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Persistent Circulation of Very virulent Infectious Bursal Disease virus (IBDV) in Egypt: Phylogenetic analysis, Pathogenicity and Immunogenicity of an immune-complex vaccine

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ABSTRACT

Infectious bursal disease (IBD) viruses continue to cause considerable economic losses in the Egyptian poultry industry. The purpose of this study was to investigate the molecular features of IBDV isolated in Egypt from 2021 to march 2022, and assess the pathogenicity, immunogenicity, and protection of the IBD immune-complex vaccinations (Transmune®-CEVA vaccine). Twenty-three field samples (bursa of Fabricius) were collected from broiler farms and a highly variable region encompassing VP2 gene was targeted for IBDV screening utilizing RT-PCR. Out of 23 tested farms, 19 were positive by RT-PCR.Six positive samples were chosen for viral isolation, sequence, and phylogenetic analysis. Phylogenetically, five of the strains under study belonged to the very virulent (vvIBDV) strains, with 95-98% resemblance to Giza 2008 belonging to Genogroup 3 of IBDV strain. The remaining strain were identified as a vaccination strain (Genotype 1) and matched the winter field 2512 vaccine strain by a similarity percentage of 96%. One day old commercial chicks were vaccinated (Transmune®-CEVA vaccine) then challenged with selected very virulent strain (OP056767). The Fabricius Bursa was examined grossly and histologically. Furthermore, the Bursal Body Weight Ratio and Bursa Index were computed. The transmune IBD vaccination was able to elicit a high ELISA mean titer of 3179 at 32 days of age (4 day post challenge). Moreover, the greater raised mean ELISA titers of 9264 (38day) and 9354 (42 day) post vaccination, indicating that the challenge IBDV serves as booster immunization. Beside the Efficacy of transmune®-CEVA vaccine in reducing mortality in comparison to Pathogenicity group. The bursal body weight ratio and index demonstrate that the IBD vaccine was able to indicate an inflammatory response in the bursa of fibricia, resulting in a better immunological response and the safety of the Transmune®-CEVA vaccine. **Finally**, our findings show the dual circulation of both G1 and G3 strain in poultry flocks, and the immune-complex vaccination is still effective in protecting commercial broiler chicks against dominant circulating vvIBD strains.

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INTRODUCTION

IBDV caused by a tiny, non-enveloped virus that belongs to the Birnaviridae family and has a bi-segmented ds-RNA genome **(Kibenge et al. 1988)**. Segment A of the viral genome encodes a precursor polyprotein, which is autoclaved into the proteins VP2, VP4, and VP3 ((**Müller and Becht 1982)**. The key immunogenic determinants are carried by VP2, which is the capsid protein **(Letzel et al. 2007; Schnitzler et al. 1993)**. Also responsible for antigenic site which neutralizing the antibodies **(Becht et al. 1988)**. Within VP2 a small area known as the variable domain **(Bayliss et al. 1990)**. Genomic segment B (2.8 kbp) encodes the viral RNA-dependent RNA polymerase (RdRp) VP1 **(Mundt et al. 1995)** as well as the nonstructural protein VP5 **(Garriga et al. 2007)**. Both segments have a role in virus replication and virulence **(Müller et al. 2003)**

IBDV, like any other RNA virus, has a high polymerase mutation rate, which leads to genetic evolution and the generation of new viruses with unique features that contribute to antigenic diversity and virulence modification **(Van den Berg et al. 2000)**. Virus neutralization (VN) tests revealed two IBDV serotypes **(Ashraf et al. 2006)**. Pathogenic strains are classified as mild, intermediate, intermediate plus, classical, variant, and very virulent in serotype 1. Serotype 2 strains, on the other hand, are mostly non-pathogenic and are mostly isolated from turkeys **(Van den Berg et al. 2000)**. Sequence studies between pathogenic and non-pathogenic strains revealed nucleotide alterations across the genome **(Absalón et al. 2017; Brown and Skinner, 1996)** which likely contribute to the virulence's mutagenic aspect **(Escaffre et al. 2013)**.

In 1975, IBDV was discovered in Egyptian broiler chickens for the first time **(Ayoub and Malek, 1976)**. The its first introduction of very virulent IBDV Since 1989 in vaccinated Egyptian flocks **(EL-BATRAWI, 1990)**. Currently, circulating variant IBDV strains have been identified from flocks vaccinated with IBDV vaccinations **(Abou El-Fetouh and Abdallah, 2018; El-Bagoury et al. 2018; Helal et al. 2012; Mawgod et al. 2014; Samy et al. 2020; Shehata et al. 2017)**. Despite the use of a high range of vaccinations, Egypt has been stricken by recurrent IBD episodes in the latest period **(Mohamed et al. 2014; Shehata et al. 2017; Zanaty et al. 2022)**

Despite strict sanitary precautions, the IBDV is very contagious, immunosuppressive, resistant, and it tends to persist in the environment. As a consequence, vaccination is seen as an important means of protecting young birds during their first few weeks of life **(Eterradossi et al. 2008; Faragher et al. 1972)**. IBDV infection may worsen existing infections with other infectious agents and reduce the bird's capacity to react to vaccination because the virus inhibits humoral and cellular immune responses **(Fan et al. 2020)**. Hyper immunization of breeders with inactivated vaccinations is used to reduce IBDV infection in chicks. Although passive immunity protects chickens well during their early weeks of life, lasting protection against IBD necessitates the use of live vaccines **(Müller et al. 2012)**. Mild vaccinations are harmless, while intermediate and hot vaccines are significantly more effective in the case of strong maternal antibodies or against particularly virulent strains of IBDV, although they can cause moderate to severe lesions in the Fabricius Bursa **(Camilotti et al. 2016)**.

As a result, novel vaccines that combine safety and efficacy, such as immune complex and recombinant vaccines have been produced to address these issues. The recombinant vaccine employs a viral vector to contain and produce the immunogenic protein VP2 of IBDV, causing the development of particular antibodies even in the presence of passive immunity **(Camilotti et al. 2016; Rage et al. 2020; Sze et al. 2016)**.The immune-complex vaccine, on the other hand, is novel in comparison to traditional live vaccines because the vaccine virus is coated with anti-IBD antibodies and its pathological effects are delayed for up to one week when administered to one-day-old chicks, during which the level of maternal antibodies is greatly reduced **(Camilotti et al. 2016; Shazali, 2008)**.

This study aims to investigate the molecular features of the currently circulating IBDV isolated in Egypt (2021-2022), and to characterize the pathogenicity and immunogenicity of IBD immune-complex commercial vaccines (Transmune®-CEVA vaccine) as well as the degree of protection afforded by those vaccinations in chickens challenged with a highly-virulent strain of IBDV.

