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Molecular and phylogenetic characterization to assess the evolutionary dynamics of the Spike gene hypervariable regions of recent Infectious Bronchitis Virus (IBV) isolates in Egypt

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ABSTRACT

Infectious bronchitis virus (IBV) remains a significant threat to poultry production despite the widespread use of vaccination, resulting in considerable economic losses due to its genetic variability. Our study aimed to investigate the genetic characterization and evolution of IBV field isolates from Egyptian broiler and layer flocks during the period of 2023 to 2024, focusing on the sequences of hypervariable regions of the S1 gene. In this study, 17 samples were collected from various poultry flocks across three Governorates between 2023 and 2024. Of these 17 samples, six tested positive for IBV using RT-qPCR. Virus propagation was conducted for the RT-qPCR positive samples in the allantoic sac of SPF-ECES. Additionally, five isolates undergo genetic characterization through RT-PCR targeting the S gene hypervariable regions 1, 2, and 3, followed by sequencing and phylogenetic analysis. Findings can be summarized as follows: three isolates were found to cluster with the variant II (Egy/var-II) strain, while two aligned with the classic vaccine strains of the Massachusetts serotype. Phylogenetic analysis revealed a divergence between field and vaccine strains, highlighting the significance of mutations in the S1 gene in viral evolution. These results underscore the necessity for continuous surveillance to monitor IBV variants and assess vaccine efficacy in Egypt.

Introduction

Avian Infectious Bronchitis (IB) is a highly contagious viral respiratory disease commonly found in poultry, caused by the infectious bronchitis virus (IBV). The disease primarily affects the respiratory tract, intestinal tract, kidneys, and reproductive system of chickens, leading to respiratory distress and/or potential renal complications. Additionally, it is linked to decreased egg production in both layer and breeder hens. The impact of IB on chicken performance can result in significant financial losses for the poultry industry on a global scale (Jackwood and De Wit, 2018).

IBV is classified under the order *Nidovirales*, family *Coronaviridae*, subfamily *Orthocoronavirinae*, which is the sole member of the genus Gamma coronavirus that affects chickens (Cavanagh, 2003; Perlman and Holmes, 2006).

Infectious bronchitis virus is an enveloped, pleomorphic virus with a diameter of approximately 120 nm. Its club-shaped surface projections, known as spikes, give the virus a crown-like appearance (Lai and Cavanaght, 1997).

The 5′ two-thirds of the viral genome encodes the viral replicase gene. At the same time, the 3′ one-third is responsible for encoding the structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N). These genes are interspersed with several non-structural proteins and are organized in the following manner: 5′-UTR-ORF1a, ORF1b, S, NSP3, E, M, NSP5, N-UTR-Poly(A)-3′ (González *et al*., 2003; Cavanagh, 2007).

The Spike (S) glycoprotein is the most abundant structural protein of IBV, consisting of two subunits: S1 and S2. The S1 subunit plays a critical role, as it contains the receptor-binding domain essential for the virus's infectivity. Furthermore, S1 enhances protective immunity, features sequences specific to various serotypes, and influences the tissue tropism of IBV (Jackwood *et al*., 2012).

The S1 gene of IBV demonstrates considerable genetic heterogeneity, as it contains three hypervariable regions (HVRs). These regions correspond to amino acid positions 60–88 (HVR1), 115–140 (HVR2), and 275–292 (HVR3), which play critical roles in the virus's adaptive evolution and immune escape mechanisms (Cavanagh *et al*., 1988; Moore *et al*., 1997).

In accordance with current classification systems, IBV is categorized into nine distinct genotypes (GI to GIX) and 38 lineages. This classification is based on the sequencing of hypervariable regions within the spike gene, as well as complete S1 gene sequencing (Rafique *et al*., 2024; Shah *et al*., 2025).

Genotype I of IBV involves 30 lineages (GI-1-GI-30) while the remaining genotypes only contain one viral lineage each (GII-1, GIII-1, GIV-1, GV-1, GVI-1, GVII-1, GVIII-1, and GIX-1) (Domanska-Blicharz *et al*., 2017; Ma *et al*., 2019; Mendoza-González *et al*., 2022; Rafique *et al*., 2024).

In Egypt, four genotypes have been identified: GI-1, GI-13, GI-16, and the most prevalent genotype is GI-23, which is further divided into two variants, Egyptian variant I and variant II. (Rohaim *et al*., 2019; Sabra *et al*., 2020; Shosha *et al*., 2024).

