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BIO-MOLECULAR IDENTITY OF CIRCULATED STRAIN OF INFECTIOUS BRONCHITIS VIRUS (IBV) IN VACCINATED BROILER CHICKEN

SABRY M TAMAM; EMAN A.M. HASAN; SAMAR S. EWIES AND HANAFY M MADBOULY

Virology Department, Fac. Vet. Medicine, Beni-Suef University, Egypt.

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ABSTRACT

Infectious bronchitis virus (IBV) is regarded as one of the most deleterious viruses, inducing high risk in the poultry industry worldwide, including Egypt. The present study aimed to detect the biological and molecular characteristics of IBV in samples collected from vaccinated broilers in Beni-Seuf Governorate during 2023. The virus propagated on SPF-embryonated chicken eggs (ECE), after the fifth passage, the characteristic pathological lesions, such as dwarfing and curling of the infected embryos, were observed. The trypsin-induced hemagglutination test (THA) and reverse transcriptase polymerase chain reaction (RT-PCR), amplification of partial Spike-1 glycoprotein, sequencing, partial identity, and phylogenetic tree were performed to achieve this aim. The amplification of a partial Spike-1 glycoprotein and phylogenetic tree assigned the isolate to genotype I, clade 23 (S1: GI-23), and submitted it to GenBank as Egypt/Beni/Suef/05 Egyptian IBV strain with accession no.: OR471645. Sequencing analysis showed a high degree of similarity to previously isolated Egyptian strains, such as OP585566 F1282-6 Egypt 2022 (100, 100%), EG/VVT.NRC_Egypt_2021 (96.9, 98.4%) and MN967774 Bens/AR07 Egypt 2019 (97.6 ,98.7%) of nucleotide and amino acid sequences, respectively. In conclusion, the present IBV strain during 2023 clustered in GI. 23 lineages are still circulating in susceptible or vaccinated broiler chickens, causing persistent infection to poultry flocks. Despite broad vaccination regimes, this strain is virulent to broilers, leading to serious mortalities and morbidity, altering the economic situation of local industry in Egypt. Efficient vaccination providing cross-protection against the circulating genotype is based on the genotyping, serotyping, and protectotyping in a collective manner, but not in a separate manner.

Key words: IB virus; molecular biology; genotyping; RT-PCR; phylogenetic tree.

INTRODUCTION

Infectious bronchitis virus (IBV) is considered the causative agent of an acute, greatly infectious disease that impacts chickens of all ages and has a significant financial impact on the poultry industry. IB virus presents in many genetically and antigenically diverse viral forms, which makes it difficult and complex to prevent and control this significant pathogen (Cavanagh, 2005 and 2007). IBV belongs to the family Coronaviridae and is categorized as a Gamma coronavirus, subgenus Igacovirus. Its enveloped virus has a diameter of around 120 nm (King *et al.,*

Corresponding author: Eman A.M. Hasan
E-mail address: vetemanali@yahoo.com
Present address: Virology Department, Fac. Vet. Medicine, Beni-Suef University, Egypt.

2018). The genome has single-stranded positive-sense RNA, which is around 27.6 kb long. (Masters and Perlman, 2013).

In addition to the primary structural proteins envelope (E), spike (S), nucleocapsid (N), and membrane (M), the viral genome is made up of two overlapping open reading frames (ORFs) that encode the polyproteins 1a and 1ab (Spaan *et al.,* 1988). Furthermore, two untranslated regions (UTRs) are present at the 5′ and 3′ ends. The most crucial protein is spike protein, because it facilitates attachment and entry into the host cell. The two subunits of the spike protein are S1 (the amino-terminal portion that binds to cells) and S2 (the carboxy-terminal portion that facilitates membrane fusion). In light of this, research has documented the significance of S1 in antigenic differentiation (Thor *et al.,* 2011; Jackwood, 2012).

Subunit S1 is a greatly variable region; thus, different antigenic strains have been circulating. (Jackwood, 2012; Jackwood and Lee, 2017). As a result, genotyping—with an emphasis on the S1 gene in particular—has replaced serotyping of IBV, which is difficult to standardize (Jackwood and de Wit, 2013).

For IBV, the variability of the entire S1 sequence has led to the proposal of a novel classification scheme. IBV strains are divided into seven genotypes (GI-GVII), each of which has a variety of genetic lineages. The GI genotype has the greatest number of genetic lineages (n = 29) (Hong *et al*., 2023). The Egyptian and numerous Middle Eastern IBV strains belong to the GI-23 lineage, which has spread to numerous nations in Africa, Asia, and Europe, whereas the Massachusetts (Mass) type is a member of the GI-1 lineage. (Chen *et al.,* 2017). A new genotype called Egypt-Beni-suef 01 (Abdel-Moneim *et al.,* 2002; Madbouly *et al.,* 2002) was isolated in 2001 and then spread throughout Egypt (Susan *et al.,* 2010). Egypt has now discovered a number of IBV genotypes, including GI-1, GI-13, GI-16, and GI-23, each of which has distinct genetic and pathogenic characteristics (Hassanein and Mohmoud, 2020).

IBV is principally spread by the direct contact between the diseased and healthy birds. It has been found that more than 200 species of birds are considered reservoirs for the spread of IBV (Saadat *et al.,* 2017). The infection can be detected by isolation of IBV with embryonated chicken eggs (Gelb and Jakwood, 1998). IBV HA activity has been induced by utilizing phospholipase-C, trypsin, and neuraminidase enzymes (Schultze *et al.,* 1994).

It has been shown that the latest advances in molecular diagnosis, which are based on the amplification of viral nucleic acids, are more specific, sensitive, accurate, and reliable in the detection of several viruses, including IBV. Among these techniques are RT-PCR and sequencing analysis (Liu and Kong, 2004). IBV has been known to exist in Egypt since 1954 (Ahmed, 1954).

This study intended to demonstrate the molecular characteristics of the current circulating strains of IBV in broiler chickens, with molecular comparison with the previously isolated IBV strains in Egypt during the last two decades.

MATERIALS AND METHODS:

1. Ethics statement: The animal protocols were approved by the Institutional Animal Care and the Use Committee of Beni-Seuf (BSU-IACUC) (024-074

2. Sample collection:
Fifty-five samples of trachea and lungs were collected from a commercial broiler farm of 28-day-old infected broilers. During July 2023, located in the Beni-Suef governorate, Egypt. The flock was vaccinated against the Massachusetts type and live Newcastle disease virus strain Clone 30 at 5 days of age using Sphereon® technology MSD Animal Health Nobile's Ma5 + Clone 30 vaccines. The chick had suspicious IBV infections and showed respiratory symptoms.

3. Sample processing:
Fifty-five samples of trachea and lungs were collected from a commercial broiler farm of 28-day old, infected broilers. Each of the five lung samples and tracheal Swabs gathered from the farm were combined in a single tube and processed as a single sample. Lung samples were homogenized with phosphate buffer saline (PBS) in a mortar and pooled with a tracheal scraping suspension as one sample. Following processing, each sample was centrifuged for three minutes at 3000 r.p.m. According to the OIE Terrestrial Manual (2008), the supernatants were gathered, treated with antibiotics (penicillin + streptomycin), and kept at -80ºC for subsequent molecular analysis.

4. Virus isolation and passage in SPF ECE:
Using the Nile SPF (Koom Oshiem, Fayoum, Egypt) specific pathogen-free (SPF) embryonated chicken eggs (ECE), the field strain was isolated using serial blind passages and virus titration. After injecting pooled samples into the allantoic sac of a 10-day-old SPF ECE, they were incubated for three more days at 37ºC and were candled every day. Three days later, allantoic fluid was gathered and used for reintroduction into ECE. Following the fifth passage, typical lesions of IBV, including dwarfing, curling, and stunting, were documented (OIE Terrestrial Manual 2008).

