Genetic evolution and phylogenetic analysis of Infectious Bronchitis Virus circulating in broiler flocks in New Valley Governorate, Egypt

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ABSTRACT: Infectious bronchitis virus (IBV) is still the etiology of serious economic losses among poultry flocks despite extensive vaccination regimes. During this study, 100 samples were collected from different poultry flocks, New Valley Governorate, Egypt in the period 2022-2023. Positive IBV samples using real time RT-PCR assay (16 samples show positivity with percentage 64%) were subjected to virus isolation through egg inoculation. IBV cytopathic effect on SPF-ECE was mainly stunting, curling, and dwarfing of the inoculated embryos after 3 to 5 passages. The samples were identified by conventional RT-PCR at 400 bp in size then directed to partial sequencing targeting the spike gene (S) (HVR-III regions). Interestingly, the sequencing analysis of S1 gene revealed eleven isolates which were diversified into three distinct genotypes, GI-1 (three isolates), GI-13 (one isolate), and GI-23 (seven isolates). The New Valley-IBV-3-2022 isolate has genetically related to IS/1494/2006 and IBV-Eg/CLEVB-2/IBV/012 with nucleotide identity percentage 89%. Moreover, New Valley-IBV-4-2022, New Valley-IBV-7-2022 and New Valley-IBV-8-2022 isolates share similarity with IBV-H120 and IBV-Connecticut with nucleotide identity percentage 89%, 87%; respectively. Likewise, New Valley-IBV-10-2022 was similar to 4/91-1998 and IBV-Eg/CLEVB-2/IBV/012 with nucleotide identity percentage 94%, 83%. The current study confirmed the co-circulation of both variants and classic IBV strains in New Valley Governorate. IBV variant-II strains clustered in GI.23 lineages is still circulating in the Egyptian field causing persistent infection to poultry flocks despite broad vaccination regimes. Our isolates are antigenically different from vaccinal strains that highlight the importance of continuous monitoring and surveillance of IBV in commercial flocks to clarify genomic characteristics of predominant strains.

KEYWORDS: Infectious bronchitis Virus, Variants, Spike gene, Sequencing, Broilers.

1. Introduction

Avian infectious bronchitis (IB) is an economically important acute viral disease in the local poultry industry. The disease is caused by infectious bronchitis virus (IBV), with severe clinical manifestations in the upper respiratory tract, reproductive tract, and kidneys. The viral Infection is highly contagious with morbidity rate reaching 100%, mortality rate from 0% - 80% and decreased egg production in layers and breeders (reaching up to 70%); depending on the age, immune status of the flocks and the circulating IBV strain [1]. In Egypt, IBV clinical manifestation is greatly observed in broilers as respiratory distress (gasp- ing, coughing, sneezing or tracheal rales). Alterations in the respiratory tract are caused by excess mucus accumulation deposited in the trachea and thickened, turbid air sacs. Importantly, nephropathogenic IBV strains cause acute to subacute nephritis of young chickens with polyuria and significant mortalities. Postmortem findings are dominated by congested, swollen kidneys and urates deposition [2, 3]

