



Genetic and Pathological Characterizations of Gumboro (IBD) in Chickens

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ABSTRACT

Gumboro, or infectious bursal disease (IBD), represents a significant threat to poultry worldwide due to its genetic and pathogenic variations. Viruses can mutate and produce new variants, which can have an impact on the epidemiological situation. The study will determine and assess the genetics, protein sequence, and pathogenicity of IBDV present in broiler flocks in different locations within Ismailia and Dakahlia Governorates, Egypt, in 2022. A total of 150 bursa samples were collected from 15 broiler flocks exhibiting symptoms indicative of IBDV infection. The VP2 (viral protein2) gene was partially detected and then subjected to complete gene sequencing in order to summarize the key genetic mutations found and histopathological examination. The results demonstrated that 12 out of 15 farms were infected with IBDV. Genetic analysis revealed that 10 of the 12 identified strains belonged to genogroup 3, which was similar to previous Egyptian strains. The remaining two strains were of genus 1. The VP2 gene's hypervariable region displayed amino acid alterations at several positions. Classic IBDV strains (genogroup 2) showed similar amino acid profiles. Amino acid alterations were identified in the VP2 gene, indicating that classic IBDV strains may cause damage to the bursa of Fabricius, leading to immunosuppression. In Egypt, vvIBDV (very virulent infectious bursal disease virus) strains have led to recurring outbreaks and high chick mortality rates. These findings are anticipated to contribute to the latest features of IBDV in the examined regions. Therefore, it is recommended that IBDV immunization programs in Egypt, where live attenuated virus vaccinations are common, be reevaluated. It is also recommended that the evaluation of clinical signs in broiler chicken flocks over time be monitored for diagnosis.

Keywords: Bursa, Chickens, Genetic analysis, Infectious Bursal Disease Virus, Pathological finding.

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INTRODUCTION

Infectious bursal disease virus (IBDV), a significant immunosuppressive virus in chickens, typically infects birds aged four to seven weeks (Jackwood, 2017). The virus induces lymphopenia by inhibiting the B-lymphocytes of the bursa of Fabricius,

thereby increasing the risk of secondary viral or bacterial infection, which results in economic losses in the poultry industry (Dey *et al.*, 2019).

IBDV belongs to the Birnaviridae family of viruses and is a non-enveloped, double-stranded RNA virus. Chickens can only be infected with serotype I

IBDV viruses, which represent one of the two known serotypes (I and II). The IBDV serotype I strains comprise typical, highly virulent, and genotoxic variations, each exhibiting different levels of antigenicity and pathogenicity (Aliyu *et al.*, 2021).

IBDV serotype I strains have been classified into three distinct categories: classic, highly pathogenic, and antigenic variations. Each category displays distinct antigenicity and pathogenicity. The updated IBDV genogroup classification approach has been defined as G1 (classic), G2 (variant), and G3 (very virulent) (Wang *et al.*, 2021). Furthermore, additional genogroups, specifically G4 to G7, were discovered and classified based on geographic or distinctive molecular characteristics that differed from those of the original strains. This indicates that the IBDV genome is subject to continuous alteration and reassortment (Hernández *et al.*, 2015; Michel and Jackwood, 2017).

The virus's genetic material comprises two RNA pieces, designated A and B. Segment A contains two overlapping parts with instructions for synthesizing two viral proteins: The viral proteins VP5 and polyprotein (PP) VP2-VP4-VP3 are encoded by the virus. Auto-proteolysis 1 separates PP into VP2, VP3, and VP4, where VP3 translates into various actions that promote the virus's growth and adhesion (Jackwood, 2017). VP4 is involved in the degradation of a larger protein, polyprotein. Segment B contains a single open reading frame (ORF), which encodes the VP1 protein. The VP1 protein is of significant importance due to its functions in gene duplication, genetic modification, and virulence activities.

The VP2 protein is composed of three distinct parts: the base, the shell, and the projection. The VP2 protein (comprising the shell and projection domains) is a crucial component of viruses. It facilitates the virus's entry into cells, determines the cells it infects, enhances the virus's pathogenicity, and enables it to alter its appearance to evade the immune system (Chen *et al.*, 2022).