5. Virus titration: The virus isolate was titrated following the protocol of Villegas and Purchchase (1989). The virus titters were calculated using the method outlined by Reed and Muench (1938) and reported as 50% embryo infectious doses (EID_{50}).

6. Trypsin-induced hemagglutination (THA) assay:
Reagent-grade trypsin working solutions (Gibco Life Technologies Company UK) containing 2.0% trypsin in PBS were produced, with the pH set to 7.2. After 72 hours post-inoculation (PI), allantoic fluid (AF) was gathered. 50 µL of the working solutions (PBS with 2% trypsin) were combined with 0.25 ml of AF from each inoculum to treat the AF directly. The samples were kept at 37°C for about 30 minutes after the trypsin was added, and then at 4°C for 5 minutes. In a micro-titration plate with 50 µL of saline, 50 µL of trypsin-treated AF, and 50 µL of a 1% solution of chicken RBCs are combined. Untreated groups with trypsin were included, and a control group (RBCs and saline) were also included (Mahmood *et al.,* 2004). Direct agglutination of the chicken RBCs was reported within 5 minutes (Corbo and Cunningham, 1959). The constant and clear HA plates were regarded positive.

7. RNA extraction, RT-PCR, and sequencing:

7.1. RNA extraction.
The QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH) was used to extract the RNA. In summary, 140 microliters of sample suspension were incubated for about 10 minutes at room temperature, with 560 microliters of viral lysis (AVL) and 5.6 microliters of carrier RNA. Following the incubation period, the lysate was mixed with 560 µL of 100% ethanol. Following that, the sample was cleaned and centrifuged in compliance with the manufacturer's guidelines. The nucleic acid was eluted using 60 µL of elution buffer.

7.2. Oligonucleotide Primers: supplied from (**Metabion Germany)** Table (1).

Table 1: Primers sequences, target genes, amplicon sizes

Target gene	Primer sequence (5'-3')	Length of amplified product	Reference
Spike SP1	IBV-HVR1-2-FW GTK TAC TACTAC CAR AGT GC	457 bp	Naguib *et al.,* 2017
	IBV-HVR1-2-RV GAA GTG RAA ACR AGA TCA CCA TTT A		

7.3. Reverse transcriptase-PCR (RT-PCR) amplification.

Primers were utilized in a 25-µl reaction containing 12.5 µl of Quantitect probe rt-PCR buffer (QIAgen, GmbH), 1 µl of each primer of 20 pmol concentration, 0.25 µl of rt-enzyme, 4.25 µl of water, and 6 µl of template, as described by the manufacturer (Millipore Sigma, a leading science and technology company). The reaction was conducted using the Biometra thermal cycler. The first denaturation phase was conducted for five minutes at 95ºC, after the application of reverse transcription for thirty minutes at 50ºC. 35 cycles of 30 seconds at 94°C, 40 seconds at 52°C, and 45 seconds at 72°C were then performed. A final extension step was conducted at 72ºC for 10 minutes.

7.4. Analysis of PCR Products.

Using gradients of 5V/cm on a 1.5% agarose gel, the PCR products were separated by electrophoresis in 1x TBE buffer at room temperature (Applichem, Germany, GmbH). Each gel slot was filled with 15 µls of the items for the gel analysis. The fragment sizes were measured using a gene ruler 100 bps ladder (Fermentas, Thermo Fisher, Germany). A gel documentation system (Alpha Innotech, Biometra) was used to take pictures of the gel, and computer software was used to evaluate the data.

6.5. Purification of PCR Products

According to the manufacturer guidelines of the QIAquick PCR Product extraction kit (Qiagen Inc., Valencia, CA), five volumes of Buffer BP1 were added to 1 volume of the PCR sample and mixed, a QIAquick spin column was placed in a provided 2 ml collection tube. To bind DNA, the sample was applied to the QIAquick column, and centrifuged for 1 min at 8000 rpm, The centrifugate was discarded, and QIAquick column was placed back in the same collection tube. For washing, 750 ul were added of buffer PE was added to QIAquick column and centrifuged for 1 min at 8000 rpm. The centrifuge was discarded, and the QIAquick column was placed back in the same collection tube. QIAquick column was placed into a clean 1.5 ml centrifuge tube.
For eluting DNA, 30 µl of Buffer EB (10mM Tris.CL PH 8.5) or water (PH 7.0-8.5) was added to the center of the QIAquick membrane for 5 minutes, then centrifuged for 1 min at 12000 rpm was done

7.6. Sequencing

Applied Biosystems 3130 (ABI 3130, USA) automated DNA sequencers were used to perform forward and/or reverse sequencing on a purified PCR product. Employing a Perkin-Elmer/Applied Biosystems, Foster City, CA, BigDye Terminator V3.1 cycle sequencing kit (Cat. No. 4336817) that was prepared for use. A BLAST® analysis (Basic Local Alignment Search Tool) (Altschul *et al.,* 1990) was initially performed to ascertain sequence identity to GenBank accessions.

7.7. Phylogenetic analysis

For phylogenetic studies, MEGA6 employed maximum likelihood, neighbor joining, and maximum parsimony (Tamura *et al.,* 2013). The sequences were compared using the CLUSTAL W multiple sequence alignment algorithm, which is version 12.1 of the MegAlign module of Lasergene DNAStar software Pairwise (Madison, Wisconsin, USA). Thompson *et al.* (1994) invented it.

RESULTS

1. Isolation of IBV on SPF eggs

According to our findings, the virus did not affect at all on the inoculated embryos throughout the first three passages. When comparing the infected embryos to the PBS-inoculated embryos in passages four and five, the infected embryos showed signs of congestion, bleeding, edema, curling, and dwarfing (Figure 1).

2. THA Assay:

After being treated with trypsin, the infected allantoic fluid was able to directly agglutinate 1% chicken RBCs (Figure 2).

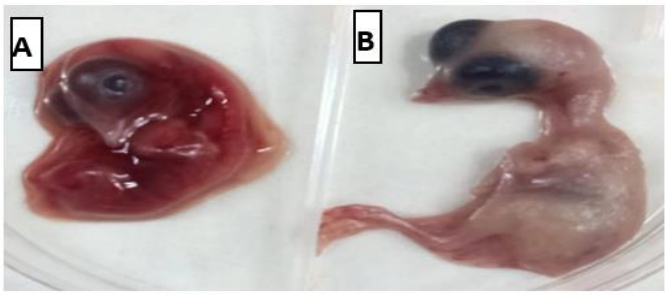


Fig. 1: (A) The infected embryos showing hemorrhage, curling, and dwarfism, compared to (B) the non-infected control embryos after passage 3-5.

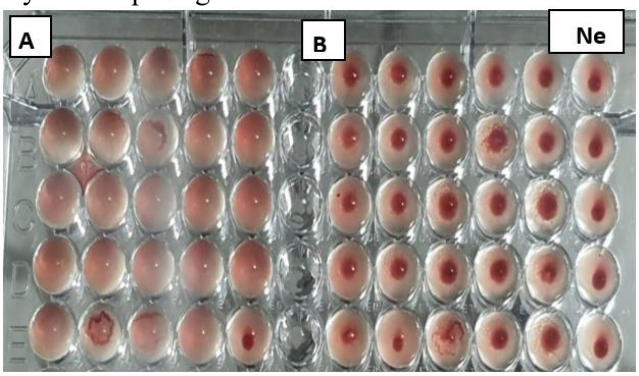


Fig. 2: (A) On the left side: IBV treated with trypsin showed a clear lattice shape (agglutinates RBCs); (B) on the right side: IBV untreated with trypsin showed a clear button shape (can't directly agglutinate RBCs); (Neg): the last column of the plate is the negative control.