IBV, recently termed as Avian coronavirus-ACoV, is an enveloped virus which belongs to the order Nidovi- rales, family Coronaviridae, subfamily Orthocoronavirinae, genus Gammacoronavirus [4]. It has positive-sense single stranded RNA genome (approximately 27.6kb in length). Obviously, the virion contains four main structural proteins: nucleocapsid protein (N), membrane protein (M), envelope protein (E), and spike (S) glycoprotein. S protein is the most variable structural protein which is cleaved into S1 and S2 subunits. Furthermore, sequenc- ing analysis of S1 gene can differentiate IBV into seven genotypes (GI-GVII), and 35 distinct lineages (1-35), and a number of inter-lineage recombinant worldwide [5, 6]. Different genotypes were isolated from poultry flocks as
S1:GI-1 genotype which contains the Massachusetts vaccine strains as Mass/Mass41/41, Mass/H120/55 and Ma5. Meanwhile, genotype S1:GI-12 includes D274 vaccine strain and genotype S1:GI-13 also contains CR88 and 4/91 vaccine strains. QX IBV strains belongs to genotype S1:GI-19, whereas Egyptian variant 1 (Egy/var I) and 2 (Egy/var II) segregate into genotype S1:GI-23 depending on the full S1 gene sequence (Valastro et al., 2016). However, other IBV lineages like GI-12 [7], GI-13 [8] and GI-16 (Q1 like strains)[9] have been dominated in Egypt governorates. The S1 gene contains three hyper-variable regions (HVR 1, 2, and 3) and it plays a vital role in virus neutralization, tissue tropism, cell attachment, and serotype specificity. During IBV replication and evolution, most mutations and recombination events in the S1 gene lead to the emergence of new variant strains [10]. In this respect, the current IBV vaccines mostly failed to provide cross-protection among these various serotypes & genotypes and IBV outbreaks in vaccinated birds have been occurring causing severe infection [11]. Importantly, continuous evolution of IBV variants worldwide remains a great concern for poultry industry with a significant economic impact. Alterations of a few amino acids in S1 gene are able to emerge newly evolving IBV variant strains. Thus, genotyping of circulating field strains is crucial to investigate any new IBV variants as well as evaluate the existing vaccination regimes [12]. Taken together, the aim of this study is to genetically characterize field circulating IBV strains within the broiler flocks and clarify the genetic diversity between these field strains and the existing commercially used vaccines in New Valley governorate, Egypt as the majority of scientific articles focused on IBV studying in both north and middle of Egypt.

2. Materials and methods:

2.0.1. Ethics statement
This study protocol and all animal experiments was designed following the guidelines for Animal Experimentation and Approved by Institutional Review Board, Medical Ethics Committee, Faculty of Medicine, Assiut university, Egypt (04-2023-200249).

2.0.2. History of flocks and sampling
A total number of 100 pooled samples from broiler flocks of 25–35 days’ old suffered from respiratory manifestations and mortalities were collected from New Valley governorate, Egypt. These examined flocks exhibited respiratory symptoms such as sneezing, nasal discharge, coughing, bronchial rales, gasping with necropsy picture as tracheitis, lung congestion, caseous materials in the trachea. There are nephropathogenic lesions such as pale enlarged kidneys and urolithiasis. Mostly, these flocks have previously vaccinated with one or more of IBV vaccines as H120, (H120 + D274), 1/96 and M41. The tissue samples included: trachea, lung, bronchi and kidneys were aseptically collected from each flock then put in plastic bags under cooled conditions within an ice box transmitted immediately to the laboratory to be frozen at −20°C until processed for isolation and identification of IBV. The samples were collected as pooled homogenates from each flock. Further, the samples were prepared as 10% w/v suspensions in sterile phosphate buffered saline (PBS) (pH 7.4) with 10% antibiotic solution containing penicillin (1000 IU/mL), streptomycin (1mg/mL), gentamycin (2mg/mL) (Sigma Chemical Co., USA). The clear supernatant fluid was subjected to cooling centrifugation at 3000 rpm for 10 minutes; the supernatants were then collected for further analysis.

2.0.3. Virus isolation
Inoculation of the tissue homogenate (0.2 mL) of each pooled sample for each flock via the allantoic cavity of 10 days old SPF-ECE (OIE, 2018). The inoculated eggs incubated for 7 days at 37 °C with daily candling to check for embryonic pathognomonic characteristic changes as stunting, curling, dwarfing, and urates deposition in the mesonephros. Un-inoculated SPF-ECE were considered as negative control. Finally, the allantoic fluids from each sample were harvested and 25 samples screened using real-time reverse transcriptase PCR (rRT-PCR) for further confirmation of IBV in the tested isolates [13].
2.0.4. Viral RNA extraction and real-time reverse transcriptase PCR

Viral RNA was extracted directly from the harvested infected allantoic fluid (n = 25) using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the kit manufacturer’s instructions. IBV identification was also confirmed by rRT-PCR based on the highly conserved N gene of 130 basepair (pb) using QuantiTect® probe RT-PCR kit (Qiagen, Hilden, Germany), with specific primers (IBV-forward): 5’- ATG CTC AAC CTT GTC CCT AGC A -3’ and (IBV-reverse) 5’- TCA AAC TGC GGA TCA TCA CGT -3’ and probe (IBV-TM) 5’-[FAM] TTG GAA GTA GAG TGA CGC CCA AAC TTC A [TAMRA] -3’ (Meir et al., 2010).

