of IBV to optimize the vaccination strategies to lay a good foundation for developing vaccines having strains genetically close related to the circulated field viruses.

1. INTRODUCTION

Infectious bronchitis (IB) is a highly contagious disease of serious economic importance in the poultry industry worldwide (Cavanagh and Gelb, 2008).

IBV replicates in various tissues of chicken and directly or indirectly causes various clinical signs lesions such as tracheal rales, dyspnea, air sacculitis, diarrhea, proventriculitis, nephritis, atrophied cystic oviducts, abnormal egg and egg-drop (Cavanagh and Gelb, 2008; Cavanagh and Naqi, 2003). Infections may lead to mortality up to 20-30% or higher at five to six weeks of age in chicken flocks (Ignjatovic et al., 2002; Seifi et al., 2010)

Its genome consists of a single-stranded positive RNA molecule (approx. 27.6 kb) that encodes 4 structural proteins: nucleocapsid (N), membrane (M), small membrane (E) and spike (S) (de Wit et al., 2010; de Groot, 2012) The spike glycoprotein on the outside of the virus contains epitopes associated with serotype differences and binding of neutralizing antibodies and it plays a role in attachment and entry into the host cell.

S glycoprotein is post-translationally cleaved into amino-terminal S1 and carboxyl-terminal S2 subunits by cellular proteases. S1 forms a receptor-binding site whereas S2 anchors S1 to the viral membrane. S1 protein involves in infectivity, contains virus neutralizing epitopes, serotype-specific sequences, and hemagglutinin activity (Casais et al., 2003)

For the control of disease caused by IBV, live attenuated viral vaccines are available and had so far been very reliable (Cavanagh, 2003; Cavanagh, 2007) These vaccines use IBV strains such as Massachusetts, Connecticut and Arkansas and combinations therefore, providing protection against almost all field strains of IBV. However, emergence of new variant more heterogeneous IBV strains (Cavanagh, 2003) leads to infectious bronchitis outbreaks in vaccinated flocks leading to significant production losses (Shimazaki et al., 2009; Xu et al., 2007)

The appearance of a large number of IBV variants in different regions is a major constraint for the practical application of serotyping (de Wit et al., 2011). It is important to note that serotyping of IBV is becoming less common today, since the serological tests require a panel of serotype specific antibodies, which is not easy to obtain. Thus, The nucleotide sequencing followed by phylogenetic analysis based on the IBV S1 complete gene that contains the main virus neutralization epitopes provide a fast and powerful method for the identification of genotypes and the prediction of serotypes. Recently, more than 20 serotypes within IBV have been identified worldwide. The complex epidemiology characterize of IB raised the control difficulty (de Wit et al., 2011; Ji et al., 2011; Acevedo et al., 2012; Kamble et al., 2014).

Vaccination against IBV in Egypt is carried out with live and inactivated vaccines containing mainly the virus of the Massachusetts (Mass) serotype, although field observations suggest poor or no protection against wild isolates because IBV outbreaks are frequently observed in vaccinated as well as non-vaccinated farms. Several IB variants have been recognized in Egypt since the 1950s with the isolation of an IB variant shown by neutralisation tests to be closely related to the Dutch variant D3128 and subsequently variants related to Mass, other Europeans IBVs and one related to an Israeli variant have been identified by genome analysis and a new variant ones were recorded by (Abdel-Moneim et al., 2006; Abdel-Moneim et al., 2012).

In the view of the lack of genetic information concerning IBV in Assiut, Egypt and the need for characterization of IBV isolates was considered necessary to determine the genotypes circulating in the region to select the appropriate vaccine for IB control. The objective was generated to investigate the genetic diversity and the phylogenetic relationships among IBV isolates associated with respiratory disease from Assiut chicken flocks, based on partial S1 phylogenetic marker.

