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Genetic Changes of S Gene during Co-inoculation of Two Infectious Bronchitis Virus Vaccines in SPF Chicks

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ABSTRACT

Despite widespread immunizations, infectious bronchitis (IB) remains a significant issue in the Egyptian poultry industry. Multiple IBV genotypes, GI-1, GI-13, GI-16, and GI-23 have been continually circulating among chicken flocks in Egypt, inducing a substantial economic loss to the poultry sector. In addition, live attenuated vaccines representing classical and variant strains can control IBV in Egypt, mainly H120 and 793B. The H120 vaccine is widely spread and offers inadequate protection against heterotypic IBVs in the field. Therefore, a homologous live-attenuated VAR2 vaccine was developed from the Egyptian variant-2 strain Eg/1212B/2012.IB. Variant II vaccine protects against the homologous IBV challenge under experimental and field circumstances. In this study, an experimental trial was performed to simulate field practices such as heterologous vaccination of day-old specific pathogen-free chicks with IBV H120 vaccine (representing GI-1) and IB Var 2 vaccine (representing GI- 23). The current study aimed to determine the existence of nucleotide and amino acid variations within the S gene in isolated viruses following ten passages in the same bird. The deduced amino acid sequence of the S gene indicated viruses isolated from the 6^{th} and 10^{th} passages were identical and shared (96 %) and (83 %) identities with the IB variant II vaccine and H120, respectively. However, amino acid substitutions were observed at 26 positions in the N terminal domain (S1) and S2 is conserved compared to IB Var 2 vaccine. Most amino acid modifications occurred in the receptor binding domain (RBD) of the S1 gene. HVR2 has seven amino acid changes compared to the IB Var II vaccine. Isolates of P6 and P10 lacked IBV glycosylation site at position 139 which was detected in IBV/EG/1212B/2012 as well as IB variant II vaccine. The study also revealed no evidence of recombination between the two used live vaccines.

Keywords: Glycosylation site, IBV, Live attenuated vaccine, Receptor binding domain, Spike protein.

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INTRODUCTION

Infectious bronchitis (IB) disease is a severe, extremely infectious chicken viral disease, with all chicken ages being susceptible. There are three primary clinical manifestations of IB; reproductive, renal, as well as respiratory, with morbidity rates reaching one hundred percent. Nevertheless, the mortality depends on the existence of secondary infection, management, immune status, flock age, and environmental factors (**Ganapathy, 2009**). Based on strain virulence, the death rate among young chicks ranges from 25 to 30 percent but may surpass 80 percent **(Jackwood and De Wit, 2013**). It causes a substantial economic loss in the global poultry sector (**Cook *et al.*, 2012; Jackwood, 2012**). IB etiological agent is infectious bronchitis virus (IBV), a gammacoronavirus member of the Coronaviridae family. The viral genome is a single-stranded, positive-sense RNA of an approximate length of 27.6 Kb (**Masters and Perlman, 2013**) containing 13 open reading frames (ORFs) in the sequence 5'-UTR-1a-1b-S-3a-3b-E-M-4b-4c-5a-5b-N-6b-UTR-Poly (A) tail-3' (**Payne, 2017**). The three ′ one-third of the viral genome codes for four structural proteins, namely; nucleocapsid (N), spike (S), membrane (M) and

envelope (E) proteins, as well as multiple non-structural proteins (**Masters and Perlman, 2013**).

Additionally, spike protein presents as trimers on the virion's surface and includes a conformation-dependent epitope (**Cavanagh, 1995**). The S protein is translated as a single polypeptide before being cleaved subsequently via host proteases to develop two subunits (globular head domain as well as N-terminal) as well as S2 (stalk domain as well as C-terminal) subunits that mediate viral entry as well as membrane fusion (**Bosch *et al.*, 2003; Belouzard *et al.*, 2012**).