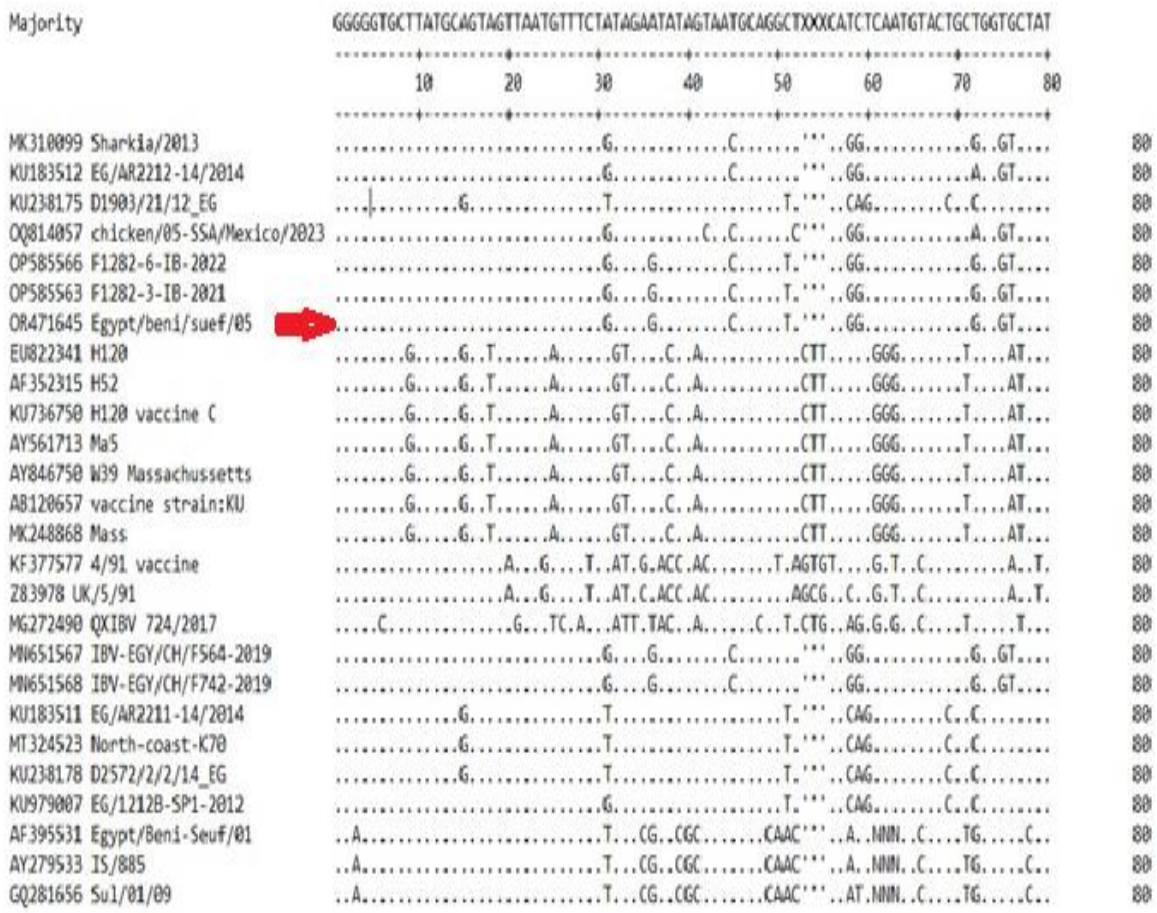
3. Reverse-transcriptase polymerase chain reaction:

Each five samples of lung and tracheal swabs were combined as a single sample of fifty-five samples, which were collected from infected and vaccinated birds, and chosen for further examination using molecular methods. About 40% of these samples were positive with RT-PCR. Two of the chosen five samples were detected in agarose gel electrophoresis, the expected band's size equivalent to 457 bp (Figure 3).

Fig. 3: PCR gel of IBV detects two positive bands (2 and 5) at 457 bp, against DNA ladder (L), positive control sample (P) with positive band at 475 bp, and negative control sample (N) with no band.

4. Sequencing and phylogenetic analysis:

Five samples tested by conventional RT-PCR, two from which were positive to IBV infection, and one of two RT-PCR IBV-positive samples went through partial sequencing for the S1 gene (Figure 4), and it was submitted to the GenBank as Egypt/beni/suef/05, under accession no.: OR471645. The amplification of a partial Spike-1 glycoprotein and phylogenetic tree assigned the new isolated strain to genotype I, clade 23 (S1: GI-23), as shown in figure (5). The percent identity showed that our isolate has a similarity about 100% and 100% with (OP585566 F1282-6 Egypt 2022), 96.9% and 98.4% with genotype GI.23 (EG/VVT.NRC_EGYPT 2021) of IBV, 97.6 and 98.7 with (MN967774Bens/AR07Egypt 2019) and (MN890132QENA-31 Egypt 2018), 92.3% and 96.1 with (OR515484_Jo/N-5-Jordan_2021) based on identity of nucleotides and amino acid sequences, respectively, as shown in (Table 2). Our findings confirm that our (Egypt/Beni Suef/05) IBV strain is clustered in GI.23 lineages and has been circulating in the Egyptian poultry farms since 2023.