2. MATERIALS AND METHODS

2.1. Field Samples

As a result of routine diagnostic activity, 21 pooled tracheal tissue samples (5 for each sample from the same farm) collected from suspect IB outbreaks in Assiut province were submitted for virological investigation. All isolates were from broilers, most of which exhibited respiratory signs of 17 days of age or older. Only live IB vaccines of the Massachusetts type (H120) had been applied.

2.2. Virus Screening with RT-PCR and Molecular Characterization using Sequencing

RNA was extracted from tracheal tissues with Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. For The IBV detection and sequencing, the amplification of the S1 gene variable region approximately 457-bp was the target with using the following primers; forward one was XCE1+ ACTGGTAATTTTTTCAGATGG and reverse one was XCE2-CCTCTATAAACACCCTTACA (Alpha DNA, Montreal, Quebec, Canada) (Cavanagh et al., 2002).

One-step RT-PCR was performed using the verso one step RT-PCR kit (Thermo) according to the procedure of the manufacturer. Reactions were performed in a 25ul volume in a programmable thermocycler. Reverse transcription PCR was carried out for 1 reverse transcription cycle of 60 min at 45°C, followed by 94°C for 5 min, then 35 PCR cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 45s, with a final extension cycle at 72°C for 5 min. Reverse transcription PCR produces a **457-bp** fragment common to all IBV.

Amplified products were separated on a 1% agarose gel and then the gel was visualized under ultraviolet light (AlphaImager; Alpha Innotech, San Leandro, California, USA). the bands were excised and eluted using a QIAquick gel extraction kit (Qiagen Valencia, CA) and then sequenced at the DNA sequencing facility at lab technology company, Cairo Egypt. ABI Big Dye Terminator version 1.1 sequencing kit (Applied Biosystems, Foster City, CA) was used, and products were run on an ABI 3730XL DNA analyzer (Applied Biosystems).

2.3. Egg Inoculation

RT-PCR positive samples were propagated in 9-days-old embryonated chicken eggs for confirmation of virus presence as the followings; tracheal samples were homogenized, diluted 1:10 with PBS,

clarified by centrifugation at 3000 rpm for 5 min and filtered with a 0.45 µm milipore filter. The filtered samples were inoculated into four embryonated eggs via the allantoic cavity (0.2 ml per egg). The eggs were candled daily to record embryo mortality, and allantoic fluid from two of the inoculated embryos was collected 72 h post-inoculation for RT-PCR amplification. After 7 days, the remaining embryos were chilled at 4°C and examined for characteristic IBV lesions such as the dwarfing, stunting, or curling of embryos.

Samples were considered negative if the embryos did not show lesions after five blind passages of 7-day duration. A positive sample was recorded if the specific lesions were observed and the RT-PCR amplification was positive.

2.4. Nucleotide Sequence Analyses

Sequences were edited and aligned with the DNA Star Lasergene 8.0 program (Madison, WI) using the Clustal W algorithm. Strains were designated as three-letter country code/sample ID/year. Phylogenetic analyses and tree construction, performed by the neighbor-joining method with 1000 bootstrap replicates, were conducted with Lasergene software.

The S1 protein gene sequences for the three obtained strains were compared for phylogenetic analysis with twenty-nine representative sequences available in GenBank including the following; vaccine strains, H120 (M21883), M41 (X4722), Connecticut (L18990), Arkansas99 (L10384), Delaware (U77298), GA/2787/98 (AF274438), JMK (L14070), Gray (L14069), Florida 18288 (AF27512), Holte (L18988), 4/91 pathogenic (AF93794), D274 (X15832), D1466 (M21971), Italy02 (AJ457137), K507-01 (AY257064), QX (AF193423), T Australia (AY775779), New Zealand (AF151958), Egypt/F/03 (DQ487085), Eg-12120s-2012 (KC533684), Eg-12197B-2012 (KC533683), EGY/1299B Sp1 gene (KC608182) Egypt-beniSeuf-01 (AF395531), Egypt-D-89-nephropathogenic (DQ487086), EGY-Qalyobia-121 Sp1 gene-2012 (KC608181), IBV-Egypt-01-13-VIR9715-2012 (KC527831), Israel-720-99 (AY091552), Israel-AY135205, Sul-01-09- Iraq (GQ281656).