MATERIAL AND METHODS

Collection and preparation of IBDV field strain:

The chicken flocks suffered from high Morbidity andmortalities with depression, watery diarrhea, ruffled feathers, and dehydration. At necropsy, the cloacal bursa is swollen, edematous, yellowish, occasionally congested. Bursal of fibricia (10 bursae/farm) were taken aseptically from 23 broiler chicken farms located in different provinces of Egypt (**Table 1**). Bursa samples were homogenized, and the supernatant was collected and filtered in accordance to **(Yovel et al. 2008)**. Following the manufacturer's instructions, RNA from the prepared samples was extracted using a QiaAmp® Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany). The RNAs were then confirmed for IBDV using AgPath-ID™ One-Step RT-PCR Reagents Kit (Applied Biosystems, USA) on the extracted RNA was performed to process the reverse transcriptase-polymerase chain reaction (RT-PCR). Forward and reverse primers were used to amplify a 620 bp included in HVR of the VP2 gene **(Metwally et al. 2009)**. ProFlex PCR System thermal cycler (Applied Biosystems, California, and USA) was used to carry out the reaction.PCR Analysis was performed by gel electrophoresis 1.5% against 100 bp Plus DNA Ladder GeneRuler™ (Fermentas).

Virus isolation and Titration

The filtrated supernatant of positive Bursa samples was injected on the chorioallantoic membrane (CAM) in embryonated SPF eggs 10-11 day old embryos According to **(Dufour-Zavala, 2008; Hirai et al. 1972)**. Then, they were incubated at 37°C with candling daily. The allantoic fluids were collected at 4-5 days post-inoculation **(Hirai et al. 1972)**, PCR testing was applied to confirm isolation **(Metwally et al. 2009)**.Then Titration of the selected dominant IBDV strain according to **(Soubies et al. 2018)**, using Specific Pathogen Free eggs (SPF). **(Van den Berg et al. 2004)**. The $EID_{50}$of local strain (EGY-IBDV-Domiedta-VV22-2022-VP2) was determined using the following formula according to **(Muench, 1938)**.

Sequence and phylogenetic analysis of VP2 gene hyper variable region

A QiaAmp purification kit (Qiagen, Germany) was used to purify the positive PCR samples. BigDye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster city, CA, USA) was used to sequence the VP2 gene. The nucleotide sequences were determined using an Applied Biosystems 3500XL Genetic Analyzer (Applied Biosystems,Foster City, CA). To edit and assemble sequences, the Bio-edit application (version 7.2.5) was used **(Hall et al. 2011)**. BLASTn was used on the NCBI website (www.ncbi.nlm.nih.gov/BLAST), to compare IBDV sequences to GenBank sequences.The sequences of the unique strains were deposited in the GenBank database (**Table 1**).

Table1. Epidemiological data and accession number of VP2 of infectious bursal disease virus isolates strains in the current study

Samples Name	Province	Collection Year	Host	Age (days)	Gene Bank Accession No.	Phenotype
EGY-IBDV-GIZA-VV21-2021	Giza	2021	Broiler	32	OK032601	Very virulent strain
EGY-IBDV-Dakahlia-VV21-2021	Dakahlia	2021	Broiler	26	OP056763	Very virulent strain
EGY-IBDV-GHARBIA-VV22-2022	Gharbia	2022	Broiler	33	OP056764	Very virulent strain
EGY-IBDV-GIZA-CL22-2022	Giza	2022	Broiler	25	OP056765	Classical
EGY-IBDV-Dakahlia-VV22-2022	Dakahlia	2022	Broiler	28	OP056766	Very virulent strain
*EGY-IBDV-Domietta-VV22-2022	Damietta	2022	Broiler	35	OP056767	Very virulent strain

*Referring to the strain used for assess the pathogenicity, immunogenicity, and protection of the IBD **immune-complex vaccinations**

The sequences under study were compared to others provided from the National Center for Biotechnology Information, including IBDV strains from different groups and vaccine strains used in Egypt. The alignment was done with the CLUSTAL-W tool and the MegAlign module of DNASTAR software (Lasergene version 7.2; DNASTAR, Madison, WI, USA **(Burland, 2000)**. The phylogenetic tree was constructed using MEGA version 6 **(Tamura et al. 2013)**, employing the maximum likelihood strategy with moderate strength and 1000 bootstrap repeats **(Kumar and Reinartz, 2016)**. The pairwise nucleotide percent identity was calculated using DNAstar software.

Experimental design for assessment of the pathogenicity, immunogenicity, and protection of transmune®-CEVA vaccine.

An Immune-complex vaccine (Transmune 2512®-CevaInc,France) was given s/c to one-day-old chicks at the hatchery. A total of 100 non-vaccinated commercial broiler chicks at one day old were used. Sera were tested individually for the presence of anti-IBD antibodies by ELISA using a commercial kit (BioChek-UK- Ltd.) according to the manufacturer's instructions, for determination of levels of IBDV antibodies in sera and the time of waning of maternal derived antibodies at various intervals (1, 7, 14, 21, 28, 32, 38, and 42 days of age).

Chicks (n = 100) were randomly sorted into three groups: GI (control negative group, n = 25), GII (challenge group, n = 50), and GIII (pathogenicity group, n = 25), all of which were raised in separate rooms in biosecurity laboratory experimental facilities. On one-day -old chicks, GII was inoculated s/c to with the Transmune®-CEVA vaccine (0.02 mL/chick). For standardization, GI and GIII were injected with 0.02 mL PBS (Phosphate Buffered Saline). At day 28 of age, GII (vaccinated group and challenged) and GIII (not vaccinated-challenged) chicks were infected with 100ul of the $10^6 EID_{50}$/ml viral inoculum via ocular route with the local field IBDV field strain (**Acc.No:OP056767**).

Data collection

On days 28, 32, 38, and 42 post-vaccinations (dpv), chickens from each experimental group were examined, slaughtered, bled by heart puncture, weighed, and necropsied for the collection and analysis of buesa of fibricia. Throughout the 14-day post-challenge (PC) monitoring period, clinical symptoms, death,

and inspection for the presence of gross lesions were all noted. Each murdered bird's bursa of fibricia and body weight (BW) were also recorded. Five separate bursae were taken at specified intervals to determine therelative Bursa Fubresia to BW ratio (B/BW ratio) $= \frac{\text{fibricia weight (g)}}{\text{Body Weight (g)}} \times 1000$ **(Raji et al. 2017; Tanimura et al. 1995)**. The bursa to body weight index (B/BW index) $=$ BB ratio of infected (or vaccinated) birds/ BB ratio of the controls **(Ng et al. 2000; Raji et al. 2017)**. Surviving birds were sacrificed humanely**.**

Histopathology

The existence of lesions in every bursa of fibricia was determined by histological investigation. Bursae were fixed in 10% buffered formalin for histopathology according to **(Banchroft et al. 1996)**, for recording histopathological lesion scoring and mean severity index. The normal physiological figure in GI chick was determined. In addition, the effect and degree of protection provided by an IBD transmune vaccination in GII, as well as the effect of an IBD field isolate challenge during a pathogenicity experiment in GIII. the lymphoid depletion score was obtained by using mathematical modeling using artificial neural networks According to the approach described by **(Morais et al. 2010)**.