Majority
GGGGGTGCTTATGCAGTAGTTAATGTTTCTATAGAATATAGTAATGCAGGCTXXXCATCTCAATGTACTGCTGGTGCTAT
10 20 30 40 50 60 70 80
MK310099 Sharkia/2013
KU183512 EG/AR2212-14/2014
KU238175 D1903/21/12_EG
OQ814057 chicken/05-SSA/Mexico/2023
OP585566 F1282-6-IB-2022
OP585563 F1282-3-IB-2021
OR471645 Egypt/beni/suef/05
EU822341 H120
AF352315 H52
KU736750 H120 vaccine C
AY561713 Ma5
AY846750 W39 Massachussetts
AB120657 vaccine strain:KU
MK248868 Mass
KF377577 4/91 vaccine
Z83978 UK/5/91
MG272490 QXIBV 724/2017
MW651567 IBV-EGY/CH/F564-2019
MW651568 IBV-EGY/CH/F742-2019
KU183511 EG/AR2211-14/2014
MT324523 North-coast-K70
KU238178 D2572/2/2/14_EG
KU979007 EG/1212B-SP1-2012
AF395531 Egypt/Beni-Seuf/01
AY279533 IS/885
GQ281656 Sul/01/09
Majority
TCATTGGAGTAAAAATTTTAGTGCTTCTTCTGTAGCTATGACAGCACCTGCTACAGGTATGTCTTGGTCAACCAGTCAGT
90 100 110 120 130 140 150 160
MK310099 Sharkia/2013
KU183512 EG/AR2212-14/2014
KU238175 D1903/21/12_EG
OQ814057 chicken/05-SSA/Mexico/2023
OP585566 F1282-6-IB-2022
OP585563 F1282-3-IB-2021
OR471645 Egypt/beni/suef/05
EU822341 H120
AF352315 H52
KU736750 H120 vaccine C
AY561713 Ma5
AY846750 W39 Massachussetts
AB120657 vaccine strain:KU
MK248868 Mass
KF377577 4/91 vaccine
Z83978 UK/5/91
MG272490 QXIBV 724/2017
MW651567 IBV-EGY/CH/F564-2019
MW651568 IBV-EGY/CH/F742-2019
KU183511 EG/AR2211-14/2014
MT324523 North-coast-K70
KU238178 D2572/2/2/14_EG
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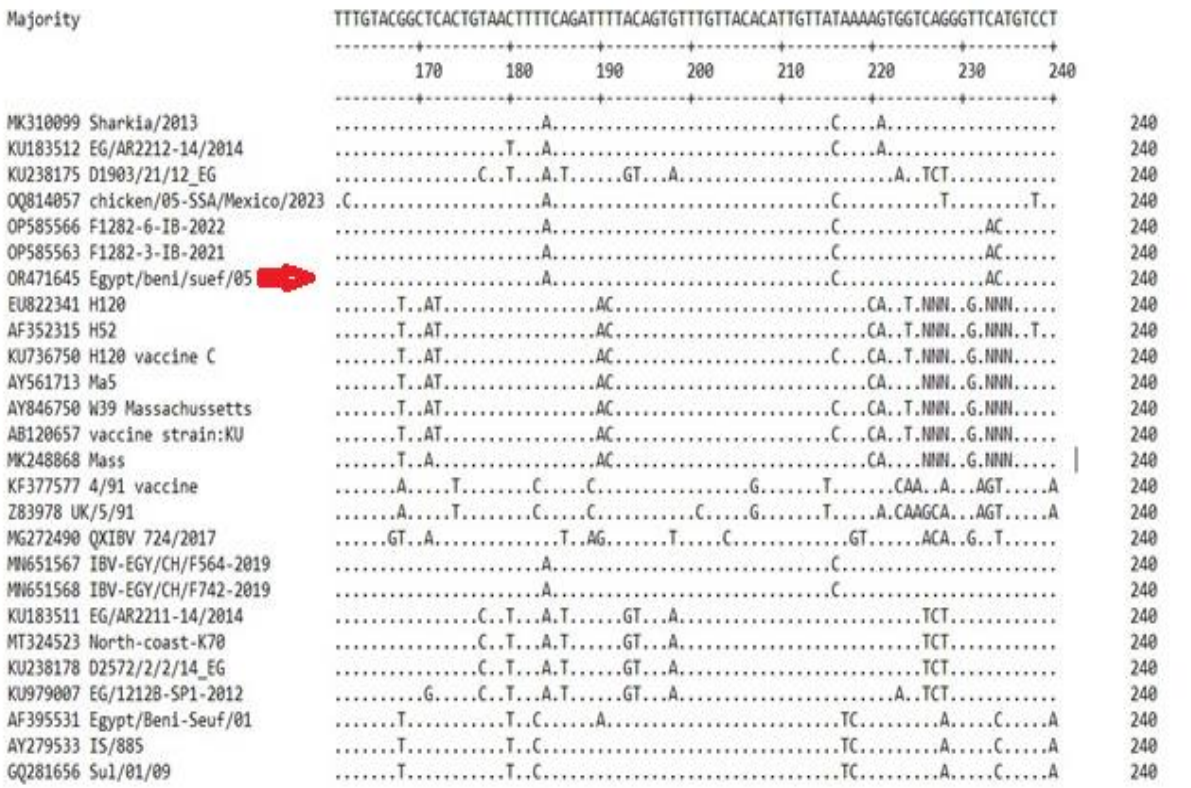
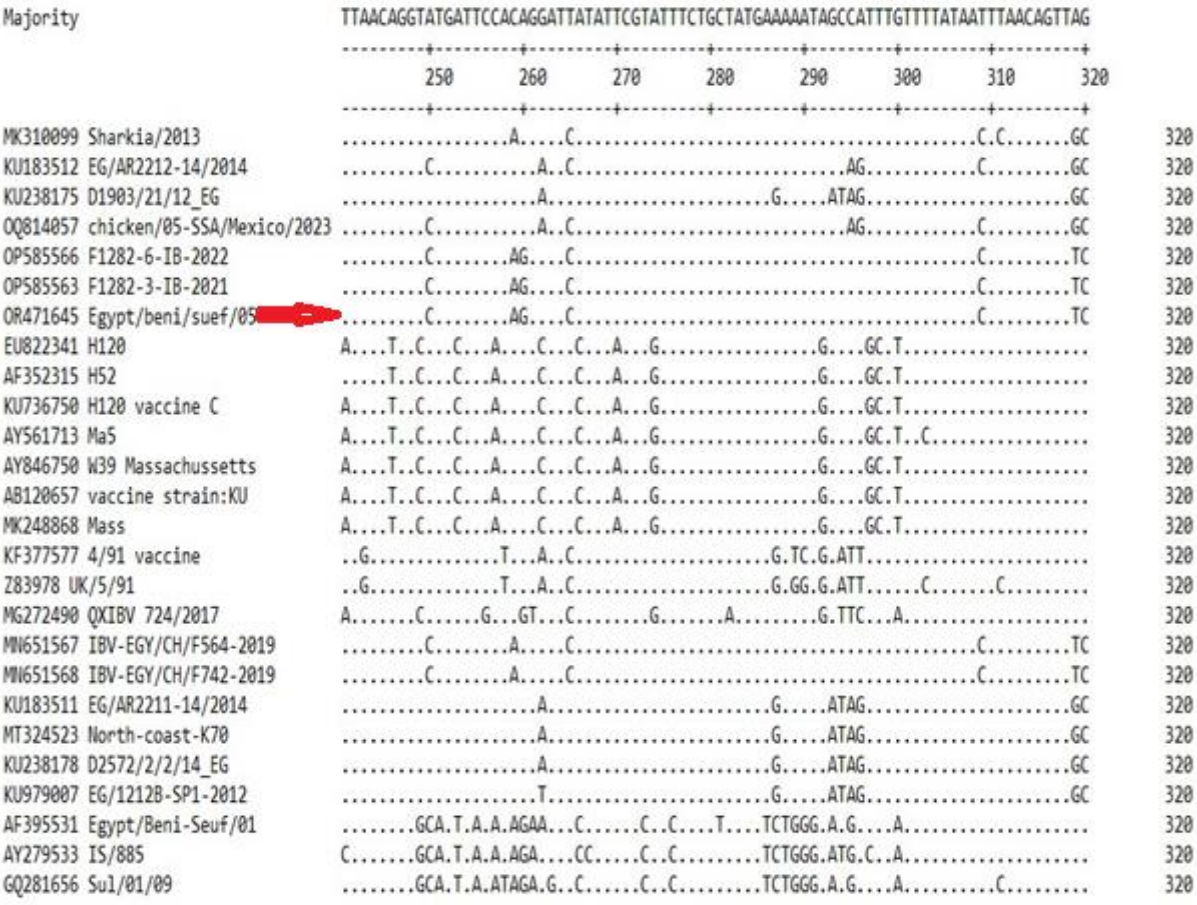
AF395531 Egypt/Beni-Seuf/01
AY279533 IS/885
GQ281656 Sul/01/09


Majority
TTTGTACGGCTCACTGTAACTTTTCAGATTTTACAGTGTTTGTTACACATTGTTATAAAAGTGGTCAGGGTTCATGTCCT
170 180 190 200 210 220 230 240
MK310099 Sharkia/2013
KU183512 EG/AR2212-14/2014
KU238175 D1903/21/12_EG
OQ814057 chicken/05-SSA/Mexico/2023
OP585566 F1282-6-IB-2022
OP585563 F1282-3-IB-2021
OR471645 Egypt/beni/suef/05
EU822341 H120
AF352315 H52
KU736750 H120 vaccine C
AY561713 Ma5
AY846750 W39 Massachussetts
AB120657 vaccine strain:KU
MK248868 Mass
KF377577 4/91 vaccine
Z83978 UK/5/91
MG272490 QXIBV 724/2017
MW651567 IBV-EGY/CH/F564-2019
MW651568 IBV-EGY/CH/F742-2019
KU183511 EG/AR2211-14/2014
MT324523 North-coast-K70
KU238178 D2572/2/2/14_EG
KU979007 EG/1212B-SP1-2012
AF395531 Egypt/Beni-Seuf/01
AY279533 IS/885
GQ281656 Sul/01/09