2.5. Virulence Assessment in Chickens

To determine the ability of IBV isolates to induce disease in chickens, the 3 isolated IBV strains were used for their testing their pathogenicity. Four groups of 15 each were randomly divided, at the age of 30 days, groups 1-3 were challenged intra-nasally with each of the three tested strains with 10^5 egg infectious dose fifty per 200µl of IBV strains. Birds in the 4th group uninfected (mock group) were inoculated with 200µl phosphate buffered saline (PBS). Birds were housed in isolators and provided feed and water ad-libitum. The birds monitored daily for three weeks after inoculation to record the observed clinical signs, mortalities and the seen post mortem lesions.

2.6. Ethical Statement

Animal experiments were carried out in strict accordance with the Ethics of Animal Experimentation of the Egyptian Government, and all efforts were made to minimize suffering. Experiments were approved by the Ethical Committee for Animal Experimentation established by the authors.

3. RESULTS

3.1. Virus Screening and Isolation

Oligonucleotides XCE1+ and XCE2- were used in an attempt to amplify a region of the S1 part of the spike protein gene (Figure 1), as these oligonucleotides had been used successfully to amplify several genotypes of IBV (Capua et al., 1999; Cavanagh et al., 2005) This was successful for the three isolates from 21 pooled samples with a percentage 14.28%, generating a product of 457 bp that was visible in an agarose gel after staining with ethidium bromide. The three isolated strains named Ch/As/Eg/IBV/2014/01, Ch/As/Eg/IBV/2014/02 and Ch/As/Eg/IBV/2014/03 with the following accession numbers (KR003438, KR003439 and KR003440 respectively).

Virus isolation was performed in specified pathogen-free chicken embryos, all RT-PCR positive samples produced characteristic lesions of IBV (curled, dwarfed embryos) with head pressed over the feet and covered by a thickened amnion after the third or fourth passage propagation (Figure 2).

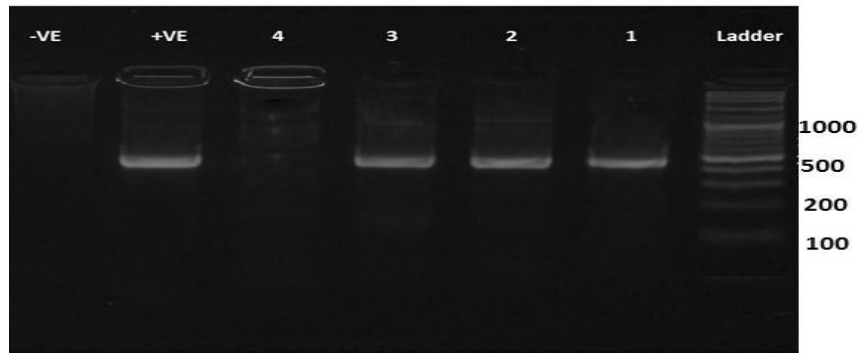


Fig1. PCR amplification for partial S1 gene for positive samples (lane 1, 2 and 3); lane 4; negative sample, lane 5; positive control H120 vaccine (IZOVAC H120 Lassato), lane 6; negative control.



Fig2. Infected chicken embryos, exhibiting dwarfing and curling compared with uninfected embryo (left side)

3.2. Alignment Analysis of the Nucleotide Sequences

The identification and genotyping of the 3 isolates were confirmed by genetic sequencing of the 457-bp region of spike1 (S1) gene followed by alignment and phylogenetic tree construction with previously characterized Egyptian and known global reference strains. Based on the nucleotide sequence, the results revealed that three isolated strains with accession numbers (KR003438, KR003439 and KR003440 respectively) were closely related to each other which the homology percentages ranged from 94.3– 99.3% which the highest similarity record 99.3% was noticed between isolate Ch/As/Eg/IB/2014/01 and Ch/As/Eg/IB/2014/02 (Table 1).