This study aimed to investigate the genetic characterization and evolution of IBV field isolates from Egyptian broiler and layer flocks during the period of 2023 to 2024, focusing on the sequences of hypervariable regions of the S1 gene.

Materials and methods

Ethics statement

The materials and procedures employed in this research received authorization from the Scientific Research and Animal Welfare Ethics Committee of the Faculty of Veterinary Medicine at Benha University, Egypt, with the approval number BUFVTM37-06-23.

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Chicken field samples and their demographic data

From January 2023 to December 2024, seventeen samples were collected from layer and broiler flocks showing respiratory issues and mortality, as detailed in Table 1.

In the examined flocks, the chickens showed clear respiratory symptoms, including nasal discharge, gasping, coughing, sneezing, and tracheal rales. Necropsy revealed mild to severe tracheitis, lung congestion, and a thick caseous plug lodged at the tracheal bifurcation. From the freshly dead chickens, 3 to 5 pooled samples of trachea and lung tissue were collected under aseptic conditions. These samples were then placed in viral transport media that contained a 10% antimicrobial solution, including 1000 IU of penicillin, 100mg of streptomycin, and 100 mg of gentamicin (WOAH, 2018).

Tissue suspensions were prepared at a concentration of 20% (W/V) from each pooled sample using phosphate-buffered saline (PBS) containing a Penicillin-Streptomycin mixture at a concentration of 10,000 U/mL and 100mg/mL, respectively (WOAH, 2018). Each processed tissue suspension was then centrifuged at 3000 RPM for 10 minutes at 4°C. Following centrifugation, the supernatants from each pool were collected and filtered using a sterile syringe, Millipore Membrane Filter with a pore size of 0.45 µm. The resulting filtrate was transferred into clean, sterile 2 mL cryotubes and stored at -20°C for subsequent testing. (WOAH, 2018).

IBV detection by quantitative real-time RT-PCR

Viral RNA was extracted directly from the tissue supernatant utilizing a Viral RNA extraction Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The IBV detection process is conducted using a quantitative single-step reverse transcriptase polymerase chain reaction (qRT-PCR) to amplify a 130-base-pair segment of the highly conserved region in the nucleoprotein (NP) gene of IBV. This is achieved with specific primers: IBV-FP (5′ – ATGCTCAACCTTGTCCCTAGCA – 3′) and IBV-RP (5′ – TCAAACTGCGGATCATCACGT – 3′), along with the probe AIBV-TM (FAM-TTGGAAGTAGAGTGACGCCCAAACTTCA-BHQ1) as mentioned by Meir *et al*. (2010).

Isolation and propagation of IBV on specific pathogen-free egg (SPF-ECEs)

Four serial passages of positive IBV qRT-PCR samples were conducted utilizing SPF-ECE obtained from the Koum Oshiem SPF chicken farm located in Fayoum, Egypt. A total of 0.2 mL of sample supernatant was inoculated into the allantoic sac of 9-day-old SPF ECE (three eggs for each sample), while the mock control group was inoculated with 0.2 mL of PBS. The inoculated eggs were incubated at 37°C, with daily candling performed. After 48-72 hours post inoculation, the allantoic fluid was harvested and stored at −20°C for further identification, and the embryos were examined for viral pathognomonic lesions (WOAH, 2018; De Wit, 2000).

Spike gene hypervariable regions genetic analysis by conventional RT-PCR

The hypervariable regions of positive isolates of the Infectious Bronchitis Virus (IBV) were amplified through conventional reverse transcription polymerase chain reaction (RT-PCR) utilizing the Qiagen One-Step RT-PCR Kit (Qiagen, GmbH, Hilden, Germany). The amplification employed the forward primer IBV-S1-F: 5-ACT ACT ACC AAA GTG CCT-3 and the reverse primer IBV-S1-R: 5-ACA TCT TGT GCA GTA CCA TTA ACA-3, which specifically target HVR1 and HVR2 (Abdelmoneim *et al*,. 2002). In addition, hypervariable region 3 (HVR3) is amplified with the forward primer IBV-S1-F: 5-TAC TGG TAA TTT TTC AGA TGG-3 and the reverse primer IBV-S1-R: 5-CAG AYT GCT TRC AAC CAC C-3 (Naguib *et al*., 2017).