RESULTS

Virus screening and isolation

Using RT-PCR, it was found that 19 of 23 field samples (83%) were positive for IBDV. The RT-PCR confirmed the presence of IBDV in the inoculated embryos. Embryos that died three dayspost-inoculation showed severe congestion; dwarfing muscle hemorrhages, and enlarged congested internal organs.

Hyper variable region phylogenetic analyses

The 620bp nucleotide and 206 amino acid sequence flank the HVR were described. Phylogenetic analyses created for HVR of the six selected IBDV sequences revealed that the sequences established two large clusters, which corresponded to the two suggested genogroups (1 and 3). Five of the sequences were grouped with the other Egyptian very virulent IBDV (vvIBDV-G3), which is often, detected worldwide **(Fig.1).** The remaining strain under study was cluster with the classic cvIBDV-G1 together with winterfield 2512sequences (acc.no: MN218126) (**Fig. 1**).

Hyper variable region mutational analysis

When the VP2 HVR of five IBDV strains was sequenced, it was determined that five strains which related to vvIBDV-G3 shared high amino acid and nucleotid similarity (94-100%) and (95-98%) respectively, with each other and to Giza 2008 strains (acc.no:EU584433.2) which represent Egyptian vvIBDV-G3 (**Table 2**). However, when compared to traditional genogroup 1 (G1) vaccine strains, (90%) identity percent was discovered (**Table 2**). The nucleotide identity percent of the classic strain **(OP056765) (Table1)** was 96 to IBDVs from G1 Winterfield 2512 (**acc.no: MN218126**) (**Table 2**). These isolates exhibited the amino acid sequence found in classical and Winterfield 2512strains **(Fig.2).** Except for the substitutions L217S and O223Pin the strain (**EGY-IBDV-GIZA-CL22-2022-VP2-OP056765**)

under research, all of the amino acid residues of the HVR (212D, 213D, 217L (loop PBC), 242V, 256V (Loop PDE), (270T and 299N) were identical to those of Winterfield 2512strains. As a result, the isolate had a higher level of amino acid similarity than the winterfield 2512 (acc.no: MN218126) (**Fig.2).**

Meanwhile, the five isolates (**OK032601, OP056763, OP056764, OP056766, and OP056767**) had amino acids characteristics identified in highly virulent IBDV strains **(Fig.2).** Furthermore, the isolatesexhibited some particular amino acid changes, including Y220 F, D213N, G254D, S315T, S317R (Loop PHI), and A321E (Loop PHI), which have been described in some Egyptian-vvIBDV strains (**Egy-Giza-2008 acc.no EU584433.2).**

In all very virulent strains, the presence of the Ssp I restriction site correlates to a substitution at residue 294 (leucine to Iisoleucine). As revealed in the amino acid alignment report, the Ssp I restriction site on VP2 has shown to be indicative of vvIBDV strains (Figure 2). The

VP2 HVR of all research isolates retains the virulence signature of serine-rich heptapeptide sequences (SWSASGS), which is proximal to (B, PHI) the major hydrophilic peak. Only the classicstrain in the current study had glutamine at position 253 rather than histidine (**Fig. 2**).

Maternal derived antibodies (MDAs) waning:

Following intervals, ELISA titers indicated the quantity of MDAs (1,7,14,21,28,32,38 and 42). MDA titers in test samples decrease by almost the same titer starting at 14Day in the GI (control group) and GII (challenge group), and rise antibodies degrading around day 28D of age. At 28 days of age, monitoring GII showed that they were susceptible, with a mean ELISA titer of 284 units for GI and 348 for GIIas the Cut off antibody titer ≥ 284.

In comparison to GI (control group) for Post-vaccination seroconversionmonitoring, GII, which was challenged at day 28 of life, showed a significant increase in IBDV antibody titer from day 32 and continued to increase until the completion of the trial at day 42**(Fig.3**).

Efficacy of Transmune®-CEVA vaccine:

Challenge with vvIBDV at 28 days of age was used to test the effectiveness of the Transmune®-CEVA vaccine. At 14-day post-challenge, 100 percent vaccine protection, with no mortalities being documented **(Table 3).**

In GIII 10^6 EID_{50}/chicken were given (not vaccinated-challenged), during the 10 day PI observation period, six chicks (28%) died on the third and fourth days PI. Ruffled feathers, white diarrhea, and shaking were among the clinical indications seen in the chicks. Enlarged swellingbursa were the gross lesions of diseased chicks.

Bursal to body weight ratios (B: BR) and Bursal index (BI):

The comparison between B:BR of GIand GII were statistical analysis, there were significant (P.value≤0.05) higher differences in GII than in GI at 14, 21, 28 and 32 days of age and lower difference at 7 days of age **(Fig 4).**Bursal index (BI), based on criteria of **(Lucio and Hitchner, 1979)** were 0.55 ،1.04 , 1.18 , 3.34 And 1.65. BI lower than 0.7 were considered to have bursal atrophy **(Li et al. 2016)**

Table 2. The Nucleotide and Amino acids similarity between infections bursal disease virus isolates under study and other Egyptian and representative reference strains obtained from NCBI.

Sequence Identity of Nucleotide																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
1	IBDV-GIZA2008-VP2		93	93	93	94	75	81%	79 %	93%	93%	97%	97%	97 %	93%	93 %	97%	95%	96%	90%	97 %	98 %	1
2	IBDV-Blue-VP2-gene	95		100	96	98	76	87%	83 %	97%	95%	94%	93%	95 %	99%	99 %	92%	91%	92%	96%	93 %	93 %	2
3	IBDV-2512-VP2-VP2-gene	95	99		96	98	76	87%	83 %	96%	95%	94%	93%	95 %	99%	99 %	90%	90%	90%	95%	89 %	88 %	3
4	IBDV-228E-VP2	95	96	96		96	77	84	82 %	97%	94%	94%	93%	94 %	95%	96 %	92%	91%	92%	93%	92 %	92 %	4
5	IBDV-Bursavac-viral-protein-2	93	97	96	96		76	85	83	97%	95%	94%	93%	95 %	97%	97 %	93%	91%	92%	94%	93 %	93 %	5
6	IBDV-vaccine-Poulvac-Bursa-F-VP2	78	80	80	80	80		88%	87 %	76%	75%	76%	76%	76 %	76%	77 %	75%	74%	75%	75%	75 %	75 %	6
7	IBDV-vaccine-Cevac-IBD-L-VP2	82	87	87	83	84	92		95 %	84%	82%	82%	81%	82 %	87%	87 %	80%	80%	80%	85%	81 %	80 %	7
8	IBDV-vaccine-Bursine-Plus-VP2	79	80	80	80	80	88	92%		82%	82%	80%	80%	81 %	82%	83 %	79%	78%	79%	81%	80 %	79 %	8
9	IBDV-D78-VP2-gene	93	96	96	96	96	80	83%	80 %		95%	94%	93%	94 %	96%	96 %	92%	91%	91%	93%	92 %	92 %	9
10	IBDV-strain-E-VP2	91	91	90	92	90	74	78%	78 %	93%		93%	92%	93 %	94%	94 %	91%	90%	91%	91%	92 %	92 %	10