2.0.5. S1 gene sequencing and phylogenetic analysis

Out of 15 RT-PCR positive isolates, the S1 gene of eleven positive isolates were amplified partially using Qiagen one step RT-PCR Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions with specific primer IBV-S1- forward 5’-CACTGGTAATTTTTCAGATGG-3’ and reverse primer IBV-S1-R 5’-CAGATTGCTTACAACCAC C-3’ (Adzhar et al., 1997). The amplicons of 400 bp in size were purified using the QIA quick Gel Extraction kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Additionally, to characterize the newly identified IBV strains, the purified PCR products were sequenced in both directions using BigDye Terminator v3.1 Sequencing Kit (PerkinElmer, Foster City, CA, USA) and an automated sequencer (ABI, 3130, Applied Biosystems, Foster City, CA, USA). The nucleotide sequences of the S1 gene of the eleven positive isolates were submitted to the GenBank with the accession numbers. Furthermore, this nucleotide and amino acid sequences were compared and aligned with other sequences of published IBV vaccines and reference strains available in GenBank Fig. 1. The phylogenetic analysis tree was designed via the maximum likelihood method using MEGA software version 7.0 with bootstrapping 1000 [14].

3. Result

3.1. Clinical and postmortem findings

Clinically examined flocks mostly exhibited respiratory manifestations signs in the form of sneezing, coughing, nasal discharges, coughing, bronchial rales, and gasping in all flocks. Postmortem examination of dead birds revealed tracheitis, lung congestion, air sacs cloudiness, caseous plugs in the trachea, and urolithiasis, enlarged kidneys (Fig. 2).

3.2. Virus isolation and identification.

Typical IBV lesions as curling, stunting, and dwarfing were observed in 80 out of 100 of examined tissue samples in inoculated embryos after three to five passages Fig. 3. Notably, our observed findings recorded that IBV was isolated from both vaccinated and non-vaccinated flocks as 5 isolates (45.4%) were from vaccinated flocks with classic vaccine while, the other 6 isolates (54.5%) belonged to non-vaccinated ones. The harvested allantoic fluids were subsequently identified for IBV using a real-time RT-PCR assay revealed 16 out of 25 samples were positive with a percentage of 64% Fig. 4. Furthermore, only 15 positive samples were amplified with one-step RT-PCR assay for S1 gene partial sequencing and molecular characterization (Fig. 5 and Table 1).

RT-PCR sequencing, and phylogenetic analysis

Eleven positive isolates were amplified partially using conventional one step RT-PCR that revealed specific amplification 400 bp in size (Fig. 5) then the isolates were submitted for S1 gene sequencing. The Descriptive data of our positive IBV isolates during 2022-2023 in New Valley Governorate, Egypt were noted in Table 1. Furthermore, the IBV amplified sequences were submitted to GenBank with accession numbers. Phylogenetic analysis among IBV strains has been conducted based on S1 gene (HVR-III regions). Interestingly, our results revealed that our IBV isolates were diversified into three distinct genotypes, GI-1, GI-13, and GI-23. Three isolates of this study were clustered as genotype I (classic of vaccine origin) and seven isolates involved in genotype 23.
(variant II) meanwhile, the last one isolate belongs to genotype 13 (variant I) (Fig. 6). The results showed that New Valley-IBV-3-2022 isolate has genetically related to EU780077-IS/1494/2006 (variant 2- isolated in Israel) and JX173488.1 IBV-Eg/CLEVB-2/IBV/012 (Egyptian isolate) with nucleotide identity percentage 89%. Moreover, New Valley-IBV-4-2022 isolate was genetically related to IBV-H120 spike protein (vaccinal strain) and IBV-Connecticut (American isolate) with nucleotide identity percentage 89%, 87%, respectively. New Valley-IBV-7-2022 and New Valley-IBV-8-2022 isolates was sharing 89%-87% similarity with IBV-H120 spike protein and IBV-Connecticut. Also, our isolate New Valley-IBV-10-2022 was similar to AF093794-strain4/91-1998 (vaccinal strain) and JX173488.1 IBV-Eg/CLEVB-2/IBV/012 with nucleotide identity percentage 94%, 83%. Besides, our study eleven isolates have a moderate nucleotide identity percent among each other ranged from 75%-88%, meanwhile 64-81% similarity based on Amino acid identity level. Regarding the amino acid identity level, New Valley-IBV-3-2022 isolate has an identity percent to EU780077-IS/1494/2006 and JX173488.1 IBV-Eg/CLEVB-2/IBV/012 with 82%. Moreover, the identity percent of New Valley-IBV-4-2022, New Valley-IBV-7-2022 and New Valley-IBV-8-2022 isolates compared to the IBV-H120 spike protein, IBV-Connecticut revealed a little higher similarity ranged from 77% to 82%. While New Valley-IBV-10-2022 showed a relative high consistency varied from 80%-91% with AF193423-QXIBV-1999 and AF093794-strain4/91-1998; respectively Figs. 1 and 6.