Majority
TTAACAGGTATGATTCCACAGGATTATATTCGTATTTCTGCTATGAAAAATAGCCATTTGTTTTATAATTTAACAGTTAG
250 260 270 280 290 300 310 320
MK310099 Sharkia/2013
KU183512 EG/AR2212-14/2014
KU238175 D1903/21/12_EG
OQ814057 chicken/05-SSA/Mexico/2023
OP585566 F1282-6-IB-2022
OP585563 F1282-3-IB-2021
OR471645 Egypt/beni/suef/05
EU822341 H120
AF352315 H52
KU736750 H120 vaccine C
AY561713 Ma5
AY846750 W39 Massachussetts
AB120657 vaccine strain:KU
MK248868 Mass
KF377577 4/91 vaccine
Z83978 UK/5/91
MG272490 QXIBV 724/2017
MW651567 IBV-EGY/CH/F564-2019
MW651568 IBV-EGY/CH/F742-2019
KU183511 EG/AR2211-14/2014
MT324523 North-coast-K70
KU238178 D2572/2/2/14_EG
KU979007 EG/1212B-SP1-2012
AF395531 Egypt/Beni-Seuf/01
AY279533 IS/885
GQ281656 Sul/01/09

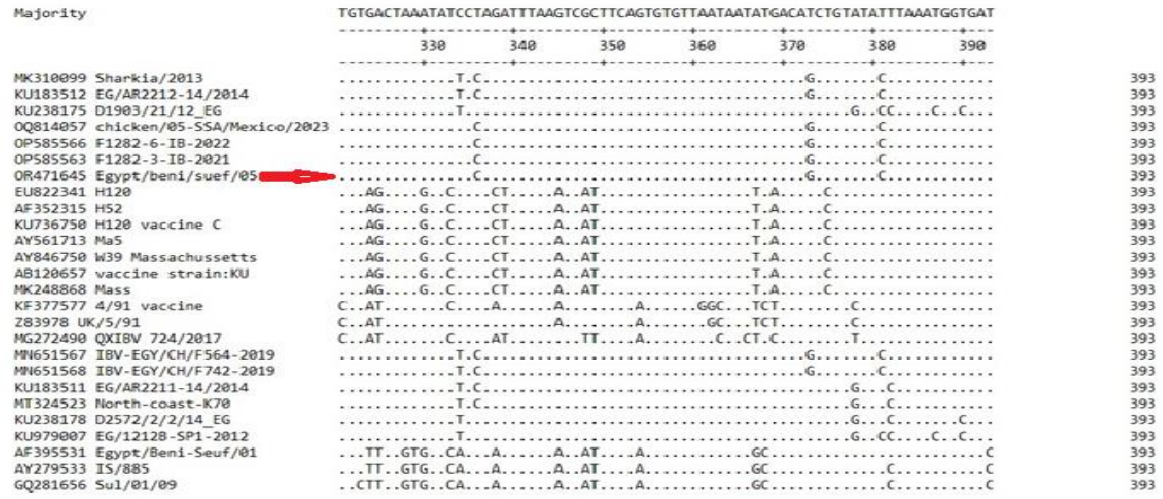


Fig. 4: Alignment of partial sequences of selected strain spike (S1) gene, compared to other strains of gene bank.

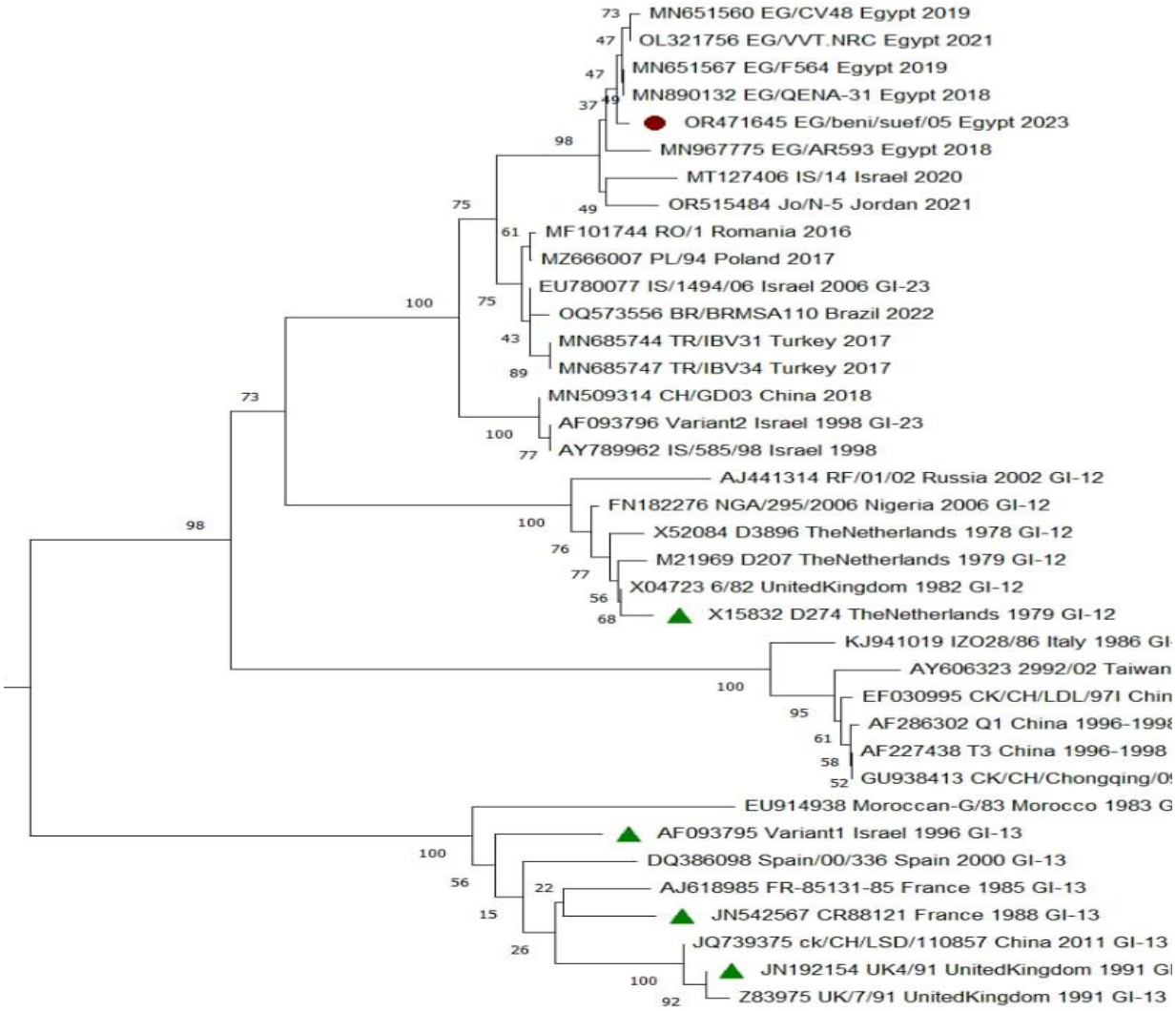


Fig 5: Phylogenetic analysis of Egypt/Beni- Seuf/05 and selected IBV reference strains in the GenBank database based on comparison of the partial S1 gene in different strains, showing the wide distribution of IBV and the isolate belongs to GI.23.