1 1	OK092295-EGY-VVIBDV-GIZA83-2021-VP2	98																					11
1 2	OK092297-EGY-CK-IBDV-MONOFIA85-2021-VP2	10 0	99 5	99 5	99 5	93	82 %	79%	93 %	91 %	98%												12
1 3	OK092298-EGY-CK-VVIBDV-DAKH87-2021-VP2	99	99 6	99 5	99 5	94	83 %	80%	93 %	90 %	98%	99 %											13
1 4	OK092296EGY-CK-IBDV-GIZA84-2021-VP2	93	99 8	99 8	99 5	95	86 %	80%	95 %	89 %	94%	93 %	94 %										14
1 5	OK092299-EGY-CK-IBDV-DAKH88-2021-VP2	95	99 8	99 8	99 6	96	86 %	81%	96 %	90 %	95%	95 %	95 %	98 %									15
1 6	OK032601-EGY-IBDV-GIZA-VV21-2021-VP2	99	99 4	99 0	99 3	92	82 %	79%	92 %	90 %	98%	99 %	98 %	93 %	94 %								16
1 7	EGY-IBDV-Dakahlia-VV21-2021-VP2	94	99 1	99 0	99 0	89	80 %	77%	89 %	87 %	93%	94 %	93 %	90 %	91 %	95 %							17
1 8	EGY-IBDV-GHARBIA-VV22-2022-VP2	96	99 2	99 2	99 1	90	80 %	77%	90 %	88 %	95%	96 %	95 %	91 %	92 %	97 %	98%						18
1 9	EGY-IBDV-GIZA-CL22-2022-VP2	89	99 3	99 6	99 0	90	83 %	78%	89 %	84 %	89%	89 %	90 %	93 %	93 %	89 %	86%	87%					19
2 0	* EGY-IBDV-Damietta -VV22-2022-VP2	99	99 5	98 9	99 4	93	82 %	79%	92 %	90 %	98%	99 %	99 %	93 %	94 %	98 %	93%	95%	89 %				20
2 1	EGY-IBDV-Dakahlia-VV22-2022-VP2	10 0	99 5	99 0	99 5	93	82 %	79%	93 %	91 %	98%	10 0%	99 %	93 %	95 %	99 %	94%	96%	89 %	99 %			21
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	

Sequence Identity of Amino acids

*Referring to the strain used for assess the pathogenicity, immunogenicity, and protection of the IBD immune-complex vaccination

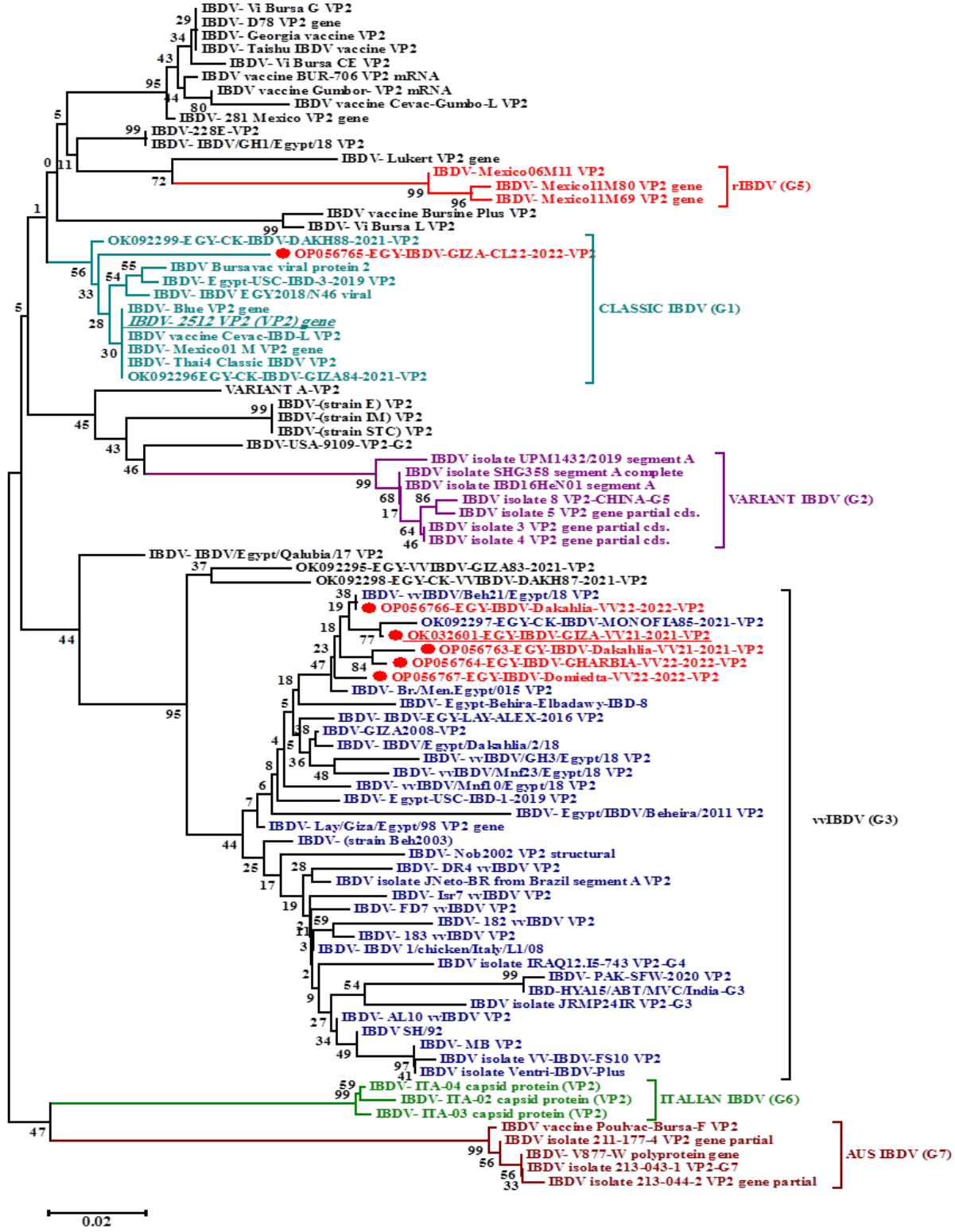

Fig. 1: Phylogenetic relationships of IBDV in Egypt. Phylogenetic tree based on VP2 genes of virus Isolated in Egypt during 2021-2022 and reference isolates from Gen Bank. A red rhomboid indicates isolates sequenced specifically for this study. The phylogenetic trees were generated using MEGA version7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates).