The reaction conditions consist of reverse transcription at 50°C for 30 minutes, followed by primary denaturation at 95°C for 15 minutes. This is succeeded by 35 cycles that include denaturation at 95°C for 30 seconds, annealing at 48°C and 50°C for 40 seconds for HVR1, 2, and HVR3, respectively, extension at 72°C for 2 minutes, and a final extension at 72°C for 10 minutes.

Sequencing and Phylogenetic analysis of spike gene hypervariable regions

The amplicons of the hypervariable regions 1, 2, and 3 were purified utilizing the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany). Sequencing hypervariable regions was conducted in both directions

Table 1. Demographic data of the examined flocks

Location	Breeds	Sample Number	Collection date	Age (Days)	Flock Number	Mortality Percent	Vaccination Program	Vaccination Age
Qalyoubia	Broiler	1	Oct-23	18	2500	5%	(GI-I + GI-12) G-23	One day old 7 days old
		2	Dec-23	25	5000	17%	GI-I + GI-13	5 days old
		3	Jan-24	20	3000	7%	G-23	5 days old
		4	Feb-24	22	4000	15%	G-23	One day old
		5	Mar-24	30	4500	14%	GI-I + GI-13	One day old
		6	Apr-24	28	5000	15%	GI-I + GI-13	One day old
		7	Jun-24	33	6000	15%	GI-23 GI-1	One day old 14 days old
	Layer	8	Oct-23	450	2000	2%	GI-1 GI-23 GI-1 + GI-13+ GI-23 (Inactivated)	5 days old 14 days old 120 days old
		9	Apr-24	400	3500	3%		
		10	May-24	500	1500	3%		
		11	Jun-24	430	1000	2%		
Menofia	Broiler	12	Oct-23	35	5500	10%	GI-1 + GI-23	5 days old
		13	Nov-23	27	6000	8%	GI-1 + GI-13	5 days old
		14	Jan-24	21	6500	5%	GI-23 GI-1	One day old 14 days old
		15	Feb-24	23	7000	9%	GI-1 + GI-23	5 days old
Sharkia	Broiler	16	Oct-23	18	5000	11%	GI-1 + GI-23	5 days old
		17	Jan-24	21	3500	12%		

using the Big Dye Terminator v3.1 cycle sequencing kit (PerkinElmer, CA), employing the amplification primers on the Applied Biosystems 3130 genetic analyzer (ABI, USA).

The nucleotide and amino acid identities of HVR 1, 2, and 3 sequences from the isolates were compared and aligned with sequences from commercial IBV vaccines, as well as several Egyptian IBV strains documented in GenBank. The comparative amino acid alignment was conducted using the Clustal W Multiple Alignment tool within Bio-Edit Version 7.0 software (Kumar *et al.*, 2018).

The phylogenetic tree was constructed utilizing the maximum likelihood method, incorporating the Kimura 2-parameter model within the MEGA 11 software, complemented by 1000 bootstrap replicates to ensure the robustness of the findings (Tamura *et al.*, 2013).

Results

IBV detection by quantitative real-time RT-PCR

In a study involving 17 flocks from the Sharkia, Menofia, and Qalyoubia Governorates, qRT-PCR testing indicated that only six samples were positive for the nucleoprotein gene (NP) of the Infectious Bronchitis Virus (IBV). Notably, five of these positive samples were from Qalyuobia Governorate, while one positive sample came from Menofia Governorate. Detailed results can be found in Table 2.

Isolation and propagation of IBV on specific pathogen-free egg (SPF-ECEs)

The embryos harvested from the inoculated samples manifest characteristic lesions indicative of the Infectious Bronchitis Virus (IBV) following four serial passages. These lesions present as notable dwarfing, pronounced stunting, and curling of the body. Additionally, there are signs of slight hemorrhage and widespread congestion, particularly when compared with the embryos from the mock negative control group, which exhibit no such abnormalities. As shown in Figure 1

Figure 1. The embryos that were inoculated exhibit dwarfism, stunted growth, body curling, and congestion when compared to the embryos in the mock control group.

Spike gene hypervariable regions genetic analysis by conventional RT-PCR

The allantoic fluid harvested from Infectious Bronchitis Virus (IBV) isolates demonstrates distinct amplification patterns when analyzed using the conventional one-step RT-PCR technique. Specifically, HVR 1 and 2 produce a clear amplification band at 570 base pairs, while HVR 3 shows an amplification band at 380 base pairs. As shown in Figure 2.