4. Discussion

IBV was a great threat causing massive economic losses in the poultry industry worldwide. Notably, IBV spread rapidly through poultry populations and undergo many genetic modifications leading to emerging of viral new variants [15, 16]. Notoriously, the spike gene mutation was resulting in emergence of new genotypes and/or serotypes that could be antigenically diverse comparable to the current classic and variant vaccinal strains. Thus, it is necessary to investigate these novel emerging IBV strain to select an efficient vaccine strategy against IBV infection [17]. IBV genotyping based on S1 gene sequencing, especially the HVRs, is the most reliable way to classify IBV isolates. Usually, IBV serotypes have a wide range of genetic variations in the S1 gene ranged from 2% to 25% [8]. In Egypt, many strains of live attenuated and inactivated vaccines used to control IBV, however, the outbreaks of the disease have continued to cause severe infections[18]. Interestingly, the present study investigates the prevalence of IBV circulating in broiler flocks with high mortality rates in New Valley Governorate, Egypt through virological examination, molecular characterization, and sequencing analysis. Additionally, we study the genetic evolutions of the circulating IBV strains seeking novel variants emerged in the field. In this study, the clinical findings among examined flocks were mainly respiratory manifestations ranged from mild to remarkable sneezing, coughing, nasal discharges, coughing, respiratory sounds, and gasping in all flocks. These observed findings were going in parallel with that previously reported clinical manifestations by[19, 20, 21]. The Gross pathology included lung congestion, caseous plugs at tracheal bifurcation, and enlarged kidneys were noticed in all examined flocks. Additionally, pneumonia and air sacs cloudiness, with or without yellow caseous exudates were reported. These results came in accordance with [20, 22]. The air sacs cloudiness with or without yellow caseous exudates is evidence of secondary bacterial infection. These results were in line with the air sacs cloudines findings of earlier research [23, 24]. Concerning egg inoculation, IBV cytopathic effect on SPF-ECE was mainly stunting, curling, and dwarfing of the inoculated embryos after three to five passages. These results came in accordance with many previous reports[25, 26, 27]. Real-time RT-PCR is a sensitive and accurate method for the molecular detection and identification of new and/or emerging IBV variants [17]. Analysis
Table 1: Flocks data sampled for IBV isolates used for partial sequencing of S1 gene characterization