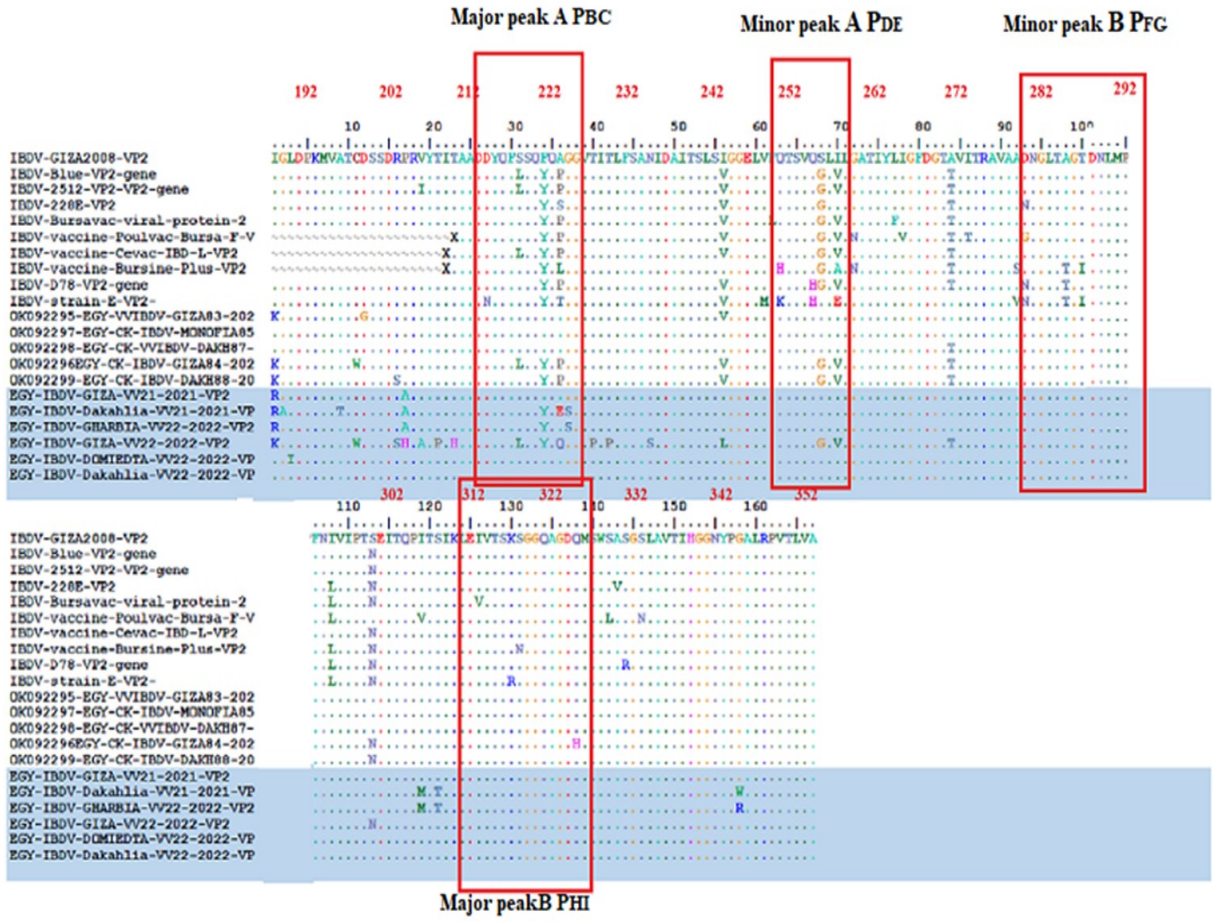

Fig.2:Amino acids alignment report of partial sequence of VP2 gene of IBDV and other Egyptian and representative vaccine strains, showing the Hypervariable region mutations of research isolates

Table 3. Efficacy of transmune IBD vaccinated broiler chicks

ID	No. of birds	Group Name	Vaccination/SC at day one of age	Challenge at day 28 of age	Sign/ No	Mortality	Protection %
GI	25	Control negative group	Phosphate Buffered Saline	No challenged	0/25	0/25	ND
GII	50	Vaccinated challenged	Transmune IBD vaccine	100ul of the 10^6 EID_{50}/ml	5/50	0/50	100
GIII	25	Pathogenicity group	Phosphate Buffered Saline	With local field IBDVfield strain (Acc. No.OP056767)	7/25	7/25	28

Challenge: SC at 1 day of age and subsequent challenges with vvIBDV at 28 days of age showing no mortality in all challenged chickens. No= number, ND= not done

Bursal histopathology:
Non vaccinated control (GI) bursae showed normal architecture of bursal follicles within the cortical and medullary regions with no evidence of histological changes at all intervals examined with 0.00 histological scores. While GII- vaccinated challenged bursae at the day 28showed histological changes that consisted of diffuse follicular atrophy, cortical and medullar lymphocyte depletion, macrophage infiltration and epithelial with average histological scores of ranged 0.07 to 3.5 with no evidence of follicular necrosis in folding**.** At 32 and 38 days (PV), showed histological changes demonstrated in **(fig.5).**

GIII- Non vaccinated challenged bursae :three bursae of dead chicks during patho typing experiment at 32 PV (which induced 30% mortalities in challenge experiment and 3 deaths occurred during 3rd and 4th days pc) revealed characteristic IBD gross and histologic lesions gross lesions showed typical IBD lesions included hemorrhagic and enlarged Bursae with perio-bursal gelatinous exudates, petechial hemorrhages on mucosal fold and pelica, petechial hemorrhages on breast, thigh muscles and proventriculus, histological lesions consisting of follicular atrophy, medullary lymphocytic depletion and interstitial oedemainfilteration with macrophages, heterophilles and epithelial in folding and interstitial edema with maximum averages of 4.0 to 5.0 histological scores**.** At 38 days (PV), showed histological changes that consisted ofsever follicular atrophy associated with medullary cystic formation and interstitial edema infilterated with macrophages and heterophiles and epithelial folding **(fig. 6).**