The base and shell regions remain unaltered, while the projection region (206-350 aa) is characterized by a hypervariable region that contains two hydrophilic segments, namely, region A (212-224 aa) and region B (314-325 aa). The determination of IBDV is dependent on the antigenicity of the hypervariable region (HVR). Numerous pathogenic variants that impact host immunity have been identified through amino acid sequence analysis (Durairaj *et al.*, 2011; He *et al.*, 2019). These variations, such as the presence of glutamine at position 253 and aspartic acid at position 279, have been demonstrated to contribute to increased virulence and pathogenicity. Furthermore, the

presence of alanine at position 284 is also considered to be of significance. Consequently, strain identification has been concentrated on genetic differences in the VP2 gene in recent years (Mató *et al.*, 2022).

The aim of this study was to determine and assess the genetics, protein sequence, and pathogenicity of IBDV present in broiler flocks in different locations within Ismalia and Dakahlia Governorates, Egypt, in 2022.

MATERIALS AND METHODS

Ethical approval

The experimental study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) in accordance with the research ethics board guidelines of the National Research Center, Egypt, and licensed under license no. (115122023).

Field inspection and sample acquisition

In this study, 150 bursae were obtained aseptically from broiler flocks aged 5 to 7 weeks that displayed signs of infectious bursal disease, including depression, sleeping with their beaks touching the ground, ruffled feathers, watery diarrhea, soiled vents, and dehydration, with morbidity rates ranging from 20% to 30% and a mortality rate ranging from a mortality rate ranging from 30% to 40%. A total of ten bursae were collected from each flock or pool. Necropsy revealed the presence of edema (bursa) and/or hemorrhage (the thigh muscles) in poultry fields in different locations in the Ismalia and Dakahlia Governorates, Egypt, in 2022.

The examined groups of birds were administered a live attenuated IBDV vaccine (classic strain) via their drinking water at 11 and 22 days of age.

Viral RNA extraction

An RNA Extraction Kit (Patho Gene-Spin; iNtRON Bio®) was employed to obtain the virus's RNA directly from pooled samples. The extraction was conducted in accordance with the manufacturer's instructions, and the resulting sample was stored at -80 °C for future analysis.

VP2 Gene Amplification

The RNA was subjected to reverse transcriptase polymerase chain reaction (RT-PCR) using the iNtRON Bio®ONE-STEP RT-PCR PreMix Reagents Kit to amplify a 620 bp fragment (Table 1). The reaction mixture comprised 1 µL of each primer (diluted to 10 pmol) from Table (1), 5 µL of PCR-grade water, and 5 µL of RNA (Metwally *et al.*, 2009).

Table 1: Primers used to amplify the VP2 gene (partial and complete)

Gene	Primer sequence	Cycling	Reference
VP2 gene (Partial gene)	F: 5'- ATG ACA AAC CTG CAA GAT CAA - 3';	45 °C for 30 min of RT, 95°C for 10 min, 40 cycles of 95°C for 30 sec, 59°C for 60 sec, then 72°C for 90 sec of PCR, and finally, a final extension cycle at 72°C for 5 min.	(Metwally <i>et al.</i> , 2009)
	R: 5'- TCT GGG CCT GTC ACT GCT G -3'		
VP2 complete gene (Part one)	F: 5-ATG ACAAAC CTG CAA GAT CAA-3	45 °C for 30 min of RT, 95°C for 10 min, 40 cycles of 95°C for 30 sec, 56°C for 60 sec, then 72°C for 90 sec of PCR, and finally, a final extension cycle at 72°C for 5 min.	(Mató <i>et al.</i> , 2022)
	R: 5-TCT GGG CCT GTC ACT GCT G-3		
VP2 complete gene (Part Two)	F: 5-GTA ACA ATC ACA CTG TTC TCA GC-3		
	R: 5-AAT GCT CCT GCA ATC TTC AG-3		

Full VP2 gene sequencing

Twelve samples were subjected to full VP2 gene sequence analysis in order to amplify the 1395 bp fragment. This was carried out in accordance with the methodology outlined in Table 1. The reaction mixture consisted of the ONE-STEP RT-PCR PreMix, 1 µL of each primer (comprising the first and second sections of the VP2 gene) (10 pmol concentration), 10 µL of PCR-grade water, and 5 µL of extracted RNA.

