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Evolutionary Analysis of the recently isolated avian infectious bronchitis virus in Egypt reveals marked genetic diversity and recombination event

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Research

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Abstract

Egypt's poultry industry has suffered significant financial losses as a result of the persistent spread of several IBV genotypes, among chicken flocks. In comparison to the typical strains that were circulating in Egypt and Israel, the 4 isolates in our investigation were all clustered in genotype I clade 23 (GI.23) within the Variant 2, according to the phylogenetic analysis of the full length S1 gene of four isolates. Whereas they were also separated from the Classic (mass-like strains) (GI-1) vaccine strains H120, M41, and Ma5. The phylogenetic data showed that the Egyptian IBV viruses GI.23 were linked to GI.23.3.A high similarity percentage (98.3% to 99.6%) was found among the research isolates based on the results of the amino acid identity analysis. While showing identity with GI.23.1and GI.23.2.1, between 91.8% and 92.4% and 94.8% to 93.1% respectively. In comparison with vaccine stains such CR88, H120, Ma5, and Connecticut-46, the tested isolates showed 77.7–79% similarity percent. S1 had 41 aa substitutions in comparison to GI-23.2.1-KU238172, HVR1 showed changes in positions 54l, 57S, 62Q, and 72H, HVR2 showed six variations, and HVR3 showed four changes. In the same context,18 locations were expected to be N-glycosylated in a predictive computational approach that was used to identify and compare posttranslational modifications such as N-glycosylation patterns in the S1 glycoprotein.

Keywords: *Phylogenetic analysis; avian infectious bronchitis; virus, S1 gene.*

Introduction

Avian infectious bronchitis virus (IBV) causes an acute highly contagious significant illness that globally affecting the poultry industry. In recent years, IBV has caused significant economic losses and commonly affects flocks that have received vaccinations as well as those that have not **Lee *et al.* (2003), Mase *et al.* (2004), Pohuang *et al.* (2009), Yan *et al.* (2009), Cook *et al.* (2012) and Jackwood (2012**). In Egypt, even though different vaccination strategies have been applied in poultry farms across Egypt, IBV appears to have established an endemic status in chicken farms, leading to significant economic losses. Respiratory diseases that are primarily suspected of being caused by IBV infections are currently plaguing poultry farms in Egypt. IB virus (IBV) serotype diversity and the frequent emergence of new variants are the main causes of vaccination failure **Yan *et al*. (2011) and Zou *et al*. (2010).**

Avian infectious bronchitis virus (IBV) is enveloped single-stranded positive-sense RNA of 27.6 kbs unsegmented genome belongs to fam-

ily Coronaviridae genus Gammacoronavirus **King *et al*. (2011) and Masters and Perlman (2013).** Four structural proteins encoded by IBV genome which are: the spike (S) glycoprotein, membrane (M) glycoprotein, nucleocapsid (N) phosphoprotein, and envelope (E) protein **Spaan *et al*. (1988)**. One of the main major structural proteins of IBV is the spike (S) glycoprotein, which is cleaved into the S1 globular and S2 stalk polypeptides following translation **Cavanagh (2007)**.Genetic variants are believed to arise from recombination and a few amino acid alterations in the spike (S1) glycoprotein of IBV **Kingham *et al*. (2000).**

The S1 subunit contains three HVRs: amino acids (38–67), 91–141, and 274–387. HVR2 and HVR1 include sequences associated with particular IBV serotypes **Binns *et al*. (1986); Kusters *et al*. (1989)** and serotype-specific neutralizing epitopes **Cavanagh *et al*. (1988); Koch *et al*. (1990); Kant *et al*. (1992)**. The only sequences that are necessary for virus characterization are HVR-III or HVR I–II regions, but for IBV genotyping, the S1 gene sequences which included three HVRs showed more precise findings **Mockett *et al*. (1984); Cavanagh *et al*. (1992).**

Previously, many poultry farms in Egypt have reported the detection of IBV strains associated with the D3128, D274, D-08880, 4/91, and Egypt/Beni-Suef/01 genotypes **Sultan *et al*. (2004); Abdel-Moneim *et al*. (2002).** Newer IBV strains that were able to undermine immunity produced by the majority of available vaccines recently caused significant losses for Egypt's commercial chicken industry **Abdel-Moneim *et al*. (2012).**