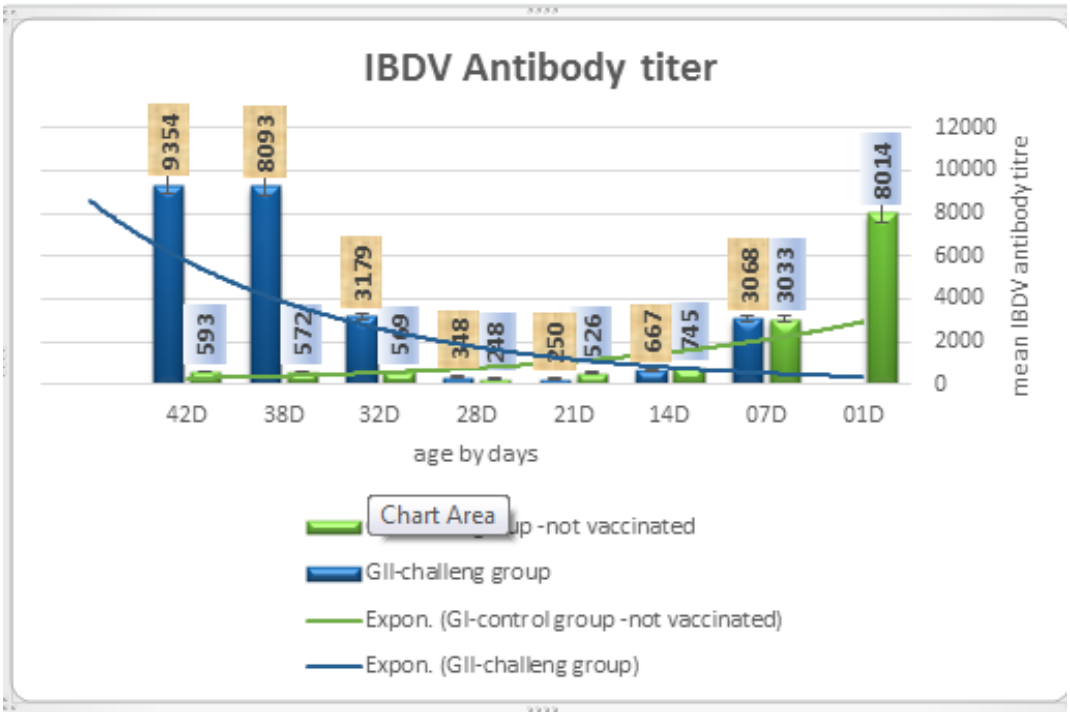

Fig 3. showing the comparison of maternally derived antibodies (MDAs) in commercial broiler chicks in GI (control) and GII (vaccinated -challenged) as detected by ELISA (Bio Check).The levels of maternal Infectious Bursal Disease antibodies in commercial broiler chicks at various ages post hatch. MDA titers decline in test samples beginning from 14D in the GI and GII by nearly similar titer, and increase antibodies degrading around day 28D of age. Monitoring GII, which challenged at day 28 of life with 100 mL of the 10^6 EID 50/mL viral inoculum by ocular route showing great increase in Infectious Bursal Disease antibodies from day 32 and still increase until the end of experiment at day 42 in comparison to GI (control group).

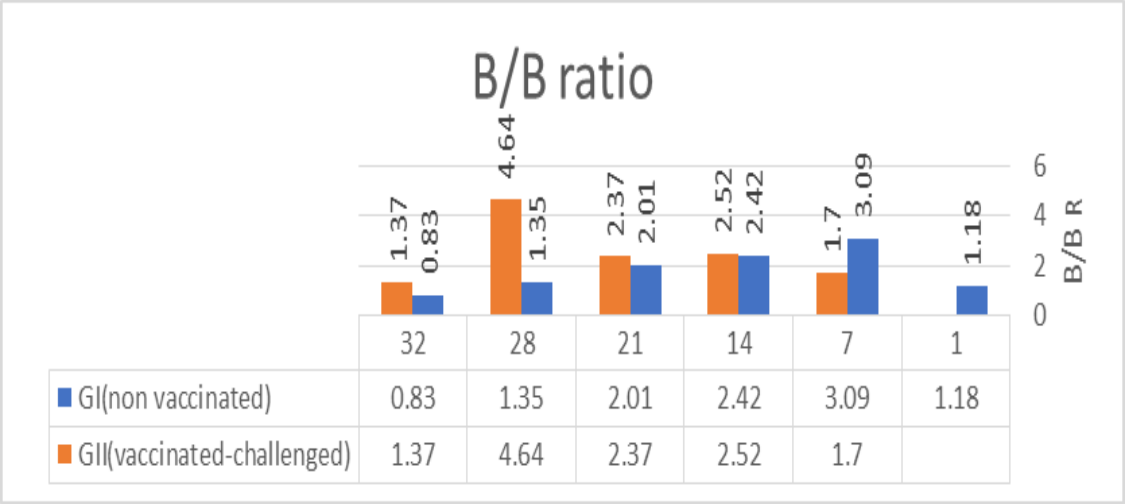

Fig.4. Comparison between bursal to body weight ratios (B: BR) ofGI (Control non vaccinated).and GII (transmuneIBD vaccinated –challenged) chicks in experimental chickens at different time points after hatchery. Chickens were vaccinated at one days of age, and challenged at day 28 of age. Results are expressed as mean ± SEM (n=3 chickens/group) and compared by using Student t test (Table 5). *there were significant (P≤0.05) higher differences in GII than in GI controls at 14, 21, 28 and 32 days of age and lower difference at 7 days of age.

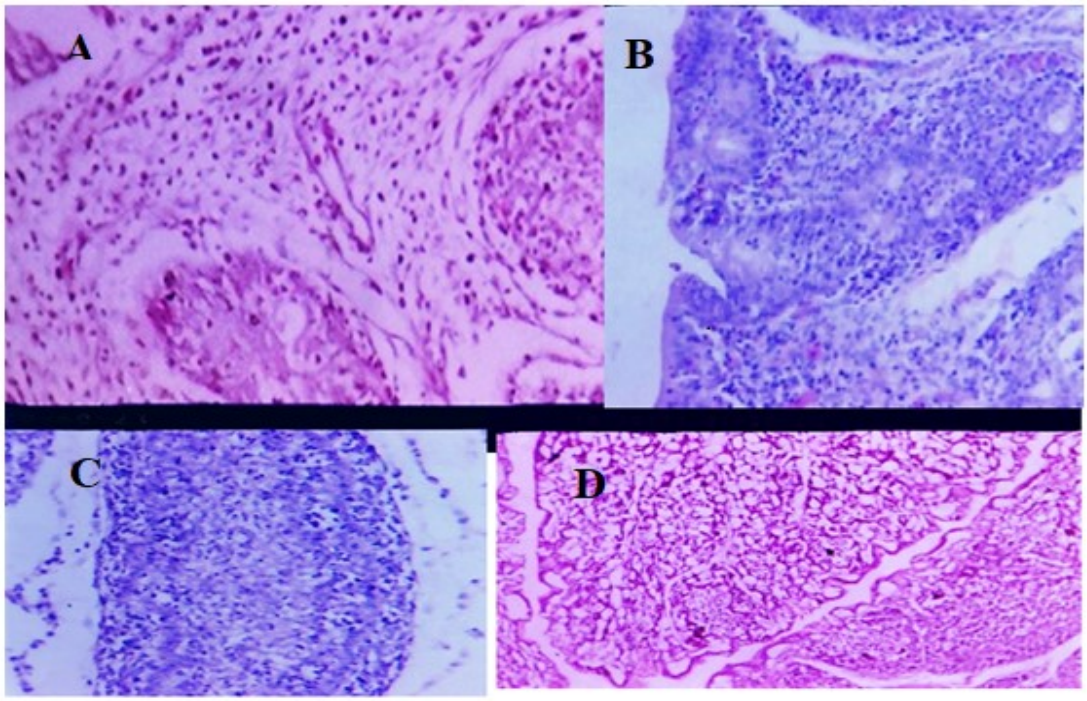

Fig.5. BF of (GII)Transmune vaccinated/challenged broiler IBDV at 4 pc at 32 D and 38 day of age showing (A) moderate inflammatory cell infiltration (H&E,X40), (B)heterophilic infiltration (H&E,X250) heterophilic infiltration (H&E,X250),(C) medullary lymphocytic depletion arrows,(D) follicular lymphoid depletion (H&E,X100).

