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MOLECULAR ANALYSIS OF FIELD INFECTIOUS BURSAL DISEASE VIRUS ISOLATED IN SULAIMANI/IRAQ

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ABSTRACT

Infectious Bursal Disease (IBD) is also known as Gumboro disease, where the illness was initially identified. It is an extremely transmissible immune suppressor virus that has a significant financial impact on the poultry sector around the world resulting in increased vulnerability to various infections, adding to its negative effects on the efficacy of vaccines. The very virulent infectious bursal disease (vvIBDV) strains that are extremely virulent and cause a large amount of fatality in hens have emerged in recent years. The present study used reverse transcription polymerase chain reaction (RT-PCR) followed by sequencing to detect and molecularly characterize the VP1 and *VP2* genes of IBDV. A phylogenetic study of the partial *VP2* gene revealed that three field isolates belonged to Genogroup 3 and clustering Poland, Hong Kong, and Iran, with the highest identities of 98.32% and 98%, respectively. Based on an analysis of the amino acid sequence deduced from the *VP2* gene at positions 242I, 253Q, 256I, 272I, 279D, 284A, 294I, 299S, and 330S, three IBDVs were shown to be very virulent strains, however, amino acid analysis of the VP1 in these two isolates showed that one of them had the distinctive vvIBDV TDN amino acid triplet, while the other isolates had a non-vIBDV HEG amino acid triplet at positions 145/146/147, respectively. The VP2 protein sequence of the circulatory vvIBDV genotype showed heterogeneity of a few amino acid substitutions within the hypervariable region with the vaccine strains ((Bursin, Cevac, and D78) that are commonly used in the vaccination program in Iraq. Conclusion: The current investigation might document the detection of highly virulent IBD virus from local farms during the outbreaks that emerged in the Sulaimani province. These data suggest that the current vaccination failure may be related to differences in the *VP2* protein at HVR of the regionally circulating IBDV strain.

Keywords: Viral evolution, viral Reassortment, *VP2*, DNA analysis.

INTRODUCTION

The severe and contagious immunosuppressive viral infectious bursal disease (IBD), which affects young chicks, has forced the poultry industry to incur significant financial losses (Ebrahimi *et al*., 2013; Mohammed *et al*., 2014; Michel *et al*., 2017). The IBD virus is a member of the *Birnaviridae* family that is most widely spread and constantly circulating, and this family includes viruses that are bipartite, non-enveloped with double stranded RNA

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genome (dsRNA) (Delgui *et al*., 2009; Jackwood *et al*., 2011; Zhang *et al*., 2022). The viral nucleic acid is subjected to genomic recombination, re-assortment of genome segments, and genetic mutations that may increase virulence and alter antigenicity, and decreasing the efficacy of vaccinations (Ahmed, 2020; Hou *et al*., 2022). These viruses can be classified into four main patho/phenotypes based on the virus's antigenicity and virulence, which were the traditional criteria for classifying IBDVs. They included "classical" (cIBDV), "attenuated" (aIBDV), "American variety" (varIBDV), and very virulent IBDV (vvIBDV) (Van den Berg *et al*., 2004; Jackwood and Sommer-Wagner, 2007). The IBDV *VP2* gene contains1356 nucleotides which encodes 452 amino acids, and its HVR has key epitope residues located in hydrophilic fields that interact closely with host immune systems and cause distinct genetic differences between genotypes (Le *et al*., 2019; Islam *et al*., 2021). The hypervariable region of the *VP2* proteins of IBDVs contains a number of important amino acids that are genetically and antigenically different, and it has been demonstrated that alterations in a few of these residues can modify the virus pathogenicity, immunogenicity, and genotyping/ genogrouping categorization. Several distinct amino acid residues within the HVR have been identified as recessive mutations and therefore are widely used as labels to distinguish between genotypes (Pikuła and Lisowska, 2022). Segments A and B make up both halves of the IBV genome: section A (3.4 kb) in addition to section B (2.8 kb). The two PP2 (48 kDa) and PP3 (32–35 kDa) capsid proteins, the viral protease PP4 (24 kDa) and the nonstructural protein VP5 (17–21 kDa) are encoded by the larger segment A, whereas VP1 is encoded by the smaller segment B (90 kDa).