Also the results showed high heterogeneity degree with the previously published Egyptian IBV strains which ranged from 41.2 to 96.1% identity (Table 1) which the highest similarity record 96.1% was noticed between Ch/As/Eg/IB/2014/03 strain and two strains (Eg-1265B and Eg-12120S) isolated in 2012 from Fayoum and Dakahlia-Egypt respectively (Table 1).

Moreover in comparison with reference global strains, the three field isolates were identified as variants which sharing less than 84.6% nucleotide identity with the reference known strains (Table 2); 46.5 to 84.6% identity with European serotypes (including D1466, D274, 793/B and Italy 02) which the highest identity percentage was recorded with D274; 68.1 to 73.9% identity with American serotypes (including Connecticut, Arkansas, Delaware, JMK, Gray, Florida and Holte); 66.9 to 70.2% identity with Asiatic genotypes (including QX); 72.7 to 75.9% identity with Australian/Pacific area strains (including T Australian,); and 66 to 71.2% identity with the middle eastern genotype (IS/720/99, IS/236 and Sul-01-09) (Table 2).

The comparison of nucleotide sequences of the isolated strains with vaccinal ones (H120, H52, MA5, (CR88), and (793/B type) 4/91) (Table 3, Figure 3) revealed low nucleotide homologies that ranged from 66.3 to 85.7%. The highest percentage of nucleotide similarity was noticed with variant vaccinal strain (D274) that ranged from 79.3% to 85.7%, in contrast to 67% to 69.8% with H120 as the most vaccinal strain used in Egypt, (Table 3).

The phylogenetic tree was constructed to ascertain the genetic diversity that currently present among the isolated strains. The topology of the phylogenetic tree indicated the isolated IBV strains with the reference examined ones were grouped into 11 clades; our isolates contained in the group A subclade AII (Figure 3). Also, as shown in the phylogenetic tree, our finding that stated the variant nature of the