<table>
<thead>
<tr>
<th>No.</th>
<th>Isolate identification</th>
<th>Flock age</th>
<th>Vaccines used in flock</th>
<th>Collection year</th>
<th>Flock location</th>
<th>Signs and postmortem lesions</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>New Valley-IBV-1-2022</td>
<td>25</td>
<td>H120</td>
<td>2022</td>
<td>New Valley/Eldakhla</td>
<td>Sever respiratory manifestations</td>
<td>OR654090</td>
</tr>
<tr>
<td>2</td>
<td>New Valley-IBV-2-2022</td>
<td>33</td>
<td>H120 + 4/91</td>
<td>2023</td>
<td>New Valley/Eldakhla</td>
<td>Kidney damage With high morbidity and mortality rates</td>
<td>OR654091</td>
</tr>
<tr>
<td>3</td>
<td>New Valley-IBV-3-2022</td>
<td>30</td>
<td>Non-vaccinated</td>
<td>2022</td>
<td>New Valley/Eldakhla</td>
<td>Kidney damage with high morbidity and mortality rates</td>
<td>OR654092</td>
</tr>
<tr>
<td>4</td>
<td>New Valley-IBV-4-2022</td>
<td>29</td>
<td>H120 + D274</td>
<td>2022</td>
<td>New Valley/Eldakhla</td>
<td>Sever respiratory manifestations</td>
<td>OR654087</td>
</tr>
<tr>
<td>5</td>
<td>New Valley-IBV-5-2022</td>
<td>34</td>
<td>Non-vaccinated</td>
<td>2022</td>
<td>New Valley/Eldakhla</td>
<td>Kidney damage with high morbidity and mortality rates</td>
<td>OR654094</td>
</tr>
<tr>
<td>6</td>
<td>New Valley-IBV-6-2022</td>
<td>32</td>
<td>Non-vaccinated</td>
<td>2022</td>
<td>New Valley/Eldakhla</td>
<td>Kidney damage with high morbidity and mortality rates</td>
<td>OR654095</td>
</tr>
<tr>
<td>7</td>
<td>New Valley-IBV-7-2023</td>
<td>31</td>
<td>H120 + D274</td>
<td>2023</td>
<td>New Valley/Eldakhla</td>
<td>Sever respiratory manifestations</td>
<td>OR654088</td>
</tr>
<tr>
<td>8</td>
<td>New Valley-IBV-8-2023</td>
<td>31</td>
<td>H120 + D274</td>
<td>2023</td>
<td>New Valley/Eldakhla</td>
<td>Sever respiratory manifestations</td>
<td>OR654089</td>
</tr>
<tr>
<td>9</td>
<td>New Valley-IBV-9-2023</td>
<td>30</td>
<td>Non-vaccinated</td>
<td>2023</td>
<td>New Valley/Elfarafra</td>
<td>Sever respiratory manifestations</td>
<td>OR654098</td>
</tr>
<tr>
<td>10</td>
<td>New Valley-IBV-10-2023</td>
<td>28</td>
<td>H120 1/96</td>
<td>2022</td>
<td>New Valley/Elfarafra</td>
<td>Sever respiratory manifestations</td>
<td>OR654099</td>
</tr>
<tr>
<td>11</td>
<td>New Valley-IBV-11-2023</td>
<td>35</td>
<td>Non-vaccinated</td>
<td>2023</td>
<td>New Valley/Elfarafra</td>
<td>Kidney damage with high morbidity and mortality rates</td>
<td>OR654100</td>
</tr>
</tbody>
</table>

Table 2: Nucleotide and amino acid identities of partial spike glycoprotein gene sequence of our eleven IBV isolates with other Egyptian strains, reference strains and vaccine strains.

Figure 1: Gene sequence
Figure 2: Gross pathology of the investigated chicken flocks

Figure 3: IBV isolation in the inoculated SPF-ECE. (a) Normal embryo and (b) curled, stunted, and dwarfed embryos infected with IBV after 3-5 passages

Figure 4: Real time PCR amplification plots. Amplification plots indicate the positive IBV control (upper arrow), and positive samples (eleven samples among positive and negative IBV controls). The negative control is not amplified, and it is under the threshold.

Figure 5: RT-PCR amplicons of IBV isolates for partial S1 gene revealed a single specific band (400bp). Lane 1: 1 kbp DNA ladder (Fermantas); Lane (1-15): positive IBV samples. Lane -ve: Negative control. Lane +ve: Positive control (H120 vaccine).