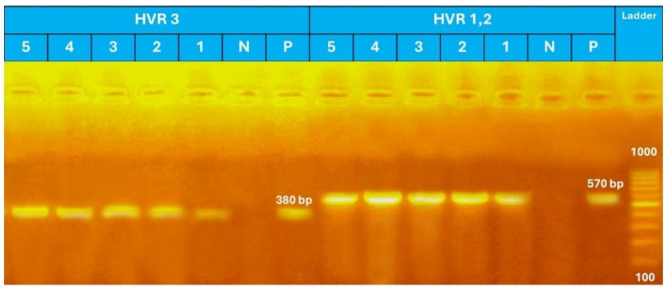

Figure 2. The electrophoretic analysis of RT-PCR products was conducted to examine the hypervariable regions of the infectious bronchitis virus (IBV) spike gene. In this assessment, the amplicon corresponding to HVR1 and HVR2 was identified at a size of 570 base pairs, whereas the amplicon for HVR3 was found to measure 380 base pairs. The results were visualized on a 1.5% agarose gel, and both positive and negative controls were included in the reaction to ensure the reliability of the result.

Sequencing and Phylogenetic analysis of spike gene hypervariable regions

The PCR amplification products of our five isolates were sequenced

Table 2. Result of IBV detection by RT-qPCR.

Location	Breeds	Sample Number	Collection date	Result	Genotype	Strain ID	Accession number	
							HVR1,2	HVR3
Qalyoubia	Broiler	1	Oct-23	Negative	-	-	-	-
		2	Dec-23	Positive	GI-23 Var II	IBV/CH/EG/Moshtohor-3/2023	PV341021	PV345595
		3	Jan-24	Positive	GI-1 Classic	IBV/CH/EG/Moshtohor-1/2024	PV341019	PV345593
		4	Feb-24	Positive		IBV/CH/EG/Moshtohor-2/2024	PV341020	PV345594
		5	Mar-24	Positive	GI-23 Var II	IBV/CH/EG/Moshtohor-4/2024	PV341022	PV345596
		6	Apr-24	Positive		IBV/CH/EG/Moshtohor-5/2024	PV341023	PV345597
		7	Jun-24	Negative	-	-	-	-
	Layer	8	Oct-23	Negative	-	-	-	-
		9	Apr-24			-	-	-
		10	May-24			-	-	-
		11	Jun-24			-	-	-
Menofia	Broiler	12	Oct-23	Positive	Not done	-	-	-
		13	Nov-23	Negative	-	-	-	-
		14	Jan-24			-	-	-
		15	Feb-24			-	-	-
Sharkia	Broiler	16	Oct-23	Negative	-	-	-	-
		17	Jan-24			-	-	-

for hypervariable regions (HVR1, HVR2, HVR3) of the spike (S) gene, and the resulting sequences have been published in the GenBank database (Table 2).

The phylogenetic analysis of hypervariable regions (HVR1, HVR2, and HVR3) derived from our isolates indicates that the five recent strains can be classified into two main clusters. The first cluster corresponds to genotype GI-1, referred to as Classic strains, which includes IBV/CH/EG/Moshtohor-1/2024 and IBV/CH/EG/Moshtohor-2/2024. The second cluster corresponds to genotype GI-23, which includes IBV/CH/EG/Moshtohor-3/2023, IBV/CH/EG/Moshtohor-4/2024, and IBV/CH/EG/Moshtohor-5/2025 (Figures 3 and 4).