Currently, both classic and mutant strains of IBV co-circulate, resulting in recurrent outbreaks of disease **Zanaty *et al*. (2016a); Zanaty *et al*. (2016b).** The GI-23 lineage is the most common lineage found in Egyptian poultry flocks **Ghetas *et al*. (2016); Moharam *et al*. (2020).**

Numerous IBV genotypes, including GI-1, GI-13, GI-16, and GI-23, have been persistently present in Egyptian chicken flocks, resulting in a significant financial loss for the poultry industry **Abozeid *et al*. (2020).** The genetic diversity of S1 may be influenced by heterologous immunization given to the same chicken, which could lead to escape mutants **Ameen *et al*. (2023)**.

So, in order to employ the best vaccination strategy feasible, it was necessary to analyze the genetic evolution in the IB virus in order to pinpoint the locations of these alterations and their relatedness in selecting the strains used in vaccine manufacture.

Material and Methods

Sample collection

A total of 40 tracheal swab and organs (Trachea, lung and kidney) field samples representing four broiler flocks were taken during 2022 – 2023 from suspected commercial farms in 4 Egyptian governorates (Alexandria, El Behera, El Gharbia, and Qalyubia governorates). Samples were taken from chickens that were exhibiting significant respiratory symptoms, such as gasping, difficult inspiration, coughing, sneezing, and high mortalities. After homogenizing the tracheal samples, they were suspended in sterile PBS and centrifuged for 30 minutes at 3000 rpm at 4ºC to clarify them.

Infectious bronchitis virus screening and detection using real-time PCR:

Following the manufacturer's instructions, RNA was extracted from the supernatant fluid using the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany). Using Specific primers and probes that target the NP gene **Meir *et al*. (2010).** The RNA was examined against infectious bronchitis virus by real time PCR using the reaction mixture prepared by Verso one-step qRT-PCR kit plus ROX (Thermo Scientific, Cat No. AB-4100/A) and the Stratagene MX3005 real-time PCR equipment (Agilent Technologies, Santa Clara, CA, USA) was used to perform the reaction.

Virus isolation:

Three 10- to 12-day-old specific-pathogen-free (SPF) embryonated chicken eggs were inoculated with 0.2 ml of supernatant fluid of each positive sample via allantoic sac. The inoculated eggs were then incubated at 37ºC for up to seven days, under daily observation.

Virus detection by conventional RT-PCR:

The S gene was amplified from IBV positive samples using the Easyscript one-step RT-PCR kit (Trans. - Cat No. AE411-02) in accordance with the manufacturer's protocol, utilizing spe-

cific primer sets **(Table 1)**. The specific amplified PCR products were then separated using 1.5% agarose gel stained with ethidium bromide and Biometra® Compact electrophoresis system (Analytik, Jena) and visualized utilising a Biometra® gel documentation system from Milan, Italy's Biometra Laboratories under ultraviolet light. and a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) was used to purify the positive PCR fragments.

Table (1). Primers used for amplification of Full spike gene by conventional PCR:

Forward primers	Reverse primers	Molecular weight	Reference
AGTBTCYACACAGTGTTAYAAGCG	GGYCTRWANKSRCTYTGGTAG	1582 bp	**Abozeid *et al.* (2017)**
TTAAATCATTTCAGTGTGTTAATAAT	CATAACTAACATAAGGGCAA	1190 bp	
GATGTCAACCAGCAGTTTGTAG	GCATACTGACTAGCATTAGCTG	1418 bp	

Gene Sequencing and phylogenetic analysis:
Using S gene specific primers, the purified PCR products were then sequenced using the Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA) and a 3500 XL DNA analyzer (Applied Biosystems).
Bio-edit, version 7.2.5 **Hall *et al.* (2011)** was used to edit and assemble the sequences. Sequence congruence between the IBV and GenBank sequences was evaluated using BLASTn on the NCBI website (www.ncbi.nlm.nih.gov/BLAST).The amino acid and nucleotide sequences were aligned using the CLUSTAL-W technique in the Bio-edit application version 7.2.5 and compared to vaccinations and other IBV strains from other groups that were obtained from the GeneBank database.
The alignment was performed using the CLUSTAL-W tool (Lasergene version 7.2; DNASTAR, Madison, WI, USA) and the MegAlign module of DNASTAR software **Burland (2000)**. MEGA version 6 generated the phylogenetic tree with 1000 bootstrap repeats and a moderate strength maximum likelihood method **Tamura *et al.* (2013) and Kumar *et al.* (2016)**. Using DNAstar software (DNAStar, Madison, WI), the pairwise nucleotide percent identity was determined **Burland (2000).**

Results

Virus screening and isolation

The samples from the four flocks in this study yielded positive results for IBV detection using rRT-PCR. The virus was isolated from the trachea and kidney homogenates of each flock. The allantoic fluid from the third passage of each sample was verified positive by rRT-PCR.