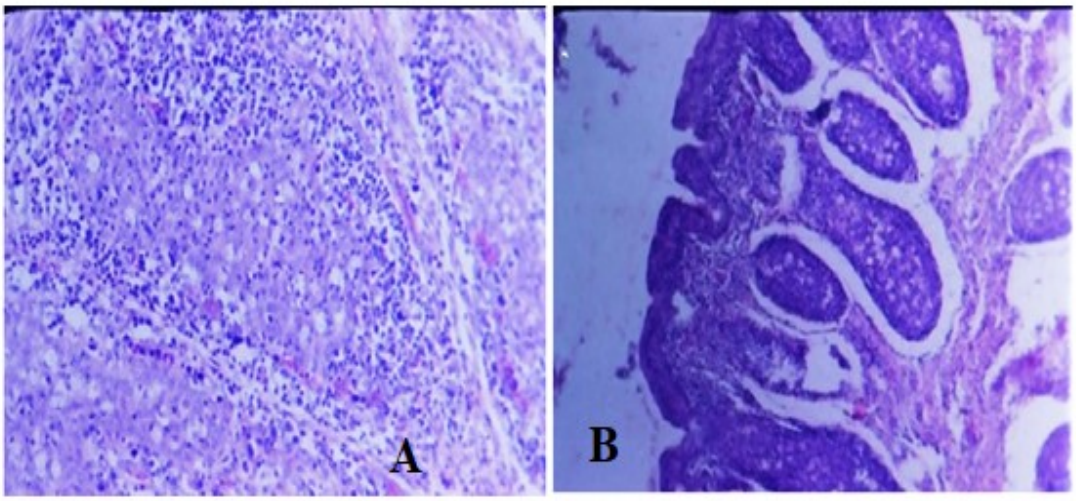

Fig.6.BF of (GIII) non vaccinated challenged broiler with IBDV at 38 days of age, bursa of dead ones During 10 days observation period PC showing cortical and medullary sever necrosis associated with interstitial oedema with macrophages and heterophills (H&E,X250),(B) sever follicular atrophy associated with medullary cystic formation and interstitial edema infilterated with macrophages and heterophiles and epithelial folding (H&E,X100

DISCUSSION

Infectious bursal disease viruses continue to cause direct and indirect economic losses in the chicken industry. Despite extensive and repeated administrations of multiple IBD vaccines, the emergence of variant and/or vvIBDV in vaccinated Egyptian flocks has raised concerns regarding IBDV vaccine regulation **(Manal et al. 2019)**. In every control method, incorrect vaccine selection is a challenge **(He et al. 2019)**. Despite the fact that vvIBDV has been known since 1989, commercial poultry growers have made no substantial alterations to their immunization schedules **(El-Batrawi and El-Kady, 1990; Hassan et al. 2004; Mawgod et al. 2014; Samy et al. 2020; Shehata et al. 2017)**.

According to a phylogenetic study, IBDV viruses are categorized into seven different genogroups (G1-G7), **(Michel and Jackwood, 2017)**. Based on HVR nucleotide sequences reported here, the strain (**OP056765**) were phylogenetically grouped with IBD viruses of genogroup 1 (G1-classical IBDVs) (**Fig.1**). Based on the mutational study and the existence of specific amino acid residues in the HVR, this strain might have been generated from vaccine strains already authorized to be used in Egyptian poultry farms **(Zanaty et al. 2022)**.

By genetic analysis of the Egyptian field vvIBDV strain circulating in 2021-2022 we identify similarities between this strain and other very virulent field strain sequences (**table. 2**). The nucleotide and amino acid sequences of the VP2 HVR from each of the five Egyptian isolates showed the greatest homology to the relevant classical and vvIBDV strains. In comparison, the nucleotide sequence analysis of the study vvIBDV isolates and the current Egyptian vvBDV strains indicated varied identities, suggesting that the virus in circulation has genetic diversity, as antigenic alterations have occurred in more recent IBDV viruses. Given that RNA viruses have a high mutation rate due to the RNA polymerase's low proofreading ability, genetic diversity is common **(Schneider and Roossinck, 2001)**.

We analysed vaccinal (Winterfield2512) strain and field strains, we determined that there are little amino acid differences, with amino acid sequence similarity between challenge and other Egyptian strains ranging from 92 to 95 percent. As a result, the selected strain (EGY-IBDV-Domiedta-VV22-2022 Acc, No

OP056767) was representing dominant circulating strains in Egypt during 2021-2022 (**Fig. 1**). The identity percent between the vaccinal strain (Winterfield 2512 acc.no: **MN218126**) and the Egyptian field strain ranged from 89% and 96% to the Variant and classic IBDV strain respectively (**Table. 2**), indicating that the vaccinal straingenetically may possibly protect flocks **(Rautenschlein et al. 2005)**.

The P domain is one of the three domains of the VP2 protein and is composed of four sub domains: P_{BC} loop structures, P_{DE}, P_{FG}, and P_{HI}. all of which may be seen on the virion's surface **(Coulibaly et al. 2005)**.

Present strains (**OK032601, OP056763, OP056764, OP056766, and OP056767**) indicated the amino acid positions (222A, 242I, 256I, 284A, 294I, and 299S) (**Fig. 2**) that are characteristic of vvIBDV **(Van Loon et al., 2002)**, confirming that these five strains belong to genogroup-3. As it is located at the tip of the PBC loop, the amino acid at position 222 is essential. A mutation in the amino acid sequence at position 222 might lead to vaccination failure **(Brown et al. 1994)**. Most of vvIBDV isolated had no substitutions at position (222) displaying A (Alanin) amino acid in this position.

In all vvIBDV strains under study, a unique SspI site on VP2 (**Fig 2**) according to **(Jackwood et al. 2011)**. As a result, this SspI site has been utilized as a genetic marker to predict a highly virulent phenotype, which must be verified *in vivo*. As revealed in the amino acid alignment report, the Ssp I restriction site on VP2 was found in all of the vvIBDV isolates shown to be indicative of vvIBDV strains but not in the classic vaccine strains (**Fig 2**). However, not all vvIBDV strains have this identifier, and some non-vvIBDV strains have been shown to carry the SspI marker **(Sapats and Ignjatovic, 2002)**.