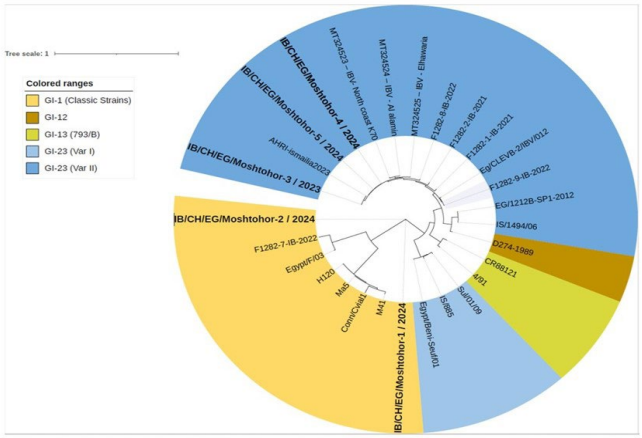

Figure 3. A phylogenetic tree of IBV strains was illustrated in a recent study alongside other Egyptian and reference vaccinal strains obtained from GenBank. The analysis was conducted using MEGA 11 software. The tree was constructed based on 380 nucleotides involving the HVR3 region, employing the maximum likelihood method and the Kimura-2 model, supported by 1000 bootstrap replicates. The tree is further refined using the Interactive Tree of Life (iTOL).
The strains are classified into four main clusters: The first cluster consists of classic strains belonging to genotype GI-1; The second cluster includes variant strains of genotype GI-13 (793/B); The third cluster comprises variant strains of genotype GI-12; The fourth cluster consists of genotype GI-23, which is subdivided into two subgroups, Var I and Var II. Our isolates are categorized under genotype GI-1 and genotype GI-23 (Var II), which are highlighted in bold.

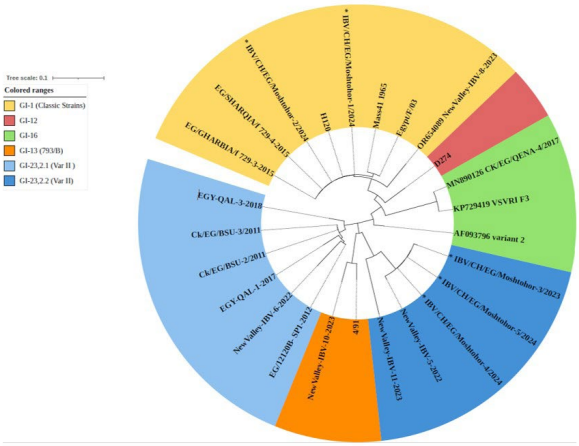

Figure 4. A phylogenetic tree of IBV strains was illustrated in a recent study alongside other Egyptian and reference vaccinal strains obtained from GenBank. The analysis was conducted using MEGA 11 software. The tree was constructed based on 380 nucleotides involving the HVR3 region, employing the maximum likelihood method and the Kimura-2 model, supported by 1000 bootstrap replicates. The tree is further refined using the Interactive Tree of Life (iTOL).
The tree are classified into five main clusters: The first cluster consists of classic strains belonging to genotype GI-1; The second cluster includes variant strains of genotype GI-12; The third cluster includes variant strains of genotype GI-13 (793/B); The fourth cluster includes variant strains of genotype GI-16 and The fifth cluster consists of genotype GI-23 (Var II), which is subdivided into two subgroups: GI-23,2.1 and GI-23,2.2. Our isolates are categorized under genotype GI-1 and genotype GI-23 (Var II), which are preceded by the (*) symbol.

The nucleotide identity analysis of HVR1, HVR2, and HVR3 shows that the strains IBV/CH/EG/Moshtohor-1/2024 and IBV/CH/EG/Moshtohor-2/2024 have a significant genetic relationship with several classical commercial vaccine strains found in Egypt. Specifically, the identity percentages for HVR1 and HVR2 are as follows: 97% for strain H120, 95% for strain M41, and 97% for strain Ma5. For HVR3, the identity percentage is 100% for strain H120 and 99% for strain M41. Furthermore, the HVR1,2 segments of IBV/CH/EG/Moshtohor-1/2024 and IBV/CH/EG/Moshtohor-2/2024 showed nucleotide identity percentages ranging from 95% to 97% when compared to the nephropathogenic strain Egypt/F/03. In contrast, HVR3 of both IBV/CH/EG/Moshtohor-1/2024 and IBV/CH/EG/Moshtohor-2/2024 demonstrated nucleotide identity percentages of 99% with the aforementioned strain (Figures 5 and 6).