The Wizard® DNA Purification Kit was employed in accordance with the manufacturer's instructions to isolate and cleanse specific bands. The PCR product was subjected to sequencing using a Sanger two-way sequencer provided by Macrogen®. The sequencing data was analyzed using Blast of NCBI and then compiled and modified with BioEdit program version 7.1.5 (Hall, 1999). Subsequently, MEGA X software was employed to conduct neighbor-joining phylogenetic analyses as part of Molecular Evolutionary Genetics Analysis (Tamura *et al.*, 2021).

Histopathological examination

Bursal samples were collected from farms with morbidity rates ranging from 20% to 30% and a mortality rate of a mortality rate of 30% to 40%. 75 bursal specimens were collected from fifteen broiler chicken flocks (five to seven weeks old) and fixed in a 10% neutral buffered formalin solution. Paraffin sections of 5µ thickness were prepared and stained with hematoxylin and eosin (H&E) and examined microscopically in accordance with the methodology outlined by Suvarna and Layton (2013). Bursal lesions were assigned a score (where 1 represents no bursal lesion, 2 represents scattered or partial follicle damage (mild), 3 represents 50% or less follicle damage (moderate), 4 means 50–75% follicle damage (intense), and 5 represents 75–100% follicle damage (severe), the severity of the histological lesions compared to those observed in non-infected chickens (controls) (SPF chickens aged 5-7 weeks purchased for comparison) (Bolis *et al.*, 2002).

Ten one-day-old SPF chicks from the Qoum Oshim SPF farm in Fayum, Egypt, were raised in chicken isolators for 7 weeks. They were kept in clean conditions and provided with feed, water, and continuous lighting. The chickens were monitored daily throughout the 7-week period.

RESULTS

Macroscopically, the bursal samples taken from commercial broiler flocks exhibited depression, sleeping with their beaks touching the ground, ruffled feathers, watery diarrhea, soiled vents, dehydration, both enlargement and edema, accompanied by hemorrhagic follicles [Fig. 1a] or atrophy, and the presence of cheesy material within the lumen. Furthermore, the thigh muscles exhibited hemorrhagic streaks (0.2–0.3 mm) [Fig. 1b], while the kidneys were enlarged and pale with hemorrhagic foci, and some cases exhibited nephrosis, i.e., grayish foci in renal tubules [Figs. 1c and 1d].

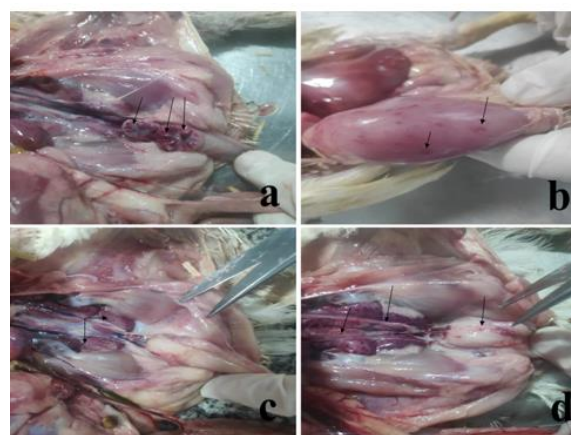


Fig. 1: a: Broiler chicken 5 weeks old naturally infected with IBDv showing enlarged bursa accompanied by exudate on the hemorrhagic follicles. b: naturally infected with IBD (very virulent strain) showing hemorrhagic streaks on the thigh muscle. c: naturally infected with IBD (very virulent strain) showing pale and swollen Renal tubules and hemorrhagic streaks. d: showing nephrosis, (grayish foci in renal tubules), and enlarged bursa with petechial hemorrhage.