Genetic and phylogenetic characterization

Sanger dideoxy nucleotide sequencing was performed on four positive RT-PCR samples from Alexandria, El Behera, El Gharbia, and Qalyubia governorates, and the sequences were submitted to GenBank with accession numbers, PQ619399, PQ619400, PQ619401, and PQ619402. The four positive samples provided amplified PCR products for the entire S1 gene, which were subsequently sequenced and analyzed. The strains under study were aligned and compared to other Egyptian IBV sequences and vaccine-like strains available in GenBank, and a phylogenetic tree was constructed using the amino acid sequences of the full S1 protein of the four isolates, which were all clustered in genotype I clade 23 (GI.23) within the Variant 2 group when compared to representative circulating Egyptian and Israeli strains. While found separated from vaccine strains like as H120, M41, and Ma5 (Classic (mass-like strains) (GI-1)) **(Figure 1).** The phylogenetic results showed that Egyptian IBV viruses GI.23 were associated with GI.23.3 **(fig. 1).**
Alignment analysis of the S1¬ for both nucleotide and deduced amino acid was done and compared to previously published reference and vaccine IBV strains routinely utilized in the field (H120, Ma5, and CR88), as well as original Egyptian viruses and Variant 1 and

Variant 2. For the comparative analysis, one virus representing each group's consensus was selected. The amino acid identity analysis results showed that the study isolates had a high identity percentage with each other, ranging from 98.3% to 99.6%. While their identity to the GI.23.1 strain ranged from 91.8% to 92.4% and 94.8% to 93.1% for GI.23.2.1 tested isolates showed 77.7-79% similarity to vaccination stains such as CR88, H120, Ma5, and Connecticut-46. **(Figure 2).**