of real-time RT-PCR assay with IBV specific primers revealed that only 16 samples show positivity with percentage 64%. This is in line with previous literature which reported a nearly similar PCR results [28, 29, 27]. Currently, our findings reported that IBV was isolated from both vaccinated and non-vaccinated flocks as 5 isolates (45.4%) were from vaccinated flocks with classic vaccine while, the other 6 isolates (54.5%) belonged to non-vaccinated ones. These results indicate little or no cross-protection of classic vaccinal strains against circulating IBV. Nearly similar results were assessed by [20] who isolated IBV from non-vaccinated and vaccinated flocks with percentages of 66.6% and 33.3%; respectively. According to our findings, only eleven positive samples were amplified at 400 bp in
Figure 6: Phylogenetic tree based on a partial nucleotide sequencing of the S1 gene, showing the relationship among our isolates and other reference IBV strains retrieved from GenBank. The tree was designed using the maximum-likelihood method with (GTR+G+I) model and 1000 bootstrap replicates using MEGA version 7 software. Our isolates are highlighted in bold font with blue color.
size then genotyped by partial sequencing of the HVR-III of the S1 gene. Notably, our IBV isolates were clustered as genotype I (three isolates), seven isolates involved in genotype 23 (variant II) while, the last one isolate diversifies into genotype 13 (variant I). The genotype 23 (the variant group) are indigenous and dominate in the Middle East region. In Egypt, IBV variant groups were subdivided into 2 subgroups based on the HVR-III sequencing [7]. The first variant subgroup is Egy/Var which is closely related to the original IBV Egyptian strain of Egypt/Beni-Suef/01 and Ck/EG/BSU-1,4,5/2011[30, 3]. Our findings showed that New Valley-IBV-3-2022 isolate was sharing 89% similarity with EU780077-IS/1494/2006 (Israeli strain) and JX173488.1 IBV-Eg/CLEVΒ-2/IBV/012 based on nucleotide identity. These recorded results agreed with many researchers [9, 22, 29], who mentioned that the circulating IBV strains were closely related to Egyptian variant II group. Accordingly, Egy/Variant II subgroup was widely spread in New Valley governorate, Egypt during 2022 and 2023. In addition, the likeness of Egyptian isolates comparable to Israeli isolates as neighboring countries representing 89% may be due to human beings uncontrolled movement and/or contraband objects through borders. cross-protection of classic vaccinal strains against circulating IBV. Nearly similar results were assessed by [20], who isolated IBV from non-vaccinated and vaccinated flocks with percentages of 66.6% and 33.3%; respectively. According to our findings, only eleven positive samples were amplified at 400 bp in size then genotyped by partial sequencing of the HVR-III of the S1 gene. Notably, our IBV isolates were clustered as genotype I (three isolates), seven isolates involved in genotype 23 (variant II) while, the last one isolate diversifies into genotype 13 (variant I). The genotype 23 (the variant group) are indigenous and dominate in the Middle East region. In Egypt, IBV variant groups were subdivided into 2 subgroups based on the HVR-III sequencing [7]. The first variant subgroup is Egy/Var-I which is closely related to the original IBV Egyptian strain of Egypt/Beni-Suef/01 and Ck/EG/BSU-1,4,5/2011 [30, 3]. Our findings showed
representing 89% may be due to human beings uncontrolled movement and/or contraband objects through borders. Finally, our findings can confirm that our various IBV lineages could have reached to Egypt poultry flocks due to uncontrolled movement of animals across borders or through the wild birds from the adjacent countries as Israel.

Conclusions

The current study confirmed the co-circulation of both variants (GI-13 and GI-23). and classic strains of IBV in New Valley Governorate. IBV variant-II strains clustered in GI.23 lineages is still circulating in the Egyptian field causing persistent infection to poultry flocks despite broad vaccination regimes. Furthermore, these variants are virulent and pathogenic to broilers leading to serious mortalities and morbidity that alter the economic situation of local industry in Egypt. In addition, our isolates are antigenically different from vaccinal strains that highlight the importance of continuous monitoring and surveillance of IBV in commercial flocks besides wild birds to clarify genomic characteristics of predominant strains. These will facilitate revising and updating the appropriate vaccination programs to choose an efficient vaccine that can provide a cross-protection against the circulating genotypes. Likewise, further investigation is needed to evaluate the genetic characteristics of IBV variants with its antigenicity, pathogenicity, tropism, shedding and evolution-driven failure in vaccine efficacy.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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