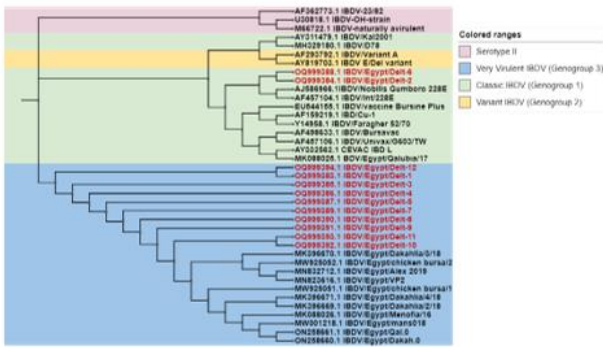


Fig. 2: Phylogenetic characterization of the nucleotide sequences in this study strains of (red font) IBDV based on VP2.

Detection of IBDV using RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction)

Fifteen broiler farms were examined, with ten bursae samples taken from each pool or flock. This resulted in a total of 150 samples being tested. RT-PCR was employed to identify the samples, with specific bands at 620 bp observed in 12 of them, indicating a positive result in 80% of the samples.

Full VP2 gene sequencing

The complete VP2 gene's 1395-bp segment was subjected to sequencing and subsequently deposited in

Genbank under the accession number. The sequence was designated OQ999383–OQ999394.

Results of phylogenetic tree analysis

The VP2 complete gene phylogenetic characterization (**Fig. 2**) revealed that ten IBDV strains (OQ999383.1, OQ999385.1, Q999386.1, OQ999387.1, OQ999389.1, OQ999390.1, OQ999391.1, OQ999392.1, OQ999393.1, and OQ999394.1) were genetically grouped in genogroup 3. Furthermore, two strains (OQ999384.1 and OQ999388.1) were clustered in Genogroup 1, which includes classic strains.

In the present investigation, the amino acid sequence of the HVR of the VP2 gene of the vvIBDV strains indicated modifications specifically at positions L217, F220, A222, I242, Q249, Q253, I256.

The amino acid sequence of the HVR of the VP2 gene of the vvIBDV strains revealed modifications at positions D279, A284, I294, S299, and A321 (**Fig. 3**) (**Table 2**).

The amino acid sequence of the VP2 gene in the two typical IBDV strains (genogroup 2) (OQ999384.1 and OQ999388.1) exhibited a high degree of similarity to the vaccine strains IBDV/Nobilis Gumboro 228E (AJ586966.1) and IBDV/Int/228E (AF457104.1).



Fig. 3: IBDV full VP2 gene amino acid alignment. This study strains red font

Table 2: The amino acid changes in the antigenic regions of the HVR-VP2 protein in the detected strains

	Detected strains	Major-Antigenic-Peak-A (210–225 aa)	Minor-Hydrophilic-Peak-1 (247–254 aa)	Minor-Hydrophilic-Peak-2 (279–292 aa)	Major-Hydrophilic-Antigenic-Peak-B (312–325 aa)
Genogroup 3 (VVIBDV)	OQ999383.1	L217, F220, and A222	I242, Q249, Q253, and I256	D279, A284, I294, and S299	A321
	OQ999385.1				
	OQ999386.1				
	OQ999387.1				
	OQ999389.1				
	OQ999390.1				
	OQ999391.1				
	OQ999392.1				
	OQ999393.1				
	OQ999394.1				

Microscopical histopathological findings

The lesions observed in the bursa of Fabricius in birds infected with IBDV exhibited distinct characteristics at different stages of infection. Initially, apoptotic and necrotic changes of lymphocytes occurred in both cortical and medullary zones, which typically resulted in lymphoid depletion with or without cyst formation, accompanied by intense hyperemia and hemorrhage within and between follicles (**Fig. 4.c**). The lesion subsequently progressed to serous exudation within the inter-medullary cystic spaces (**Fig. 4.d**). The follicles, which had lost their lymphocytes, became edematous and were infiltrated by inflammatory fluids containing heterophils, as depicted in **Fig. 4a**. Subsequently, due to intense heterophils accumulating inside the cystic cavities of the follicles and the interfollicular tissues, suppurative inflammation progressed (**Fig. 4b**). The follicles exhibited distinct stages of pathological alterations, culminating in the proliferation of fibrous tissue, which commenced within the interfollicular tissue and extended to replace necrotic and depleted follicles (**Fig. 4.c, Fig. 5**). The covering epithelia exhibited a range of variations, including hyperplasia, acute cellular swelling, and vesicle formation. The size of the follicles varied depending on the stage of the disease. The follicles showed greater length due to the accumulation of exudate, but this was later reduced due to the reabsorption of the exudate and fibrosis, in comparison with the control group (**Fig. 4.d**). There were no significant pathological differences between flocks infected with either the very virulent or the classic strains (**Table 3**).