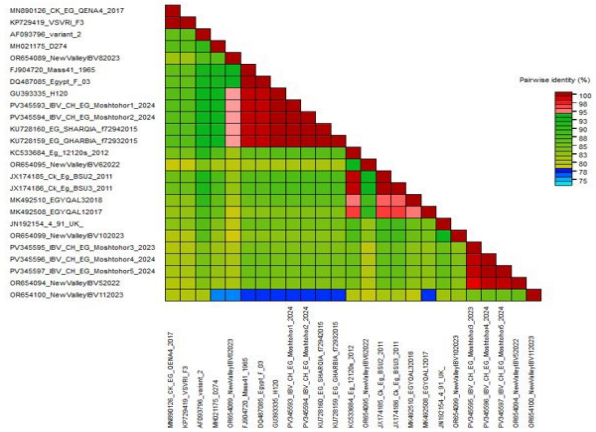

Figure 5. The pairwise nucleotide sequence percent identity of hypervariable regions 1 and 2 (HVR 1 and 2) has been colour-coded for recent isolates of Infectious Bronchitis Virus (IBV), in comparison to reference Egyptian strains and vaccines obtained from the GenBank database. This graphical representation was generated using Sequence Demarcation Tool version 1.3 (SDTv1.3)

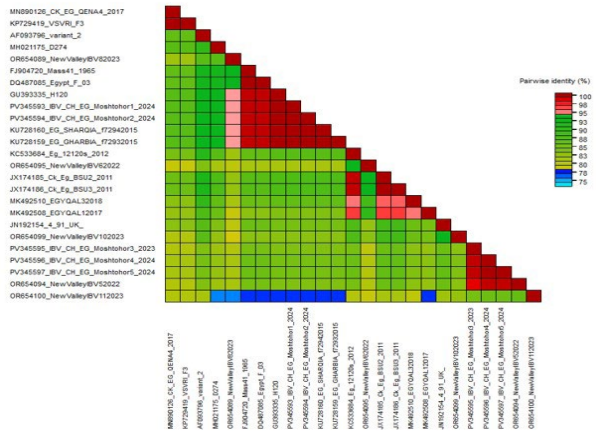

Figure 6. The pairwise nucleotide sequence percent identity of hypervariable region 3 (HVR 3) has been color-coded for recent isolates of Infectious Bronchitis Virus (IBV), in comparison to reference Egyptian strains and vaccines obtained from the GenBank database. This graphical representation was generated using Sequence Demarcation Tool version 1.3 (SDTv1.3).

Overall, the identity percentages of IBV/CH/EG/Moshtohor-1/2024 and IBV/CH/EG/Moshtohor-2/2024 compared to recently identified classical strains in Egypt varied between 94% and 99%.

The nucleotide identity percentages of HVR1 and HVR2 for the strains IBV/CH/EG/Moshtohor-3/2023, IBV/CH/EG/Moshtohor-4/2024, and IBV/CH/EG/Moshtohor-5/2025 were reported to be between 82% and 83% when compared to commercial Var II vaccine strains in Egypt. Additionally, these strains exhibited a nucleotide identity percentage ranging from 95% to 99% with Egyptian Var II strains. As shown in Figure 5.

The nucleotide identity percentages of HVR3 for the strains IBV/CH/EG/Moshtohor-3/2023, IBV/CH/EG/Moshtohor-4/2024, and IBV/CH/EG/Moshtohor-5/2025 were reported to be between 85% when compared to the commercial Var II vaccine strains in Egypt. Furthermore, these strains demonstrated a nucleotide identity percentage ranging from 84% to

86% when compared to CK/EG/BSU/-2/2011 and CK/EG/BSU/-3/2011, respectively. Overall, the identity percentages of the strains IBV/CH/EG/Moshtohor-3/2023, IBV/CH/EG/Moshtohor-4/2024, and IBV/CH/EG/Moshtohor-5/2025 compared to recently identified variant 2 strains in Egypt ranged from 92% to 98% (Figure 6).

Amino acid (aa) alignments of the hypervariable regions HVR1 and HVR2 in variant II-related strains identified (13:15) amino acid substitutions in comparison to the EG/1212 B-SP1-2012 strain. The strains IBV/CH/EG/Moshtohor-4/2024 and IBV/CH/EG/Moshtohor-5/2024 exhibit 13 substitutions: A69S, Y71H, A79S, Q87V, N88T, S118G, S121Y, M127L, O150R, Y152H, and R159Z. Additionally, IBV/CH/EG/Moshtohor-3/2023 presents the same substitutions as listed above, along with two additional substitutions: S77N and S117N.

Amino acid alignments of the hypervariable region HVR3 in the strains IBV/CH/EG/Moshtohor-3/2023, IBV/CH/EG/Moshtohor-4/2024, and IBV/CH/EG/Moshtohor-5/2024 reveal five amino acid substitutions when compared to the EG/1212 B-SP1-2012 strain. These substitutions are detailed as follows: H281S, N284S, H288S, I290F, and S292T (Figure 8).