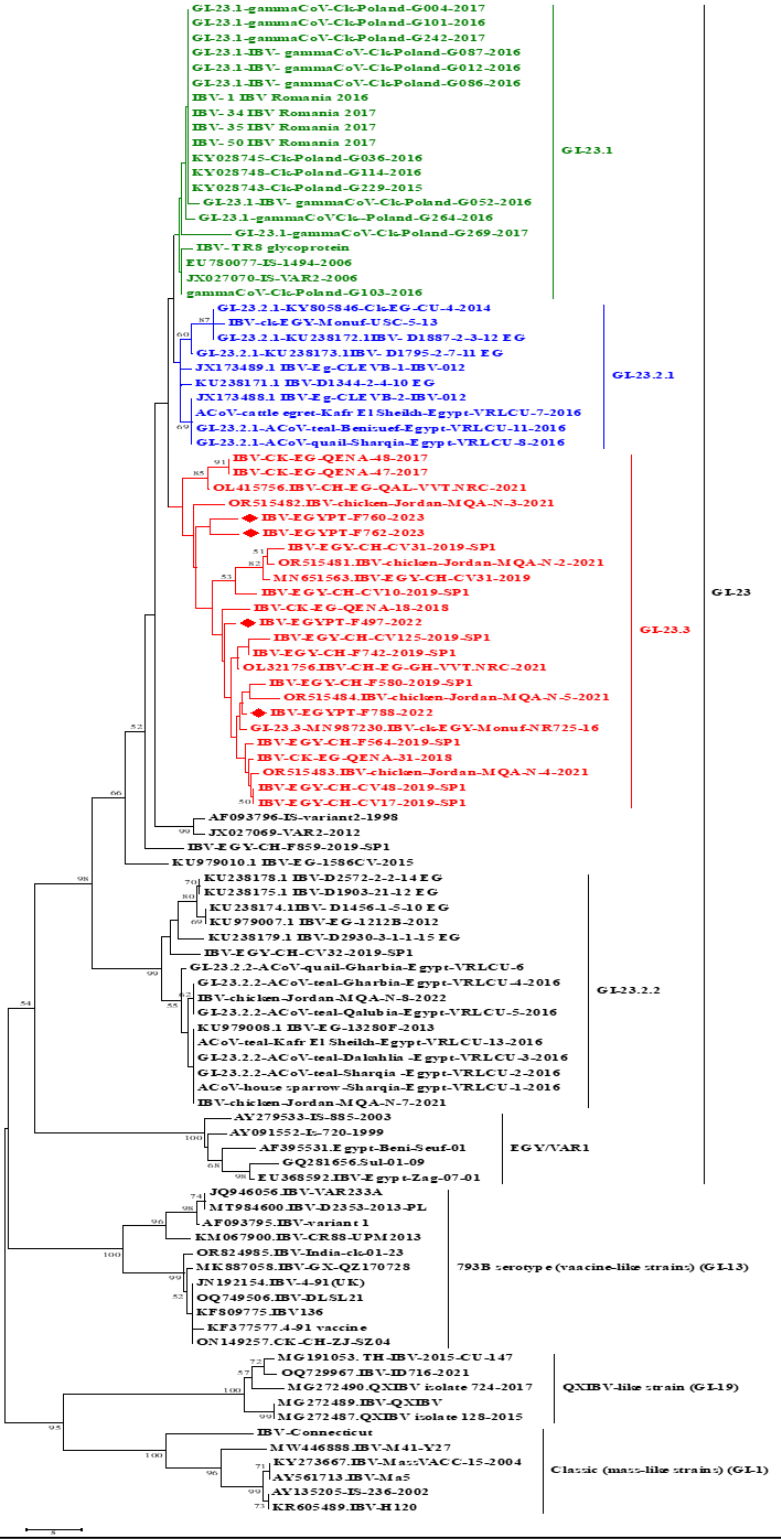

Figure (1). Phylogenetic tree of the S1 gene of IB viruses isolated in Egypt during 2022–2023 and reference isolates from GenBank. Phylogenetic analysis was conducted by using the neighbor-joining algorithm with the Kimura 2-parameter model and the reliability of Phylogenetic tree inference at each node was estimated by the bootstrap method with 1,000 replications. Evolutionary analysis was conducted by using MEGA6.A red rhomboid indi-cates isolates sequenced specifically for this study. ♦ indicate the tested isolates in this study

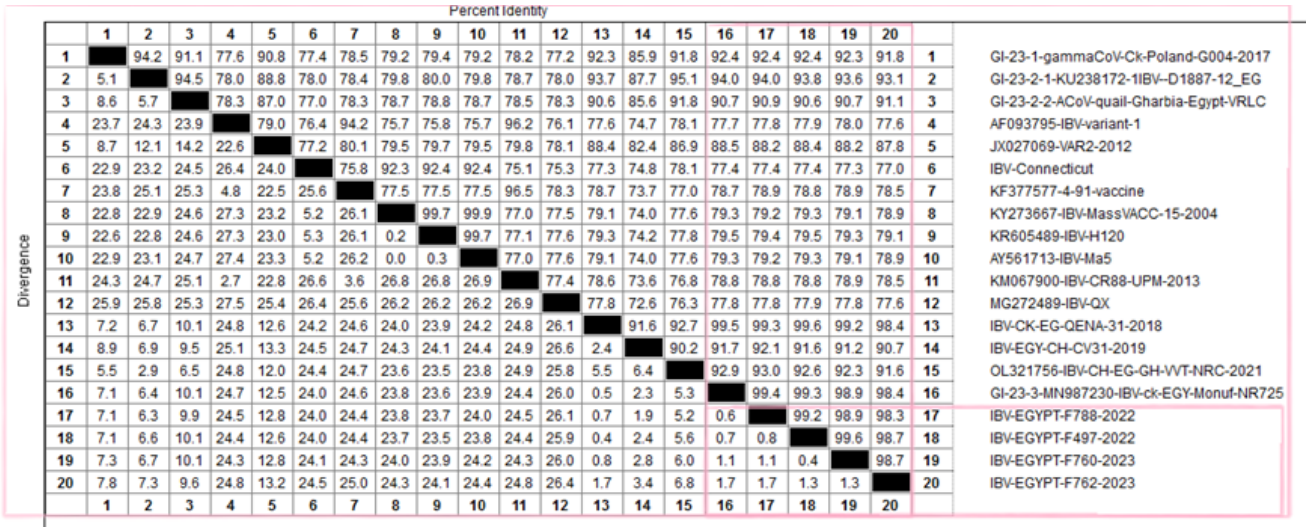

Figure (2). Nucleotide identities and divergence of Sequenced viruses compared to other selected strains.