serial	Seq	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
1	*OR471645 EG/beni-suef/05 Egypt 2023*	ID	100.0 %	98.4 %	98.7 %	98.7 %	83.5 %	96.1 %	93.8 %	80.4 %	94.8 %	91.7 %	95.3 %	94.1 %	94.6 %	93.8 %	92.9 %	73.2 %	73.7 %	82.8 %	73.5 %	73.7 %	74.3 %	Nucleotides
2	*OP585566 F1282-6 Egypt 2022*	100.0%	ID	98.4 %	98.7 %	98.7 %	83.5 %	96.1 %	93.8 %	80.4 %	94.8 %	91.7 %	95.3 %	94.1 %	94.6 %	93.8 %	92.9 %	73.2 %	73.7 %	82.8 %	73.5 %	73.7 %	74.3 %	
3	*OL321756 EG/VVT.NRC Egypt 2021*	96.9 %	96.9%	ID	99.7 %	99.7 %	84.6 %	97.4 %	93.3 %	80.7 %	95.8 %	92.8 %	95.8 %	94.8 %	95.6 %	94.8 %	93.9 %	73.5 %	74.0 %	83.8 %	74.0 %	73.7 %	74.3 %	
4	*MN967774 Bens/AR97 Egypt 2019*	97.6 %	97.6%	99.2 %	ID	100.0 %	84.3 %	97.4 %	93.5 %	80.4 %	95.6 %	92.5 %	96.1 %	94.8 %	95.3 %	94.6 %	93.6 %	73.5 %	74.0 %	83.5 %	73.7 %	74.0 %	74.5 %	
5	*MN890132 QENA-31 Egypt 2018*	97.6 %	97.6%	99.2 %	100.0 %	ID	84.3 %	97.4 %	93.5 %	80.4 %	95.6 %	92.5 %	96.1 %	94.8 %	95.3 %	94.6 %	93.6 %	73.5 %	74.0 %	83.5 %	73.7 %	74.0 %	74.5 %	
6	*KU979007 EG/1212B Egypt 2012*	83.0 %	83.0%	84.6 %	84.6 %	84.6 %	ID	85.1 %	83.8 %	76.6 %	85.3 %	85.8 %	85.3 %	85.3 %	86.6 %	85.3 %	84.5 %	69.4 %	69.9 %	90.0 %	73.2 %	72.2 %	74.8 %	
7	*OR515484 Jo/N-5 Jordan 2021*	92.3 %	92.3%	94.6 %	94.6 %	94.6 %	83.8 %	ID	91.5 %	79.9 %	93.8 %	91.7 %	94.1 %	93.8 %	93.5 %	93.5 %	92.4 %	74.3 %	74.8 %	83.8 %	74.5 %	74.0 %	75.0 %	
8	*OR233139 409101 Israel 2022*	93.0 %	93.0%	91.5 %	92.3 %	92.3 %	86.1 %	86.9 %	ID	81.4 %	94.6 %	92.0 %	95.1 %	93.8 %	94.6 %	94.1 %	92.4 %	71.5 %	72.0 %	82.3 %	74.3 %	73.5 %	74.3 %	
9	*OQ116341 AC-28569/01 Iraq 2022*	77.4 %	77.4%	76.6 %	76.6 %	76.6 %	76.6 %	75.1 %	78.1 %	ID	80.4 %	81.9 %	80.2 %	80.2 %	80.7 %	79.9 %	80.9 %	68.1 %	67.6 %	73.9 %	71.4 %	70.9 %	70.6 %	
10	*MN685744 IBV31 Turkey 2017*	92.3 %	92.3%	93.0 %	92.3 %	92.3 %	86.1 %	87.6 %	93.8 %	78.9 %	ID	95.6 %	99.2 %	98.2 %	98.7 %	97.9 %	96.4 %	72.5 %	73.2 %	85.6 %	75.5 %	75.0 %	76.3 %	
11	*MN509314 GD03 China 2018*	87.6 %	87.6%	87.6 %	87.6 %	87.6 %	86.1 %	85.3 %	90.0 %	81.9 %	92.3 %	ID	95.3 %	94.3 %	95.8 %	94.8 %	94.1 %	74.0 %	74.3 %	86.6 %	76.3 %	75.5 %	77.3 %	
12	*MF101744 IBV/1 Romania 2016*	93.8 %	93.8%	93.0 %	93.8 %	93.8 %	86.9 %	88.4 %	95.3 %	78.1 %	98.4 %	92.3 %	ID	98.7 %	98.7 %	97.9 %	96.4 %	73.0 %	73.7 %	85.8 %	75.3 %	75.3 %	76.5 %	
13	*MZ666004 43 Poland 2017*	90.7 %	90.7%	90.7 %	90.7 %	90.7 %	86.1 %	88.4 %	92.3 %	76.6 %	96.1 %	90.0 %	96.9 %	ID	97.6 %	97.1 %	95.4 %	73.0 %	73.7 %	85.6 %	75.3 %	75.0 %	76.3 %	
14	*OQ573556 BR/BRMSA110 Brazil 2022*	92.3 %	92.3%	92.3 %	92.3 %	92.3 %	88.4 %	87.6 %	93.8 %	78.1 %	96.9 %	92.3 %	97.6 %	95.3 %	ID	97.9 %	97.2 %	72.7 %	73.5 %	86.1 %	75.3 %	74.5 %	76.3 %	
15	*OR475237 BRMSA2926(328/22) Brazil 2022*	89.2 %	89.2%	90.0 %	89.2 %	89.2 %	85.3 %	86.9 %	91.5 %	77.4 %	95.3 %	89.2 %	94.6 %	93.0 %	93.8 %	ID	96.2 %	72.7 %	73.5 %	85.1 %	75.3 %	74.0 %	76.0 %	
16	*OR529309 LB001 Mexico 2023*	89.3	89.3%	89.3	89.3	89.3	84.0	86.3	90.9	77.4	93.9	90.9	94.6	92.4	95.4	92.4	ID	71.4	72.1	84.5	74.6	73.4	75.6	
17	*AY561711 M41 USA 1941 GI-1*	64.8	64.8%	64.8	64.8	64.8	61.0	66.4	62.5	61.6	64.1	65.6	64.1	63.3	64.1	63.3	61.6	ID	95.8	72.0	70.9	71.5	70.4	
18	*FJ888351 H120 TheNetherlands 1960 GI-1*	67.9 %	67.9%	67.1 %	67.1 %	67.1 %	64.1 %	68.7 %	66.4 %	64.6 %	67.1 %	68.7 %	67.1 %	66.4 %	66.4 %	66.4 %	64.6 %	90.6 %	ID	72.2 %	71.7 %	72.2 %	71.2 %	
19	*X15832 D274 TheNetherlands 1979 GI-12*	81.5 %	81.5%	81.5 %	81.5 %	81.5 %	86.9 %	80.0 %	83.8 %	73.6 %	86.9 %	88.4 %	86.9 %	85.3 %	87.6 %	83.8 %	84.8 %	64.1 %	64.8 %	ID	74.3 %	72.7 %	75.3 %	
20	*JN542567 CR88121 France 1988 GI-13*	71.7 %	71.7%	72.5 %	71.7 %	71.7 %	72.5 %	72.5 %	72.5 %	69.1 %	74.8 %	74.0 %	74.0 %	73.2 %	74.0 %	74.8 %	72.1 %	63.3 %	64.1 %	71.7 %	ID	93.8 %	96.1 %	
21	*JN192154 UK4/91 UnitedKingdom 1991 GI-13*	71.7 %	71.7%	70.9 %	71.7 %	71.7 %	71.7 %	70.2 %	70.9 %	69.1 %	72.5 %	72.5 %	73.2 %	71.7 %	72.5 %	70.2 %	69.1 %	61.8 %	62.5 %	70.9 %	87.7 %	ID	93.1 %	
22	*AF093795 Variant1 Israel 1996 GI-13*	72.5 %	72.5%	71.7 %	72.5 %	72.5 %	73.2 %	72.5 %	72.5 %	69.1 %	74.0 %	74.0 %	74.8 %	73.2 %	74.8 %	73.2 %	72.9 %	63.3 %	64.1 %	72.5 %	94.6 %	88.5 %	ID	
		Amino Acid																						

Table 2: percent identity of nucleotides and AA. Sequences of Egypt/Beni- Seuf/05 and selected IBV strains in the GenBank database. (The strain marked in red color represented by numbe

DISCUSSION

Since its first discovery in the 1930s, IBV has still been circulating worldwide with the ability for evolution and genetic diversity. According to Madbouly *et al.* (2002) and Sediek (2010), one of the primary problems with IBV is the frequent emergence of novel variable strains. Detection and identification of these new strains are essential for disease control (Nakamura *et al.,* 1996). The existence of variable strains of IBV in Egypt dates back to the 1950s, when researchers identified a strain that was closely related to the Dutch variant (D3128) based on neutralization tests (Sheble *et al.,* 1986; Eid, 1998).