Two strains, designated IBV/CH/EG/Moshtohor-1/2024 and IBV/CH/EG/Moshtohor-2/2024, which belong to the classic group, demonstrated no amino acid mutations in hyper variable regions 1, 2, and 3 when analyzed against the reference strains in H120, as well as the Ma5 and/or M41 strains.

Discussion

Infectious bronchitis virus (IBV) remains a significant concern for chicken flocks in Egypt. Despite the application of extensive IBV vaccination protocols within the poultry industry, IBV strains are still circulating in the country and having effects on poultry flocks (Zanaty *et al*., 2016).

Sultan *et al.* (2019) Indicated that vaccination protocols represent the most effective preventive measure for mitigating infections caused by IBV. Nevertheless, these protocols periodically encounter challenges due to the evolutionary mechanisms of IBV, and the emerging probability of a new IBV variant can occur from a minor amino acid mutation in the hypervariable regions of the S1 subunit of IBV (Adzhar *et al*., 1997). Therefore, continuous genetic characterization of recent IBV isolates from Egypt is essential for tracking revolutionary patterns and updating the prevalence map, which are crucial for implementing effective preventive measures against IBV disease (Sultan *et al*., 2015).

To achieve the stated objective, we collected seventeen pooled trachea and lung samples from broiler and layer flocks that exhibited respiratory symptoms and were gathered from various regions of Qalyuobia, Menofia, and Sharkia governorates. These findings matched with common signs observed in IBV-infected flocks in the field (Mostafa and Ali, 2019). The collected samples were analyzed by using qRT-PCR, and we identified six positive samples, resulting in a detection rate of 35%. No-

Strains	HVR 1 (60 -88)																										
Nucleotide Position	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88
Var II	G	S	X	X	Q	C	T	A	G	A	I	Y	W	S	K	N	F	S	A	A	S	V	A	M	T	A	P	Q	N
IBV/CH/EG/Moshtohor-3/2023	.	.	.	.	.	.	.	.	.	S	.	H	.	.	.	.	.	N	.	S	.	.	.	.	.	.	.	V	T
IBV/CH/EG/Moshtohor-4/2024	.	.	.	.	.	.	.	.	.	S	.	H	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	V	T
IBV/CH/EG/Moshtohor-5/2024	.	.	.	.	.	.	.	.	.	S	.	H	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	V	T

Strains	HVR 1 (60 -88)																										
Nucleotide Position	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88
H120	G	S	S	S	G	C	T	V	G	I	I	H	G	G	R	V	V	N	A	S	S	I	A	M	T	A	P	S	S
Ma5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
IBV/CH/EG/Moshtohor-1/2024	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
IBV/CH/EG/Moshtohor-2/2024	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Strain	HVR 2 (115 – 140)																										
Nucleotide position	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	1311	132	1333	134	135	136	137	138	139	140	
Var II	Y	K	S	S	S	G	S	C	P	L	T	G	M	I	P	O	Y	Y	I	R	I	S	A	M	R	N	
IBV/CH/EG/Moshtohor-3/2023	.	.	N	G	Q	.	Y	.	.	.	.	.	L	.	.	R	D	H	.	.	.	.	.	.	z	.	
IBV/CH/EG/Moshtohor-4/2024	.	.	.	G	Q	.	Y	.	.	.	.	.	L	.	.	R	D	H	.	.	.	.	.	.	K	.	
IBV/CH/EG/Moshtohor-5/2024	.	.	.	G	Q	.	Y	.	.	.	.	.	L	.	.	R	D	H	.	.	.	.	.	.	K	.	

Strain	HVR 2 (115 – 140)																										
Nucleotide position	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	1311	132	1333	134	135	136	137	138	139	140	
H120	Y	K	H	V	G	-	-	C	P	I	T	G	M	L	Q	O	H	S	I	R	V	S	A	M	K	N	
Ma5	.	.	.	G	.	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
IBV/CH/EG/Moshtohor-1/2024	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
IBV/CH/EG/Moshtohor-2/2024	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	

Figure 7. A comparative analysis of the deduced amino acid sequences of hypervariable regions 1 and 2 (HVR1 and HVR2) of the Spike protein from our isolates, as reported in this study, against the vaccine strains currently in use in Egypt. Note that, dot indicates an identical amino acid, and a dash indicates an amino acid deletion.