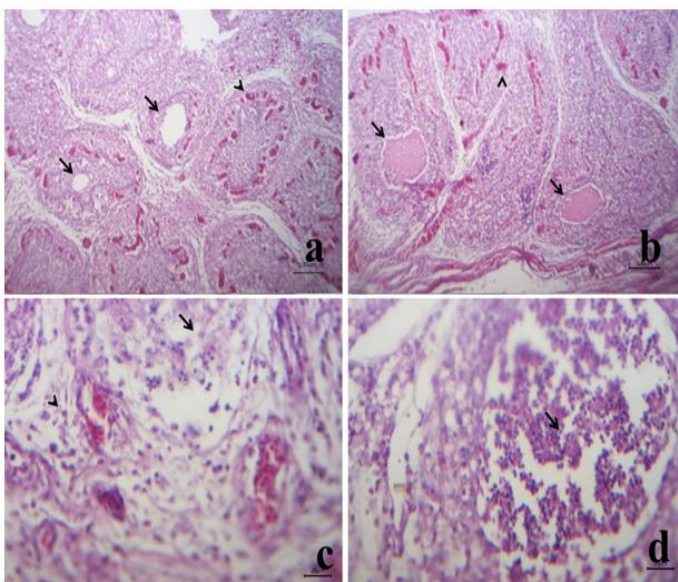


Fig. 4: a: Bursa of Fabricius of broiler chicken 5 weeks old naturally infected showing intense necrosis of lymphocytes in the cortex and medulla of follicles (arrow) and replaced by cystic spaces beside hyperemic blood vessels (arrowhead) (H&E) 100 um. b: Bursa of Fabricius of broiler chicken 5 weeks old naturally infected (very virulent strain) showing cystic spaces containing serous exudate inside some follicles (arrow) and intra-follicular hemorrhages (arrowhead) (H&E) 100 um. c: Bursa of Fabricius of broiler chicken 5 weeks old naturally infected (very virulent strain) showing edema admixed with heterophils and siderocytes which replace necrotic follicles (arrow) beside inter-follicular hyperemic blood vessels (arrowhead) (H&E) 100 um. d: Bursa of Fabricius of broiler chicken 5 weeks old naturally infected (very virulent strain) showing suppurative stage characterized by intense heterophils aggregates within cystic cavities of the follicles (arrow) (H&E) 100 um.

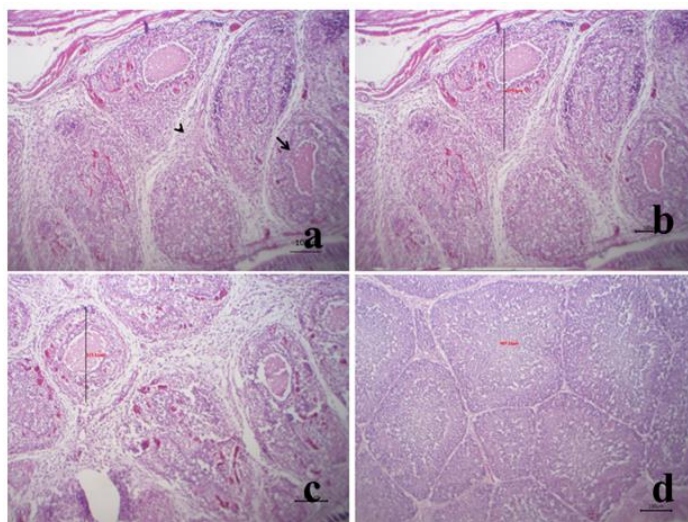


Fig. 5: a: Bursa of Fabricius of broiler chicken 5 weeks old naturally infected (classic strain) showing inter-follicular edema and fibroplasia (arrow) (H&E) 100 um. b, c: Bursae of Fabricius of broiler chicken 5 weeks old naturally infected showing histomorphometric analysis of some follicle sizes (dimension) of infected birds in comparison with control negative (H&E) 100 um. d: Normal bursa of Fabricius of broiler chicken 5 weeks old showing the normal structure of bursal follicles and normal follicular length (H&E) 100 um.