Mutation analysis of spike gene

Forty-one aa substitutions were located in S1 in comparison to GI-23.2.1-KU238172, the changes in positions 54l, 57S, 62Q, 72H showed in HVR1, while six variation were observed in the HVR 2, HVR 3 included four changes

Prediction of Potential N-Glycosylation Sites

A predictive computational analysis was performed to identify and compare posttranslational modifications such as N-glycosylation motifs in the S1 glycoprotein, where 18 sites were predicted to be N-glycosylated (24 NETV, 51 NVSV(HVR1), 74 NFSA, 102 NFTD(HVR2), 145 NLTV, 164 NMTA, 179 NDTK, 213 NGTA, 238 NFSD, 248 NSSL, 265 NTTL, 272 NFTF, (277 NESN, 280 NASP, 307 NFSF(HVR3), 426 NITL, 448 NVTD, 514 NETG)

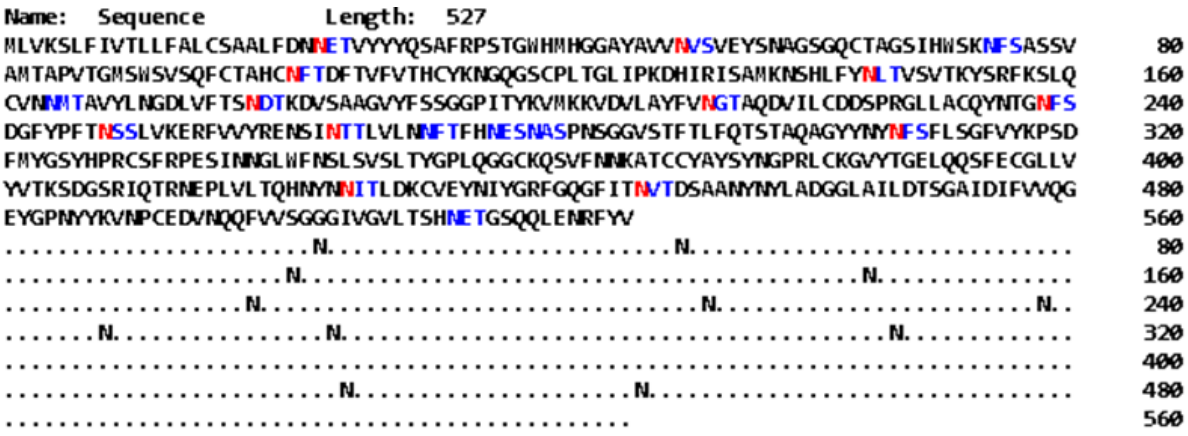

Figure (3). The S1 glycoprotein's posttranslational modifications, including N-glycosylation motifs, were found and compared using a predictive computational approach.

Selection Pressure Analysis of S1 Glycoprotein

The selection pressure examination was conducted by a web server called SELECTION. It estimates the selection pressure by using a Mechanistic Empirical Combination (MEC) model at certain codons. The MEC model acts by consideration of the different rates of aa changes. The positive selection was observed at different aa residues all over the S1 glycoprotein. The positive selection was detected in a total of 37 aa residues (7%), while the rest were subjected to purifying selection. Furthermore, it was shown that most aa residues subjected to positive selection were in and/or near the residues forming the three HVRs of S1 glycoprotein

Discussion

Over the past two decades, IBV has been persistently spreading throughout Egyptian chicken flocks, resulting in significant financial losses for the poultry industry. In Egypt, several IBV genotypes with distinct genetic and pathogenic characteristics have been identified, including GI-1, GI-13, GI-16, and GI-23 **Abozeid *et al*. (2020)**.