The S1 subunit is a highly variable subunit that contains the major neutralizing epitopes (**Moore *et al.*, 1997; Wickramasinghe *et al.*, 2011**) and the receptor binding domain (RBD) crucial for tissue tropism as well as a viral attachment (**Belouzard *et al.*, 2012**). To elude the host's immune response, these epitopes may change fast, especially inside the hypervariable regions (HVRs) of the S1 gene **(Belouzard *et al.*, 2012).** The spikes of coronaviruses are heavily adorned with glycans on the residues of asparagine (N) (N-glycosylation, consensus sequence N-x- S/T) and are consistent among various genotypes **(Shang *et al.*, 2018; Zheng *et al.*, 2018).** The spike's glycosylation in human coronaviruses has been shown to be a protein folding determinant **(Tortorici *et al.*, 2019; Walls *et al.*, 2019)**; receptor interactions **(Han *et al.*, 2007)** and the masking of neutralizing epitopes on the spike, hence impacting the immune system of the host **(Zhou *et al.*, 2010)**.

Three HVRs are present among the amino acids (38–67), (91–141) and (274–387) the S1 subunit **(Cavanagh *et al.*, 1988; Moore *et al.*, 1997)**. HVR2 and HVR1 include sequences linked with specific IBV serotypes **(Binns *et al.*, 1986; Kusters *et al.*, 1989)** as well as serotype-specific neutralizing epitopes **(Cavanagh *et al.*, 1988; Koch *et al.*, 1990; Kant *et al.*, 1992).** Virus characterization only depends on the HVR-III or HVR I-II regions' sequence. However, the S1 gene sequences, including three HVRs, revealed more accurate results for IBV genotyping **(Mockett *et al.*, 1984; Cavanagh *et al.*, 1992).** Many different IBV serotypes and genotypes are continually reported worldwide **(Bijlenga *et al.*, 2004; Cavanagh, 2007; Jackwood, 2012).**

Mutations such as recombination, substitutions, deletions, as well as and insertions among various strains regularly take place in nature **(Adzhar *et al.*, 1997; Jackwood, 2012; Hewson *et al.*, 2014)**, resulting in novel variant virus emergence **(Cavanagh, 2007; Jackwood, 2012; Abolnik, 2015).**

Classical and mutant IBV strains co-circulate in Egypt, generating periodic disease outbreaks **(Zanaty *et al.*, 2016a; Zanaty *et al.*, 2016b).** The most prevalent lineage circulating in Egyptian poultry flocks until now is GI-23 lineage **(Ghetas *et al.*, 2016; Moharam *et al.*, 2020)**. Live-attenuated vaccines have effectively contributed to controlling IB in the field **(Cook *et al.*, 1999; De Wit *et al.*, 2011).** However, live-attenuated IBV vaccines may generate variant IBV strains via recombination and/or mutations **(Toro *et al.*, 2012; Abozeid *et al.*, 2017).** Live-attenuated vaccines are primarily used to immunize broiler chickens, future prime layers, and breeder flocks (**Schijns *et al.*, 2008; Abozeid and Naguib, 2020).** In Egypt, IBV infection in broilers is characterized by respiratory distress such as gasping, coughing, sneezing, or tracheal rales**.** Live-attenuated classical and variant vaccine strains, primarily 793B and H120 are utilized to control IB in Egypt. These vaccinations offer excellent protection against most variant viruses. However, they offer inadequate protection against certain variant field viruses of the GI-23 lineage **(Sultan *et al.*, 2019)**.

Lately, a homologous live-attenuated VAR2 vaccine has been generated from the Egyptian variant-2 strain Eg/1212B/2012 (GenBank accession number JQ839287). It has been used widely in Egyptian poultry farms to control IB **(Ali *et al.*, 2018).** The VAR2 vaccine has experimentally offered protection against homologous IBV challenge **(Ali *et al.*, 2018).**

In this study, it was reported that the genomic variations occurring in S gene following 10 passages of simultaneous *in vivo* inoculation of Classic and Variant II vaccines in the same chicken intermittently obtained tissues, as well as swabs, had been subjected to IBV RT-PCR that targets the N gene **(Meir *et al.*, 2010).** IB VAR2 vaccine, reisolated virus of passage six and passage ten were sequenced and analyzed for amino acid alterations as well as single nucleotide polymorphisms (SNPs).