Since 2001, partial molecular analysis of the S1 gene for two isolates of IBV has been done. The first one of them revealed a new genotype named Egypt-Beni Suef 01 that varied from the previously described worldwide genotypes (Abdel-moneim *et al.,* 2002; Madbouly *et al.,* 2002). The second isolate, Dutch strain 274, showed close similarity to Dutch variants D274 (98% amino acid similarity and 99.4% nucleotide similarity) and D3896 (94% amino acid similarity and 97.8% nucleotide similarity) (Madbouly *et al.,* 2002). This is the first emerging of a variant strain from the Dutch strain that was introduced in Egypt by an illegal route for controlling IBV.

There are two primary categories that rely on functional testing, which examines the virus's biological function, and non-functional tests, which analyze the genome of the virus (Lohr, 1988). Antigenic types and protecto-types (epitope types and serotypes) are results of typing using functional tests. Tests that regarded the genome caused the genotypes (De Wit 2000). It's becoming evident that a variety of IBV strains with unapproved classifications are a problem for many nations.

To differentiate certain IBV strains from those grouped under Israeli Variant 1, some authors in Egypt and the Middle East assigned some of these strains as Israeli Variant II (Abdel-Moneim *et al.,* 2002; Meir *et al.,* 2004). The Egyptian isolates were also divided according to their hypervariable region 3 (HVR3) sequences. These results yielded the Egyptian Variant 1, which was defined using the reference strain Egypt/Beni-Suef/01 as a basis. This strain shares 97.6% of its sequence with the nephron-pathogenic strains (IS/720/99), and it has eight nucleotide and six amino acid changes. The nephrogenic Israeli variant strain IS/885/00 and the Egyptian variant-1 strain (the first variant subgroup is Egy/Var, which was closely related to the original IBV Egyptian strain of Egypt/Beni-Suef/0 and Ck/EG/BSU-1,4,5/2011) shared 96.6% of their amino acid identities (Abdel-Moneim *et al.,* 2012). On referring to this classification offered by Abdel-Moneim *et al.* (2012). They based their classification on the Israeli classification by Meir *et al.* (2004), which depends upon the amino acid substitution between strains isolated in 1999 and 2004. This cohesive link between Israeli Var I and Var II with Egyptian Var I and Var II is questionable. In reality, there is no full, complete sequence of nucleotides accessible for the three Egyptian strains (Egypt Beni-Suef 01, Variant I, and Variant II) to date, as described by Valastro *et al.* (2016), and they did not involve them in G1-23.

The classification of IBV strains into Variant I and Variant II, which was adopted by Abdel-Moneim *et al.* (2012), was not based on any standard criteria for classification. Consequently, the incorrect classification of the worldwide and Middle East IBV different strains, which results in the confusing coexistence of multiple genotyping schemes, allows Valastro *et al.* (2016) to propose that inference of full S1 gene analysis and pairwise sequence comparisons were not as appropriate as phylogenetic relationships alone when it comes to sequence classification. This enabled them to identify six genotypes, which, when combined, account for 32

different lineages of virus and a large number of inter-lineage recombinants.

Indeed, to achieve the International Committee on Taxonomy of Viruses Executive Committee (ICTV) criteria for the most accurate virus classification, complete sequence comparisons using both phylogenetic relationships and pairwise sequence similarity have emerged as one of the main sets of features used to define and identify virus taxonomy. (Lefkowitz *et al.,* 2017; ICTV, 2020).

The highly variable protein, which ranges in amino acid sequence from 2% to 25% between strains, was found in the S1 subunit (Lai and Cavanagh, 1997). Therefore, any change of the S1 gene by insertions, deletions, RNA recombination, and/or point mutations was usually related to the emergence of new IBV serotypes and genotypes (Wang *et al.,* 1994).

In our opinion, genotyping groups are the most used system, which has mainly replaced protectotyping and serotyping if based on fixed standards of molecular tests and defined sizes of the S1 gene analysis. Currently, the most important concern is that using different molecular tests, different locations and sizes of the S1 region produces different sequences used for comparing strains, which makes it exceedingly challenging to understand the results, and these concerns need a standardized method for genotyping. (De Wit *et al.,* 2011).

Otherwise, any classification into variants according to the variation of nucleotides and amino acid substitution in the HVR of the S1 gene is apart from the main criteria maintained above. Conclusively, typing a great number of IBV strains into variants is now a global problem, and the selection of a protective vaccine against the unclear classification of worldwide variants, either by serotyping or genotyping, is in a state of complexity.

In the present study, the Egyptian strain (EGY/beni/Seuf/05/2023) was isolated from a lung and tracheal tissue pool with a history of respiratory disease ranging from mild to severe coughing and nasal secretions, sneezing, gasping, and respiratory sounds in the flocks as clinical manifestations (Ghanem *et al.,* 2019). This IBV strain was isolated from a vaccinated broiler flock. These results indicate minimal to nonexistent cross-protection between traditional vaccination strains and circulating IBV. Even with the implementation of extensive vaccination programs in Egypt and other countries, new strains of the virus continue to develop on occasion. The high rate of IBV mutation that leads to a wide range of circulating IBV strains makes mass routine vaccination for IBV control difficult (Cavanagh, 2007). In Egypt, to control IBV, numerous strains of live, attenuated, and inactivated vaccinations are employed, but the disease's outbreaks continue to cause severe infections that cause significant economic losses (Madbouly *et al.,* 2002; Sabra *et al.,* 2020). And this weak protection may depend upon different factors, like the imperfect selection of vaccines according to their homology on the level of genotyping and antigenicity; escape of mutant strains due to intensive vaccination programs; point mutation; incorrect vaccine application; the state of maternal and/or acquired immunity during vaccination; the variable ages of the birds; and other stress factors (Madbouly *et al.,* 2002).

Additional indirect factors could be the virus's evolution in non-native hosts, intragenic recombination between local and global strains in migratory and wild birds, or the virus in other domestic and wild animals. The discovery of IBV and IB virus-like strains in non-chicken bird species, like geese, ducks, and pigeons (Cavanagh, 2005), may also have contributed to the global spread of IBV strains. Particular IBV strains, which were capable of infecting migratory birds, would spread more readily across long distances than a strain that these bird species are unable to replicate. Even though the

involvement of wild birds in IBV is unclear and mostly unknown, more research and attention should be paid to this field.

Another point of attention includes that the protection process did not depend mainly on the number of replaced amino acids, but also on the types of changed amino acids, even if they were low in number. (De Wit *et al.,* 2011). The newly isolated (EGY/beni/Seuf/ 05/2023) and Egypt/BeniSeuf/01 strains shared the same genotype (G1.23). In a previous study, Madbouly *et al.* (2002) found great variation between the used vaccinal strains (H120 and M41), which were used in the poultry flocks and the isolated field strain Egypt/BeniSeuf/01. When they compared the identity between Egypt/BeniSeuf/01 with these two vaccine strains, they showed considerable high divergence to H120 (29.8% amino acid divergence and 25.8% nucleotide divergence) and M41 (30.4% amino acid divergence and 25% nucleotide divergence). The chicken vaccinated with H120 shows 20% protection when challenged with Egypt/BeniSeuf/01 (Abdel-Moneim *et al.,* 2002).

Another limitation on a vaccine's predictive usefulness against a field challenge strain in a field setting is based on S1 gene homology (De Wit *et al.,* 2011).

Furthermore, inadequate protection was more probable to result from improper vaccine administration than from antigenic drift of the field virus (De Wit *et al.,* 2011), and the significance of administering IB vaccinations with caution was also confirmed by Jackwood *et al.* (2009). In our opinion, the selection of vaccine strains against field-challenged strains could depend on the genotyping and serotyping (antigenicity) together of the selected vaccine.