All research isolates of VP2 HVR preserve the virulence characteristic of serine-rich heptapeptide sequences (SWSASGS), which are located around the primary hydrophilic peak (B, PHI). Furthermore, the strains containing glutamine at position 253 are more pathogenic than those with histidine at position 253. Recently, there has been a lot of focus on amino acids at position 253, where histidine or glutamine might be detected. Due to the prolonged vaccination campaigns conducted in the field using live attenuated viruses, the viruses used may mutate and hence lose their pathogenic potential **(Zierenberg et al. 2001)**.

Because MDAs can neutralise standard immunizations, the immune-complex vaccine (CEVAC® Transmune IBD) combines the Winterfield 2512(**acc.no: MN218126**) strain of IBDV live virus with IBD immunoglobulins. These immunoglobulins prevent MDAs from neutralising the vaccination **(Kabajani, 2018)**. The antigen-antibody complexes linked to follicular dendritic cells by vaccine do not last forever **(Ertl and Xiang, 1996)**. MDA levels drop with time as a result of catabolism. As a consequence, the vaccination virus will be free of antigen-antibody complexes, allowing it to create protective antibodies against gumboro disease. On farms, broilers were protected against IBD for the rest of their lives without needing to be revaccinated **(García et al. 2021)**.

Commercial broiler chicks were shown to have antibodies obtained from their mothers. According to the data presented in this research, enzyme-linked immunosorbent of 1: 2000, 1:4000, or 1: 8000 was related with low, medium, or high IBDV titers, respectively **(Kreider, 1991)**, This categorization was utilized to classify 1-day-of-age titers throughout our experiment to observe MDA's titer. As a result, the chicks in our trials (GMT=**7569**) exhibited a high degree of IBDV passive immunity **(De Herdt et al. 2000; Haddad et al. 1997a)** (**fig.3**). MDAs dropped as individuals became older, eventually becoming negative and inconsequential after 28 days **(De Herdt et al. 2000)**. The mean ELISA titer for GI was 248 ELISA units (cutoff titer 284 was interpreted as negative by the Manufacturer Company); these data are compatible with **(Iván et al. 2005)**.

In the GII-vaccinated-challenged group, the outcomes of such changing MDA levels are

demonstrated in (**Fig.3**). MDAs mean titer were observed at weekly intervals of 3068, 667, and 250 ELISA units at 7, 14, and 21 days of life, respectively. At 28 days of age, the ELISA mean titer in the vaccinated group reached 348, followed by fast sharply increasing titers of 3179, 8093, and 9354 at 32, 38, and 42 days of age, respectively. When compared to the unvaccinated control group (GI) (**fig 3**), this implies that the IBD challenge virus may elicit a high serological immune response, and that the challenge might be used to supplement vaccination **(Sajid and Mohsin Gilani, 2021)**.

Rather than focusing on a post-vaccination serological reaction, the efficiency of immunization was assessed using a challenge 28 days after immunization, which is the most vulnerable and susceptible age for IBD infection. found that trasmune IBD immunization gave hundred percent protection against clinical signs and mortalities following challenge ages **(Gómez et al. 2018)**.

In order to evaluate the severity and pathotyping of the IBDV, 106 EID_{50}/chick SC were inoculated at day 28 of life in commercial unvaccinated broilers (GIII), which showed a 28% death rate during a 14-day observation period (pi) (**Table 3**). Based on the evidence supplied **(Eterradossi and Saif, 2013; Hassan et al. 2002)**, this data with significant mortality in broilers with IBD maternal antibodies might possibly characterise the IBDV as vvIBDV, where vvIBDV causes substantial mortality in meat type hens of up to 20-30%. Death usually occurs on the second and third days following infection, peaks on the fourth day, and then rapidly diminishes **(Van den Berg et al. 2000)**.

The GII Bursa to Body weight ratio (B: B-R) is larger than the GI at weekly intervals and has a decreased difference at 7 days of age when comparing the B:BR of Group I (non-vaccinated control) and Group II (vaccinated control) at weekly intervals (**Fig 4**). This increased B:BR in vaccinates is later linked to histopathologic findings, which are explained by infiltration of inflammatory cells (macrophages and heterophils) in the fibricia bursa, implying that the larger the bursa post-vaccination, the better the immune response in the vaccinated challenged group **(Eterradossi and Saif, 2013; Kebede et al. 2021)**. Bursal Index less than 0.7 was used to indicate bursal atrophy **(Xu et al. 2020)**. The bursal to body weight index (BI) only showed a significant and temporary index value of 0.55 seven days post vaccination, this finding may indicate that the IBD vaccine virus invaded the bursa of fibricia in some of the individual birds with very low MDAs of (919) and 1 ELISA units at 1 and 7 days of age**) (Perozo et al. 2009; Rosales et al. 1989; Xu et al. 2020)**, causing temporary damage to the bursa of fibricia, followed by rapid regression **(Aricibasi, 2010)**. Thus, indicate the safety of Transmune®-CEVA vaccine.

This study investigated the histological features of the bursa of fibricia in challenged and vaccinated broilers who were challenged at 28 days of age and examined 4 days following IBDV exposure**.** Gross bursal lesions with minimal histological abnormalities consisting of moderate heterophil infiltrations were seen in pathogenicity testing of (GI) unchallenged controls. The GII (vaccinated-challenged group) had cortical and medullary changes, lymphoid depletion, and follicular atrophy in their bursae. Low to moderate quantities of inflammatory cells, predominantly macrophages were found **(fig. 5).** These changes were similar to those reported by **(Haddad et al. 1997a; Singh et al. 2015)**.

Bursal changes were observed in non-vaccinated challenged (GIII) chickens, which were linked to a 28% mortality rate following challenge. histologically, the bursae examined exhibited changes consistent with the IBDV virus **(fig 6).** The severity of widespread follicular necrosis and cystic formation, follicular atrophy, medullary lymphocytic depletion, and interstitial edema, infiltration with macrophages, heterophiles, and epithelial infoldings **(Myint et al. 2021; Tsukamoto et al. 1995)**, were all rated on a scale of 0.5 **(Haddad et al. 1997b)**.

In general, we concluded the dual circulation

of both Genotype1 and Genotype 3 of infectious bursal disease virus in Egypt as recently mention before. Also, this study clearly suggest that the transmune IBD vaccination is protective in commercial broiler chicks at one day of age. The vaccine is similarly effective when given to broilers with varied quantities of IBDV-MDAs at 1 day of age, with a single injection which is a cost-effective strategy to avoid the problem of unexpected levels of maternal IBDV antibodies, which interfere with standard live vaccinations.

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