MATERIALS AND METHODS

Vaccines

IB VAR2 and H120 are two live attenuated IBV vaccines commercially available in the market and were used in the study. All immunizations were administered intranasally at the dosage advised by the manufacturer’s instructions.

Experimental Design

Thirty specific pathogen-free (SPF) chicks were required for ten passages of the two live attenuated vaccines (IB H120 and IB VAR2) in the same bird, three chicks for every passage. SPF chicks were obtained from Kom-Osheim SPF Project,

Fayoum, Egypt and placed in a rearing isolator in Reference Laboratory for Quality Control on Poultry Production (RLQP), Dokki, Egypt (Biosafety Level 3 facilities). According to the manufacturer's recommended doses, three one-day-old chicks were vaccinated intranasally with the two preceding live attenuated vaccines. Tracheal swabs and organs (kidney, trachea and lung tissues) were collected from these birds five days post-vaccination. Tracheal and kidney tissues were individually homogenized in MEM with glucose. The supernatants were collected and regarded as the viral stocks of the initial passage. The viral stock of the first passage was inoculated in another three chicks and the method outlined for the first passage was repeated. This was deemed the second passage. This procedure was repeated until the tenth passage, with all viral stocks being tested for the presence of viral RNA and its quantity utilizing RT-qPCR targeting the N gene. All individual viral stocks were stored at −80 °C for subsequent S gene sequencing.

Virus detection by real-time RT-PCR

Extraction of viral RNA from the samples was done directly utilizing the Easy Pure viral RNA/DNA extraction kit (Trans - Cat No. ER201-01) in accordance with the manufacturer's instructions. The extracted viral RNAs were stored at -20°C till examination. The virus identification was made through Real-Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) for the existence of the N gene sequence of the IBV utilizing Verso one-step qRT-PCR kit plus ROX (Thermo Scientific, Cat No. AB-4100/A), via particular primers as well as probes that target the N gene **(Meir *et al.*, 2010).**

Sequencing of the S gene

Subsequently, IBV-positive samples were examined using particular primer sets (Table 1) to amplify the S gene using Easyscript one-step RT-PCR kit (Trans. - Cat No. AE411-02) according to the manufacturer's protocol. Amplicons were excised and purified from gels using the QIAquick gel extraction kit- Cat No.28704 (Qiagen Inc. Valencia CA). The sequencing of the purified RT-PCR products was done using Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA) as well as a 3500 XL DNA Analyzer (Applied Biosystems). Genetic changes in isolates of both P6 and P10 were compared with IBV H120 vaccine published in the GenBank database with gene bank accession No. (FJ888351.1) and IB Var 2 vaccine sequenced in this study. BioEdit version 7.0 was used to read the output sequences and to create multiple sequence alignments **(Hall, 2004).**

Table 1: The sequence of primers of conventional RT-PCR for the identification of full spike gene.

Nucleotide position	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)	**Ref.**
18,990–20,572	AGTBTCYACACAGTGTTAYAAGCG	GGYCTRWANKSRCTYTGGTAG	**(Abozeid et al., 2017)**
20,819–22,009	TTAAATCATTTCAGTGTGTTAATAAT	CATAACTAACATAAGGGCAA	
21,850–23,268	GATGTCAACCAGCAGTTTGTAG	GCATACTGACTAGCATTAGCTG	

3D Visualization of mutations in the S gene

The majority of S1 protein's receptor binding domain and neutralizing epitopes were dispersed in the three HVRs frequently utilized for the classification of IBV genotypes as reported by previous studies **(Moore *et al.*, 1997; Wang and Huang, 2000).** To establish a model structure for further investigation of mutations in the newly identified IBV strains as well as the HVRs, the S protein's 1140 aa of IB VAR2 vaccine was used. By comparing the S protein's deduced amino acid sequences of passages with those of the two vaccines, an alignment was established. Using the Swiss-Model repository's best-fit template (6cv0.1.A) for a cryoelectron microscopy structure of the IBV spike protein, the 3D structure was constructed. The structure of the spike protein was retrieved and utilized as a model for the S monomer and trimer. The genetic changes of recovered viruses (P6 and P10) were detected and visualized using Geneious prime (Version 2020.1.2) (https://www.geneious.com/prime/).