Table 3: Bursal lesion scoring of field infection with IBD virus

Serial	Lesion	Intensity	Control (-ve)
1	Hyperemic and hemorrhage	+++	-
2	Apoptosis and necrosis	++++	-
3	Cystic cavities	++	-
4	Serous exudates	+++	-
5	Heterophils aggregation	+++	-
6	Fibroplasia	+	-

DISCUSSION

IBDV is regarded as a significant threat to the global poultry industry, particularly in Egypt, due to its potential for widespread infection. The chickens from various broiler flocks displayed a range of clinical signs, including depression, diarrhea, unclean cloacas, dehydration, weakness, anorexia, and tremors. These signs were similar to those documented by previous studies, including those by **Oluwayelu et al. (2002)**, **Omer and Khalafalla (2022)**, and **Damairia et al. (2023)**. Moreover, the hemorrhage on the thigh and pectoral muscles was previously mentioned due to the inertia of the virus with the normal blood clotting mechanism (**Zeryehun et al., 2012**).

Necropsies revealed that the Fabricius bursa exhibited edema, hemorrhage, and occasional atrophy, as previously reported by **Khan et al., (2009)**. The atrophied bursa develops subsequent to infection with IBDV. The size of the bursal follicles varies depending on the stage of IBD. In the initial stages, the bursa is characterized by inflammation and swelling. However, in subsequent phases, it undergoes atrophy. These findings are consistent with those reported by **Damairia et al., (2023)**. The thigh muscles exhibit a variety of injuries resulting from the disintegration of thrombocytes, which in turn leads to coagulation deterioration (thrombocytopenia) and thrombocyte apoptosis. These results completely agree with those

presented by **Toro et al., (2009)** and **Rania and Enas (2021)**. Furthermore, the report by 24 indicated that the kidneys exhibited swelling, pallor, and hemorrhagic foci.

The present study found that 80% of the field samples (12/15) tested positive for IBDV by RT-PCR, which is consistent with the findings of **Alkhalefa et al. (2018)**. The study recorded an 85% positive rate for IBDV (n = 17/20). The majority of IBDV-positive flocks had received vaccinations, indicating that gene reassortment in field IBDV strains was causing the vaccination breaks, as previously observed (**Abou El-Fetouh and Abdallah, 2019; El Samadony et al., 2019**).

A significant issue in Egypt is that IBDV continues to spread despite the wide availability of vaccines. The inefficacy of vaccines may be attributed to changes in VP2, the primary agent that protects against IBDV. The genetic recombination of different strains or immunological pressure may be accountable for these vaccination breaks (**Hon et al., 2006; Fathy and Abd El-Moneam, 2018**).

Moreover, these techniques (PCR and sequencing) provide researchers with a powerful tool to track phylogenetic and epidemiological changes among IBDV subgroups. The amino acids present in the HVR of VP2 provide valuable genetic data on the natural

variability of strains and the changes in antigenicity and/or virulence resulting from attenuation in different strains. This study therefore analyzed the partial gene sequencing of the HVR of VP2 (**Jackwood and Sommer-Wagner, 2005**).

Based on the recent IBDV typing of the VP2 HVR, this study identified ten strains of IBDV (OQ999383.1, OQ999385.1, OQ999386.1, OQ999387.1, OQ999389.1, OQ999390.1, OQ999391.1, OQ999392.1, OQ999393.1, and OQ999394.1, which were inferred to possess amino acid genetic markers for vvIBDV viruses (genogroup 3). These markers are widely distributed worldwide, with a particular prevalence in Egyptian isolates (L217, F220, A222, I242, Q249, Q253, I256, D279, A284, I294, S299, and A321).

The amino acid residue 222 plays a pivotal role in the recognition of the virus by the immune system and facilitates the differentiation between various virus genogroups. In the vvIBDV virus strains found in Egypt, the residue A222 is specifically identified (**Jackwood and Sommer-Wagner, 2005**). However, it was discovered in the 1980s in the USA that the altered P222 amino acid residue in classical strains caused a shift and led to vaccine failure (**Brown et al., 1994**).