Strains	HVR 3 (275 – 292)																	
Nucleotide position	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292
Var II	H	N	E	S	N	A	H	P	N	N	G	G	V	H	T	I	-	S
IBV/CH/EG/Moshtohor-3/2023	.	.	.	.	.	.	S	.	.	S	.	.	.	S	.	F	-	T
IBV/CH/EG/Moshtohor-4/2024	.	.	.	.	.	.	S	.	.	S	.	.	.	S	.	F	-	T
IBV/CH/EG/Moshtohor-5/2024	.	.	.	.	.	.	S	.	.	S	.	.	.	S	.	F	-	T

Strains	HVR 3 (275 – 292)																	
Nucleotide position	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292
H120	H	N	E	T	G	A	N	P	N	P	S	G	V	Q	N	I	Q	T
M 41	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
IBV/CH/EG/Moshtohor-1/2024	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
IBV/CH/EG/Moshtohor-2/2024	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Figure 8. A comparative analysis of the deduced amino acid sequences of hypervariable region 3 (HVR3) of the Spike protein from our isolates, as reported in this study, against the vaccine strains currently in use in Egypt. Note that, dot indicates an identical amino acid, and a dash indicates an amino acid deletion.

tably, five of the six positive flocks were located in Qalyobia governorate, with one additional positive flock from Menofia governorate. These findings align with prior studies conducted in Egypt, which reported prevalence rates of Infectious Bronchitis Virus (IBV) between 30% and 50% in flocks presenting with respiratory symptoms (Awad *et al*., 2023).

After that, the positive IBV samples are propagated for four passages in nine-day-old SPF-ECES, resulting in the induction of dwarfism, stunting, and congestion in SPF embryos, which are considered a pathogenomic lesion similar to previous findings with IBV Egyptian variant viruses (Amer *et al*., 2024). Moreover, molecular analysis and phylogeny of the S1 subunit serve as effective tools for tracking the genetic evolution of IBV, providing a solid foundation for its genetic classification (Wibowo *et al*., 2019).

Based on the sequences of HVR1, HVR2, and HVR3 of the S1 gene, the IBV strains identified in this study were phylogenetically categorized into two distinct IBV genotype clusters, referred to as mass-like and variant 2 (Var II). The isolates identified as being part of mass-like clusters are IBV/CH/EG/Moshtohor-1/2024 and IBV/CH/EG/Moshtohor-1/2024. This research indicates that mass-like strains account for two out of the five isolates included in this investigation.

Furthermore, the sequence alignment of mass-like strains shows nucleotide identity ranging from 94%-99%, which indicates they are genetically homologous with reference H120, Ma5, and M41 vaccinal strains in Egypt. This finding suggests stable circulation of classic strains, consistent with reports from Egypt (El-Samahy and Mourad, 2021; Awad *et al*., 2023). On the other hand, by looking at the result of this study, three isolates (IBV/CH/EG/Moshtohor-3/2023, IBV/CH/EG/Moshtohor-4/2024, IBV/CH/EG/Moshtohor-5/2024) clustered with other Egyptian variants 2 strains reported by Amer *et al*. (2024) and Zanaty *et al*. (2016), which form genotype GI-23 of IBV, together with the Egyptian variant I detected in 2001 (Rohaim *et al*., 2019).

Isolates of the Var II strain display an amino acid mutation and show 82%-83% sequence homology when compared to the reference vaccine strain of Var II (EG/1212 B-SP1-2012). However, they exhibit a closer homology of 95–99% with Egyptian field strains. This observation indicates that these isolates are pathogenic variants that have been circulating within natural populations.

Ultimately, these findings support the hypothesis that the evolution of IBV is influenced by regional selection pressures, such as vaccine implementation and host adaptation. The co-circulation of various genotypes poses challenges for disease control, as highlighted by Valastro *et al*. (2016) in their comprehensive global meta-analysis of IBV diversity.

Conclusion

Our study highlighted the coexistence of mass-like and variant 2 strains in our isolates, drawing attention to the diversity of IBV in the Egyptian field. Consequently, it is essential to conduct prevalence studies to monitor viral persistence, as well as to perform genetic analyses to detect and comprehend the evolution of IBV and its relationship to both circulating and vaccine strains in Egypt. Ultimately, our findings contribute to the understanding of IBV epidemiology as well as mutation, which can enhance strategies for mitigating the virus.

Conflict of interest

The authors have no conflict of interest to declare.

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