Prediction of N-linked glycosylation sites in IBV S protein

N-glycosylation sites' gain or loss in the spike protein was predicted using the online server at http://www.cbs.dtu.dk/services/NetNGlyc

RESULTS

Virus detection by Real Time RT-PCR

Using RT-PCR assay, which targets the N gene, isolates of P6, P10 and used vaccines were detected positive for IBV.

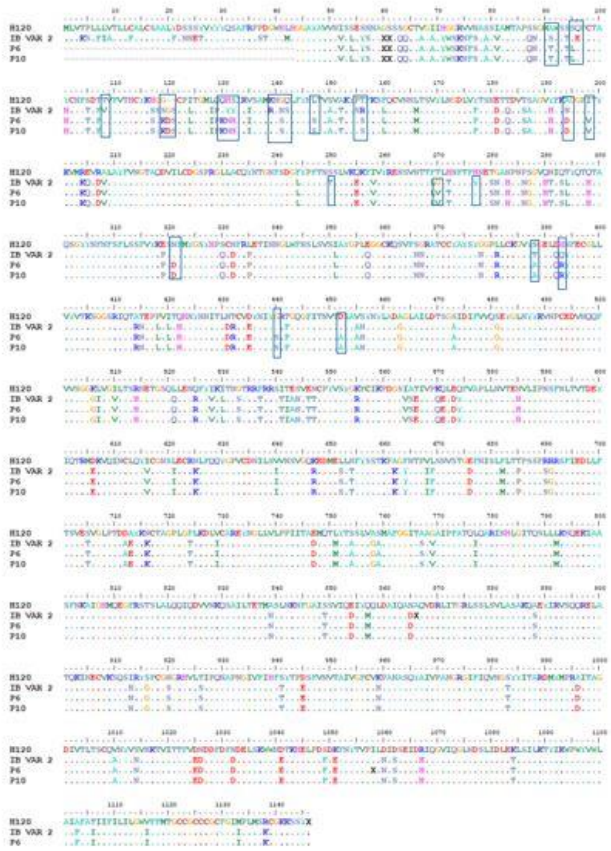
Comparative amino acid analysis between currently used vaccines, P6 and P10 isolates

S gene of the IB VAR2 vaccine and reisolated viruses of P6 and P10 were sequenced for diversity analysis. The resulting amino acid sequences and nucleotides were aligned and matched to vaccinal strains. The characterized viruses of P6 and P10 in this study indicated that they shared nucleotide as well as amino acid sequence identities compared to the IB VAR2 vaccine currently in use, 98% and 96%, respectively. These isolates showed 55 nucleotide mutations, 23 substitutions were silent and 32 nucleotide mutations led to 26 changes in amino acid residues Table (2).

Table 2: Amino acid changes Spike protein in P6 and P10 compared to IB VAR2 and H120 vaccines.

Serial	Position	H120 vaccine	IB Variant II Vaccine	P6 and P10
1	90	A	S	T
2	94	S	S	L
3	95	Q	E	Q
4	107	T	V	T
5	117	G	S	G
6	118	G	S	K
7	119	-	G	D
8	129	Q	Q	K
9	130	H	Y	N
10	131	S	Y	H
11	138	K	R	K
12	140	G	N	S
13	141	Q	S	Q
14	146	L	L	S
15	154	P	S	P
16	155	T	R	S
17	193	A	N	D
18	197	I	I	V
19	249	S	T	S
20	269	T	G	V
21	276	H	Y	H
22	320	N	N	D
23	387	S	T	A
24	392	H	Q	R
25	439	G	G	S
26	451	D	D	A

Amino acid differences scattered throughout the mature S1protein (aa19–532) as following: S90T, S94L, E95Q, V107T, S117G, S118K, G119D, Q129K, Y130N, Y131H, R138K, N140S, S141Q, L146S, S154P, R155S, N193D, I197V, T249S, G269V, Y276H, N320D, T387A, Q392R, G439S, D451A. HVRs in the S1 gene demonstrated unique patterns as well as substitutions of prominent amino acids, seven and one substitution, which were recorded in HVR2 and HVR3, respectively compared to the VAR2 vaccine. No mutations were observed in nucleotides or amino acids throughout the S2 gene (Fig.1)



3D Visualization of Mutations

Significant amino acid changes were found in the primary receptor binding domain (aa 19–252) and close to HVR I and HVR II areas, as indicated by the 3D protein modeling in Fig. 2.