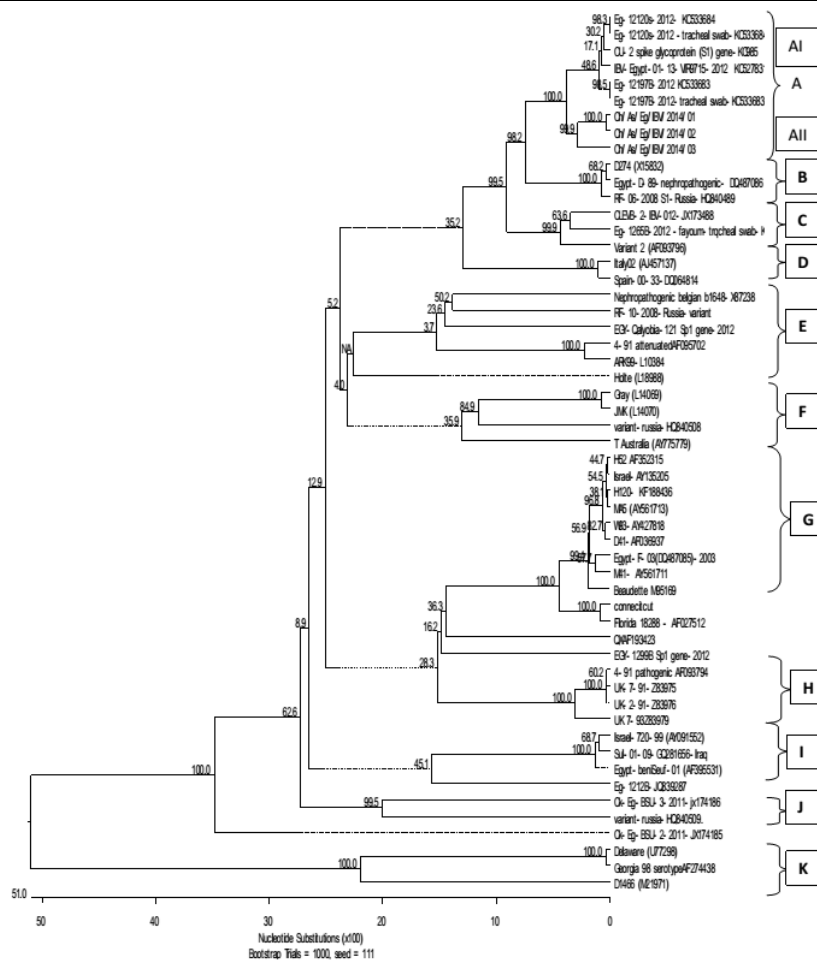


Fig3. Tree showing phylogenetic relationships of isolated IBV strains including isolates (Ch/As/Eg/IBV/2014/01, 2, 3), reference, vaccinal and local strains (based on partial S1 gene sequences S1 gene using the MegAlign program with the Clustal W method (DNASTar, USA).

3.3. Clinical And Pathological Observations

Control (PBS-treated) chickens did not exhibit any clinical symptoms or and gross lesions. All infected chickens, showed signs of head shaking and depression at 24 to 48 h after virus inoculation. Respiratory signs (occulo nasal discharge swelling of eyelids, gasping of air sneezing and conjunctivitis) (Figure 4A-D) were predominant in all of the inoculated groups and were observed as early as 32 hours after challenge in chicks from groups 1 and 2 and persisted until 10 dpi while the clinical signs in group 3 were observed after 24 hours after challenge. The clinical signs in the diseased birds tended to disappear gradually after 10 days of challenge. Deaths start at 4 to 10 days after challenge and were variable; 20% for group 1 and 2 and 30% for group 3 infected with Ch/As/Egy/IBV/2014/03 strain.

Necropsy of the dead and the sick birds revealed that gross lesions were mainly confined to the respiratory system; congestion and catarrhal exudates in the tracheal lumen with formation of caseous plugs on the distal parts of the trachea, cloudiness and caseous materials on the air sacs and congestion of lungs were noted. Hemorrhagic lesions of the cecal tonsil were observed in some of the affected chickens (Figure 5A-E).

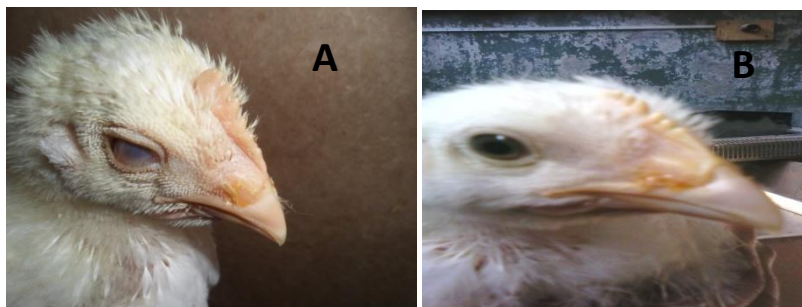




Fig4. Clinical signs showed from chickens experimentally infected with infectious bronchitis virus (IBV). A) slight swelling of eye lids and third eye lid. B) Obvious nasal discharge. C) Opening of the mouth. D) Closed eye and swelling of eye lids