Concerning egg inoculation, IBV pathological lesions on SPF-ECE were mainly curling, stunting, and dwarfing of the inoculated embryos after three to five passages, as shown in Figure (1); these results were parallel to Madbouly *et al.* (2002), Noussieur (2018), Mahmoud *et al.* (2019), and Sallam *et al. (2023).*

The newly isolated IBV strain displayed hemagglutinating properties after treatment with trypsin (2%), as shown in Figure (2). It is subsequently identified as IBV by RT-PCR using the S1 gene primers, and the PCR gel of IBV detects the positive samples at 457 bps, as shown in Figure (3). The isolated strain has been submitted to GenBank, given accession number OR471645, and named Egypt/Beni/Suef/05. The Egyptian variants, variant 1, represented by Egypt/Beni-Suef/01, Ck/Eg/BSU-1/2011, Ck/Eg/BSU-4/2011, and Ck/Eg/BSU-5/2011, were undescribed genotypes (Abdel-Moneim, 2012). The newly isolated EGY/beni/Seuf/05/2023 and Egypt/BeniSeuf/01 (related to Israel720/99) strains share the same genotype (G1.23). It was found that Israel720/99 was related (97.6%) to Egypt/Beni-Seuf/01 but showed 6 amino acid and 8 nucleotide substitutions from Egypt/Beni-Seuf/01 (Madbouly *et al.,* 2002). The IBV strains isolated in 2012 and named EGY VAR II, including both Ck/Eg/BSU-2/2011 and Ck/Eg/BSU-3/2011, showed 89% amino acid sequence identity in HVR3 to Beni-Suef/01 (EGY VAR I), and this means that the selection of IBV strains related to EGY VAR II is not protective.

In the present study, the amplification of a partial Spike-1 glycoprotein and phylogenetic tree assigned the new isolated strain to genotype I, clade 23 (S1: GI-23), as shown in Figure (5). The S1 gene nucleotides and their assumed amino acid sequences of IBV were analyzed by comparing with 21 respective reference sequences obtained from the GenBank (Table. 2). The newly isolated (EGY/beni/Seuf/05/2023) strain that belongs to G1-23 differed to a great extent from the currently used vaccinal strains (H120 and M41) and strains of Var. II showed 89% amino acid sequence identity in HVR3 to Beni-Suef/01 (EGY Var I) and 81 nucleotide

substitutions from Egypt/Beni-Seuf/01, and this denotes the great difference between these two strains. Therefore, the selection of the Beni-Suef/01 strain for vaccine production is not a good decision.

Multiple sequence alignment showed that our isolate (EG/Beni/Seuf/05) shares similarity about 100% and 100% with (OP585566 F1282-6 Egypt 2022), 96.9% and 98.4% with genotype GI.23 (EG/VVT.NRC_EGYPT 2021) of IBV, 97.6 and 98.7 with (MN967774Bens/AR07Egypt 2019) and (MN890132QENA-31 Egypt 2018), 92.3% and 96.1 with (OR515484_Jo/N-5-Jordan_2021) based on identity of nucleotides and amino acid sequences respectively. Our findings prove that our IBV strain clustered in GI.23 lineages and has been circulating in Egyptian poultry farms since 2023. All the isolated Egyptian IBV strains from 2018 to 2023 were clustered in the same sub-clade of G1-23 and nearby strains isolated from Israel in 2020 and Jordan in 2021, but far from strains isolated from European, Asian, and Brazilian countries during the period between 2016 and 2022 and the Israeli strain Var.II 1998 and related to the Egyptian Var.II. Therefore, any imported vaccines from these countries are not protective against the circulating field strains.

Therefore, further studies are needed to establish the perfect choice strain as a selective vaccine strain against the field-circulated strains for either live attenuated or inactivated vaccines. In the current time, any selected vaccine strains, either for live attenuated or inactivated vaccines, must be used from the recently isolated strain, as the most candidate strain (EGY/beni/Seuf/05/2023).

CONCLUSIONS

IBV GI.23 strains are still circulating in the Egyptian field, causing persistent infection to poultry flocks despite broad vaccination regimes. This strain is virulent and pathogenic to broilers, leading to serious mortality and morbidity that alter the economic situation of local industry in Egypt.

Many laboratories sequence a portion of S1, which concludes the greatly varying regions I and II; the others do not have. Selected molecular tests and full size of S1 gene are fixed and standardized by the ICTV for perfect genotyping of newly emerged IBV strains. Finally, the protection against IBV needs appropriate selection of the vaccine strain based on the genotyping, serotyping, and protectotyping collectively. We need further studies with great attention to continuous surveillance and monitoring of IBV in commercial flocks.

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الهوية الجزيئية الحيوية لسلالة فيروس التهاب الشعب الهوائية المعدي المنتشرة بين دجاج التسمين الملقح

صبري محمد تمام ، إيمان علي محمد ، سمر سيد عويس ، حنفي محمود مدبولي

Email: vetemanali@yahoo.com Assiut University web-site: www.aun.edu.eg

يعتبر فيروس التهاب الشعب الهوائية المعدي أحد أكثر الفيروسات ضررًا والتي تسبب مخاطر عالية في صناعة الدواجن في جميع أنحاء العالم بما في ذلك مصر. تهدف الدراسة الحالية إلى الكشف عن الخصائص البيولوجية والجزيئية لعينات فيروس التهاب الشعب الهوائية المعدي التي تم جمعها من دجاج التسمين المحصن من محافظة بني سويف خلال عام ٢٠٢٣. حيث تم عزل الفيروس من كتاكيت التسمين المصابة على بيض دجاج أجنة بعد التمريرة الخامسة مع علامات مرضية مميزة مثل تقزم وتجعيد الأجنة المصابة. تم استخدام اختبار التجلط الدموي المستحث بالتربسين وتفاعل البلمرة المتسلسل العكسي وتكثير جزء من بروتين (Spike 1) الجليكوبروتين الجزئي وشجرة التطور النوعي لتحقيق هذا الهدف. أدى تكثير هذا الجزء من الجليكوبروتين الجزئي وشجرة التطور إلى انتماء المعزولة إلى النمط الجيني الأول، الفرع ٢٣، وتم تسجيلها في بنك الجينات كمعزولة مصرية لفيروس التهاب الشعب الهوائية المعدي برقم OR471645 تحت مسمي مصر/بني/سويف/٠٥. وأظهر تحليل التسلسل درجة عالية من التشابه مع السلالات المصرية المعزولة سابقًا، مثل OP585566 F1282-6 Egypt 2022 (١٠٠،١٠٠٪) و EG/VVT.NRC_Egypt_2021 (٩٦,٩، ٩٨,٤) و MN967774 Bens/AR07 Egypt 2019 (٩٧,٦، ٩٨,٧) من تسلسلات النوكليوتيدات والأحماض الأمينية، على التوالي.لذلك، فإن معزولة فيروس التهاب الشعب الهوائية المعدي الحالية خلال عام ٢٠٢٣، والتي تتجمع في السلالات الجينية الأولى، الفرع ٢٣، لا تزال منتشرة في دجاج التسمين القابل للإصابة أو الملقح، مما يسبب عدوى مستمرة لقطعان الدواجن على الرغم من أنظمة التطعيم الواسعة. هذه السلالة شديدة الضراوة لدجاج التسمين مما يؤدي إلى انخفاض في الإنتاج، وهذا المرض الخطير يغير الوضع الاقتصادي للصناعة المحلية في مصر. يعتمد التطعيم الفعال، الذي يوفر حماية متبادلة ضد النمط الجيني المتداول، على التنميط الجيني، والتنميط المصلي، والتنميط الوقائي بشكل جماعي، وليس بشكل منفصل.

الكلمات المفتاحية: فيروس التهاب الشعب الهوائية المعدي؛ البيولوجيا الجزيئية؛ التنميط الجيني؛ تفاعل البلمرة المتسلسل العكسي؛ شجرة التطور الفرعي.