One of the main structural proteins of IBV is the spike (S) glycoprotein, which is translated into the S1 globular and S2 stalk polypeptides **Cavanagh (2007).** High levels of genetic variability are present in the S1 gene, which has three hypervariable regions (HVRs) that trigger neutralizing and serotype-specific antibodies **Moore *et al*. (1997) and Cavanagh *et al*. (1988)**. There are 32 distinct lineages and six IBV genotypes that have been found globally, and the S1 gene sequence can be utilized to distinguish between them based on phylogenetic analysis **Valastro *et al*. (2016).** There have been several reports of point mutations, insertions, deletions, and recombination between strains **Adzhar *et al*. (1997), Hewson *et al*. (2014)**. It has been found that the glycoprotein's N-glycosylation sites contribute to cellular tropism and virulence **Li *et al*. (2000).**

The S1 region of the S gene has the highest level of nucleotide variability, which is mostly contained within three distinct hypervariable regions (HVRs), which correspond to amino acids 38–67, 91–141, and 274–387 (HVR1, HVR2, and HVR3, respectively) **Shan *et al*. (2018).**

It was reported that four genetic lineages, GI-1, GI-23, GI-16, and GI-13, were co-circulating in chicken farms in Egypt. GI-1 contains both the vaccine-like strains and the classic wild strains. GI-23 includes two Egyptian variation subgroups: Egy/Var-1 and Egy/Var-2. GI-16 contains the recently reported Q1-like strains. GI-13 contains 4/91-like strains that are thought to have developed from the 4/91 vaccination strain that is now in use **Abozeid *et al*. (2020)**.

In our study, 4 isolates from the different governorates were sequenced for full length S1 gene and the sequences were submitted to GenBank with accession numbers, PQ619399, PQ619400, PQ619401, and PQ619402. The phylogenetic analysis of full length S1 gene of 4 isolates revealed that the isolates were all clustered in genotype I clade 23 (GI.23) within the GI.23.3 in comparison to typical strains that were circulating in Egypt and Israel. whereas they were separated from the vaccination strains H120, M41, and Ma5 (Classic (mass-like strains) (GI-1)) as well. Egyptian IBV viruses GI.23 were linked to GI.23.3, according to the phylogenetic data **(fig.1).**

The S1 was aligned for both nucleotide and deduced amino acid, and the results were compared to original Egyptian viruses, Variants 1 and 2, and previously published reference and vaccine IBV strains often used in the field (H120, Ma5, and CR88).

According to the results of the amino acid identity analysis, the study isolates had a high similarity percentage (98.3% to 99.6%), while revealing their identities to the GI.23.1ranged from 91.8% to 92.4%. and 94.8% to 93.1% for GI.23.2.1 **(Figure 2).** Similarities between our isolates with vaccination stains such CR88, H120, Ma5, and Connecticut-46 ranged from 77.7 to 79%.

There were 41 aa substitutions in S1 compared to GI-23.2.1-KU238172; HVR1 displayed alterations in positions 54l, 57S, 62Q, and 72H, while HVR 2 revealed six variations; HVR 3 included four changes.

To find and compare posttranslational changes like N-glycosylation patterns in the S1 glycoprotein, a predictive computational analysis was carried out; 18 sites were predicted to be N

-glycosylated.

The results of our study were in agree with **Zanaty *et al*. (2016a)** who reported that Despite vaccination, the IBV variants continue to cause persistent infection on farms, Due to the high rate of mutation and recombination, which raises the possibility of IBV vaccination failure, new mutations are constantly appearing and various types are widely circulated, making it difficult to control IB in Egypt.

Naguib *et al*. (2017) reported the continuous circulation of IBV variant II by the phylogenetic analysis of partial S1 gene of the three HVRs of IBV strains which revealed that they were closely related to the currently circulating Egyptian variant II. Our findings were in accordance to phylogenetic study of **Naguib *et al*. (2017)** which revealed that the Egyptian IBVs are divided into two groups: the variation group (field strains) of the GI-23 genotype and the classic group (vaccine strains), which resembles the GI-1 genotype.

Findings of **Ameen *et al*. (2023)** showed that one factor contributing to S1 genetic diversity that could lead to escape mutants is heterologous immunization in the same chicken.

Conclusion

Further investigations are needed to study the impact of the genetic variation of recent IBV as pathogenicity and antigenicity, also study the cross protection with commercial vaccine is needed.

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