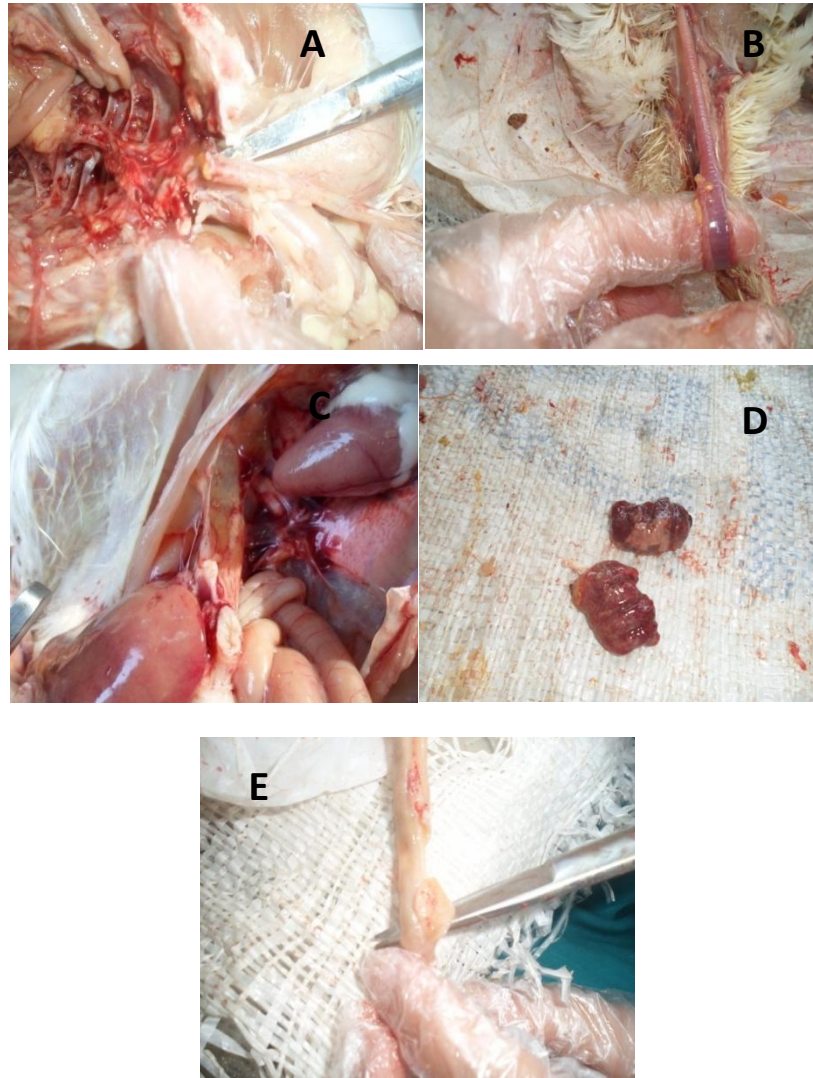


Fig5. Gross lesions from chickens experimentally infected with infectious bronchitis virus (IBV). A) Caseated material occluded the distal part of trachea and bifurcation. B) The tracheal mucosa shows slightly hyperemia and petechial hemorrhages. C) fibrinous airsacculitis. D) Congested hyperemic lung. E) Enlargement of cecal tonsils with petechial hemorrhages

4. DISCUSSION

Infectious bronchitis (IB) is one of the most common and difficult-control poultry diseases. Natural outbreaks of IBV often are the result of infections with strains that differ serologically from the vaccine strains. Come to the rapid and complicated evolutionary of IBV, it is imperative to learn profoundly the circulating IBVs, facilitate selecting the candidate vaccine strain against the infections (Liu et al., 2009). In this study, trials for detection of IBV in commercial chicken flocks that had a history of respiratory signs, and higher than expected mortality rate from Assiut/Egypt from last quarter of 2013 to the first quarter of 2014 were done using RT-PCR technique as by far the most

used tool in this field, having largely replaced serotyping IBV strains (**Jackwood et al., 2010**). The results revealed IBVs from 3 samples out of 21 pooled tracheal samples with a percentage of 14.28 %.