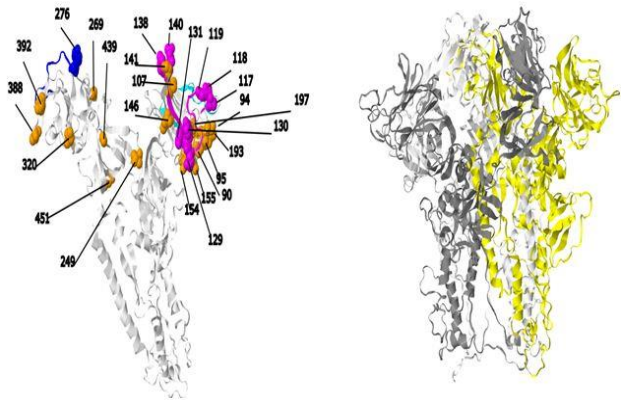


Fig.2**:** The 3D structure was modeled using the Swiss-Model repository's best-fit template (6cv0.1.A) of a cryoelectron microscopy structure of the IBV spike protein. The 3D structure was visualized by Geneious prime. HVR I is cyan, HVR II is purple and HVR III is blue. The 3D structure on the left is a spike monomer showing the mutation on one chain and that on the right is a spike trimer.

Prediction of N-linked glycosylation sites in IBV S protein

The analysis predicts several glycosylation sites in S1 protein. Passages 6 and 10 had 17 glycosylation sites and lacked an IBV glycosylation site at position 139 which was observed in IBV/EG/1212B/2012 and IB VAR2 vaccine and glycosylation site at position 284 compared to H120. H120 had 18 glycosylation sites and lacked IBV glycosylation site at position 139. The predicted glycosylation sites in S1 protein of IB VAR2 vaccine are 18 and lacked glycosylation site at position 284 compared to H120 Table (3).

Table 3: Glycosylation sites predicted in S1 using NetNGlyc-1.0 in H120, IB VAR2 vaccine and both passages.

Serial	H120	IB VAR2 Vaccine	Isolates of P6 and P10
1	51	51	51
2	77	74	74
3	103	102	102
4	-	139	-
5	145	145	145
6	164	164	164
7	179	179	179
8	213	213	213
9	238	238	238
10	248	248	248
11	265	265	265
12	272	272	272
13	277	277	277
14	284	-	-
15	307	307	307
16	426	426	426
17	448	448	448
18	514	514	514
19	531	531	531

DISCUSSION

Vaccination is the most efficient method for decreasing IBV-related economic losses. However, decreased cross-protection, as well as maximum antigenic variability between the circulating genotypes, require the use of various vaccine combinations to expand the protection spectrum **(Li *et al.*, 1994; Hussein *et al.*, 2014).** In the field, live attenuated vaccines have effectively controlled IB **(Cook *et al.*, 1999; De Wit *et al.*, 2011)**. They are characterized by ease of application and high protection against disease **(Cook *et al.*, 2012).** Utilizing live attenuated vaccines carries the risk of vaccine breakdown or reversal to virulence **(Hopkins and Yoder, 1986; Thor *et al.*, 2011).** Using different serotypes/variants provide low or no cross-protection against each other **(Cavanagh, 2007).** Vaccines that do not provide adequate protection due to serotype differences can increase the shedding level and the emergence of viral escape mutants **(Arinaminpathy *et al.*, 2012; Lin and Chen, 2017).**

This study established an *in-vivo* infection model to identify genetic variation within the S gene after 10 passages of two live attenuated IBV vaccines H120 and IB VAR2, co-inoculated in the same chicken. P6 and P10 in this study were identified as the IB VAR2 vaccine with amino acid identity (96%) suggesting an early and dramatic predominance of the IBV VAR2 vaccine over the replication of H120 vaccine within the same chicken. Regarding the two IBV vaccine serotypes, **Ball *et al*., 2017** revealed that the maximized 793B strains' ability to replicate at a higher rate over the Mass strains within the same host and that the Mass1 strain replicated faster than the D274 strain **(Ball *et al.*, 2017).**