The vvIBDV strain is distinguished by the amino acids A222, I256, and I294, which remain unchanged in addition to I242, I256, I294, and S299 (**Maqbool et al., 2020**). These molecular markers are essential for the differentiation of classical IBDV strains from variant strains of vvIBDV (**Banda and Villegas, 2004**).

It has been demonstrated that alterations in the protein composition, specifically amino acids Q253 and A284, play a crucial role in the virulence of the virus. The presence of serine-rich amino acids at positions 326 to 332 (SWSASGS) indicates that these isolates belong to the vvIBDV genogroup 3 (**Ellakany et al., 2019**).

Additionally, the VP2 gene's P-loop domain in Egyptian viruses is similar to those in vaccine strains used in Egypt, including Bursin Plus and CEVAC-IBD-L. Furthermore, the amino acid residues Q249 and Q253 in antigenic site 2, as well as D279 and A284 in antigenic site 3, are also similar. The amino acid mutations H253Q and T284A were demonstrated to contribute to enhanced virulence in vvIBDV (**Li et al., 2015**). Furthermore, the alteration of amino acid position 321 was demonstrated to be pivotal in preventing the proliferation of escape mutant strains (**Samy et al., 2020**).

However, the VP2 gene amino acid sequence of the two classic strains of IBDV (genogroup 2) (OQ999384.1 and OQ999388.1) exhibited a similar

amino acid profile to the vaccine strains AJ586966.1 IBDV/Nobilis Gumboro 228E and AF457104.1 IBDV/Int/228E. The substitution of three amino acids (Q253, A284, and S330) in the strains resulted in a loss of their virulence, as evidenced by the absence of disease manifestation (**Mundt, 1999**).

Histological examination of the bursae revealed hyperemia and hemorrhage between depleted follicles. The observed histopathological changes were predominantly fibrotic and atrophic in nature. Subsequently, the depleted follicles became edematous, and the necrotic lymphocytes were replaced by inflammatory fluids containing heterophils. As previously described by **Hoque et al. (2001)**; **Rudd et al. (2001)**; **Islam et al. (2008)**; and **Kulsum et al., (2018)**, the follicles displayed a range of pathological alterations, including mild to severe lymphoid depletion, follicular atrophy, cystic development, and bursal hemorrhage. Furthermore, the histomorphometric analysis of select follicle sizes demonstrated an increase in length during the early stage due to exudation, followed by a decrease due to exudate reabsorption and fibrosis, in contrast to the control group. This result is consistent with the findings of **Toro et al., (2009)**. Furthermore, the histomorphometric analysis revealed a notable depletion of lymphocytes, which is indicative of IBDV infection.

CONCLUSION

This study provides a molecular update on IBDV infections in commercial broiler flocks, with an 80% detection rate of vvIBDV. The coexistence of classical IBDV, which is thought to originate from a vaccine, implies that vaccine viruses exist in the wild. This may be attributed to the potential for intermediate-plus (hot) vaccines to elicit robust post-vaccination responses or to instances of suboptimal vaccine administration, which could result in the generation of antigenic variants. The potential emergence of mutants under immunological pressure, with subsequent alterations in their pathogenicity, necessitates a reassessment of IBD immunization programs in Egypt. This is because live attenuated virus vaccination programs are commonly employed.

Recommendations

Further examination of additional samples from various locations in Egypt, including those derived from backyard farming, is recommended. Furthermore, a complete genome sequence is recommended for this virus in order to provide genetic information that could be useful for controlling the disease.

Competing interests

The authors declare no Competence of interest.

Author contributions

All authors contributed to the study’s conception and design. Material preparation, sample collection, and analysis were performed by **Aalaa Saad, Hanaa A. Elsamadony, Rania Mohamed, Neven Ramzy, Hoda Mekky, Asmaa Zin ELdin, and Ahmed EL-Shemy** supervision, conceptualization, reviewing, and editing **Hanaa A. Elsamadony, and Aalaa Saad**. The first draft of the manuscript was written by **Aalaa Saad and Rania Mohamed** and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

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