The finding of IB prevalence is lower than previously reported in production farms within neighbouring countries, for example 84%, 58.8% and 42.8% were recorded in Iran, Jordan and Nigeria respectively (**Seyfi Abad Shapouri et al., 2004; Roussan et al., 2009; Owoade et al., 2006**). However both studies sampled chickens within a higher density environment, which may have contributed to the higher prevalence rate compared to the flocks subjected to sampling and examination.

The S1 protein determined the serotypic evolution, the phenotype change and the genetic diversity of IBVs (**Mondal and Cardona, 2007**). In the present study, nucleotide of S1 gene of the 3 field strains were aligned and compared to the representative global, reference, vaccinal strains, to determine the relationship of circulating field isolates with other global strains.

The isolated strains shared between 94.3 to 99.3% nucleotide sequence similarity with each other, higher similarity than the vaccine strains and other representative IBVs, high nucleotide similarities between Ch/As/Eg/IBV/2014/01 and 2 strains from nt identities.

Recent phylogenetic studies have shown that IBV is continuously evolving, with viruses of different genotypes undergoing simultaneous changes (**Liu et al., 2009; Dolz et al., 2008; Worthington et al., 2008; Han et al., 2011**). The results presented here indicate that isolated IBV are variant strains that significantly diverged from viruses representing all known IBV strains and genotypes (Table 2 and Figure 3).

Comparison of the nucleotide sequence of the three field isolated strains with vaccinal strains revealed low (67% to 69.8%) S1 identity values with H120 vaccine (Table 3). (**Gelb et al., 2005**) mentioned that the protection is limited when identity of the S1 protein of the field isolate is less than 85% compared with the vaccine virus which may be considered a cause of the disease in the case of this study as mentioned previously (**Ji et al., 2011; Sun et al., 2011**). So, when possible it is important to periodically evaluate the cross-protective capabilities of vaccines versus recently recovered field isolates.

The emerging of new genotype strains in this study may be due to the following; administration of live vaccines to IBV-infected birds, or infection of chickens with heterologous field isolates shortly after vaccination which is a favorable environment for natural recombination between vaccine and field strains, use of multiple strains for vaccination (Cavanagh, 2007). In addition to transcription of IBV RNA genomes has a high error rate (**Lee et al., 2004**). So, the results of this study may partially explain one of the possibilities for failure of vaccines in the face of infection due to emerging new serotypes that push us to revise the Egyptian vaccination program against IB.

To ascertain what effect of the genetic changes on the pathogenicity of isolated IBV variant strains the challenge test was done. After 24 hours postinoculation, the challenged infected chickens exhibited nasal discharge, gasping of air, and opening of mouth (Fig. 4A-D) which in agreement with the findings that have been described previously (**Seifi et al., 2010**). Twenty four hours after exposure cloudy and edematous abdominal and posterior thoracic air sacs were observed, then 3 days later thickening and filling with clear bubbly exudates were seen (Fig.5C). The lungs also appeared congested in all infected birds (Fig.5D). All of the previously mentioned respiratory lesions were in accordance with some reported observations (**Ignjatovic et al., 2002; Grgiæ et al., 2008**). Also, severe conjunctivitis, associated with abundant lacrimation, oedema, and cellulitis of the periorbital tissues at 48 hours following challenge with IBV were showed that were in accordance with **Terrigino et al., 2008**. In post mortem examination, the predominant lesions were localized in the trachea, lung and cecal tonsils. These results were similar to the findings had been described previously (**Mahdavi et al., 2007; Gaba et al., 2010**).

In summary, this is the first study that identified the genetic changes in the IBVs isolated from chicken broilers in Assiut-Egypt using partial S1 gene sequences. Our results indicated the variant natures of the circulating IBV strains which are genetically distinct from all other known reference IBV and vaccinal strains. Thus the introduction of IBV vaccines foreign to this region is not advisable and manifests the importance of continuing surveillance and characterization of the circulated IBV strains to prepare local vaccines that give the best protection

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Conflict of Interest

The authors declare that they have no conflict of interest.

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