Viruses P6 and P10 demonstrated 55 single nucleotide polymorphisms (SNPs), 23 SNPs were synonymous and 32 SNPs were nonsynonymous (led to 26 changes of amino acid residues) compared to IB VAR2 vaccine. These genetic changes identified in S1 of both passages suggest that competition for replication of the two vaccines may have impacted the genetic variability in the sequenced strain of IB VAR2 vaccine. However, further study is needed to prove this hypothesis by investigating the genetic alterations of recovered isolates over 10 passages from a group vaccinated with VAR2 vaccine only. **Ball *et al.*, (2016)** reported that the individual 793B vaccine appeared to have greater stability.

However, in the presence of Mass, there was a marked increase in 793B SNPs per 100 bp, with the majority now causing a change in the amino acid composition. Live IBV vaccine viruses, administered separately or in combination with day-old commercial broiler chicks, revealed that most SNPs found within the recovered IBV vaccine strain resulted in amino acid alterations **(Ball *et al.*, 2017).** The S1 protein contained HVRs linked to virus-neutralizing epitopes and serotype specificity and located within the amino acid residues 38–67, 91–141, and 274–387 **(Moore *et al.*, 1997; Wang and Huang 2000).**

The prominent amino acid substitutions within HVR II and III compared to the IB VAR2 vaccine were as follows in HVR2, the mutations were S117G, S118K, G119D, Q1129K, Y130N, Y131H, R138K, N140S, S141Q and in HVR3 was only a single mutation Y276H. It is evident that even a minor alteration in the amino acid sequence of the spike protein may contribute to generating new serotypes, or genotypes, which are antigenically different from the current classic as well as variant vaccine strains

(Zanaty *et al.*, 2016a; Abdel-Sabour *et al.*, 2017; Abozeid *et al.*, 2017). Additionally, modifications as small as 5% in the S1 gene were found to change the vaccine's protective capacity **(Wang and Huang 2000).**

Both passages (6^{th} and 10^{th}) have different amino acids compared to the IB VAR2 vaccine. Asparagine and glutamine within this region at positions 131 and 142 (130-141 in H120) instead of Tyrosine and Serine in the IB VAR2 vaccine and Histidine and Glutamine in H120, respectively. The same positions have changes in Egypt/F/03 strain that are closely related to the Mass serotype and they have been suggested to be responsible for pathogenicity alteration, elucidating the high virulence of Egypt/F/03 and the inability of H120 to provide satisfactory protection against challenges **(Abdel-Moneim *et al.*, 2006; Abozeid and Naguib, 2020).**

P6 and P10 have four prominent amino acid substitutions approximate to the HVR I/II (S90T, S94L, E95Q and V107T) that could contribute to the change of virus antigenicity and facilitate the emergence of variant strains primarily because of localization of these mutations within the RBD. In the current study, the obtained results align with the findings of **Rohaim *et al.*, (2020)** who localized three amino acid substitutions in the spike protein of IBV strains compared to their parental origin at positions 94, 95 and 105 within the RBD. Passages 6 and 10 lacked IBV glycosylation site at position 139 which was detected in IBV/EG/1212B/2012 and IB VAR2 strain compared to H120 which also lacked this glycosylation site. This finding may affect spike protein modification and folding, viral receptor binding, or virus-cell and cell-cell fusion.

CONCLUSION

Heterologous vaccination in the same chicken may be a factor in S1 genetic variability that may result in escape mutants. A further study is needed to prove this speculation by investigating the genetic changes resulting from passaging of IB VAR2 vaccine individually ten times in SPF chicks to know whether the competition between both vaccines is the main cause of variability. Also, it is needed to work hard on combinations of vaccines and their impact on the evolution of IBV.

Conflict of interest

The authors declare that they are not involved in any potential conflicts of interest.

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