The *VP2* amino acid (aa) locations from 206 to 350 correspond to significant conformational and neutralizing antigenic areas named the hyper-variable region (HVR) (Dačić *et al*., 2018), which has four hydrophilic regions, including aa 210-225 (A Peak), 247-254 (Minor Peak 1), 281-292 (Minor Peak 2), and 312-324 (B Peak) (Legnardi *et al*., 2022). The IBD virus has two different serotypes: pathogenic serotype-1 and non-pathogenic serotype 2. Chickens are infected with serotype 1, but serotype 2 is inherently virulent. Serotype 2 antibodies are more frequently found in turkeys than in chickens or ducks (Letzel *et al*., 2007; Yilmaz *et al*., 2019). As the virus is extremely infectious and highly durable to disinfectants and heat deactivation, elimination of IBD viruses on the infected farms is not feasible (Hamza *et al*., 2022; Samad *et al*., 2022). The IBDV predominantly affects chickens, but it could also infect other wildfowls including ducks, pheasants, quails, guinea fowls, and oysters without causing any visible signs of illness (Mahgoub, 2012). Detection of IBDs requires the history of the flock, clinical symptoms and presence of injuries (Franciosini, 2022). Clinical symptoms and postmortem results of sick birds can help to detect IBD, but laboratory testing is required to confirm it (Banda, 2002). Hens under three weeks old do not have clinical symptoms of the illness, whereas birds older than three weeks do exhibit clinical signs (Kegne and Chanie, 2014). The RT-PCR followed by nucleotide sequencing is widely used to further characterize the IBDV strains (Corley *et al*., 2022). The vaccine and vaccination programs differ greatly depending on various local factors, such as type of production, biological security levels, local diseases, maternal antibodies status, vaccine availability, costs, and potential losses (Getachew and Fesseha, 2020; Saidi *et al*., 2020). Despite the existence of IBDV vaccines in Iraq and utilizing them by poultry farms, the release of new IBDV strains could threaten the global chicken industry and result in considerable financial losses. It is crucial to identify genetic evolution and the severity differences in the virus variants to reduce the financial burden of the particularly harmful serotype. Some studies confirm that a

comprehensive molecular analysis of both genome segments is essential for accurately identifying genetic reassortment in genome segment B and amino acid substitutions in genome segment A, occurrences commonly observed in vvIBDV (Wang *et al*., 2019). In Iraq, systemic genetic grouping is needed to classify all IBDV strains collected periodically, especially now many modified and reassigned variants exist and are currently distributed across the country. An improved prevention and control strategy for the five decades since the Gumboro illness was first discovered in Iraq will be built on extensive knowledge of Iraq's IBDV genetic groups. This study focused on analyzing mutations located within the hyper-variable region of the VP2 protein and determining the triplet amino acid positions in VP1. The objective of this study was to identify and characterize the IBDV strains present in both Iraqi commercial and backyard chicken populations. Additionally, the study aimed to determine whether the identified vvIBDVs were true vv strains or if they were re-assorted vvIBDVs.

MATERIALS AND METHODS

Samples Preparation

Tissue samples (liver and bursa of Fabricius) were collected from five clinically infected broiler chickens aged 18 to 23 days and those who had received vaccinations and medication, along with one suspected backyard chicken in Sulaimani province. The samples were obtained throughout March to June of 2018 from different districts in Sulaimani province (Figure 1). The specimens were delivered directly to the veterinary diagnostic laboratory in Sulaimani, and then the total RNA extraction was done and maintained at -20°C.

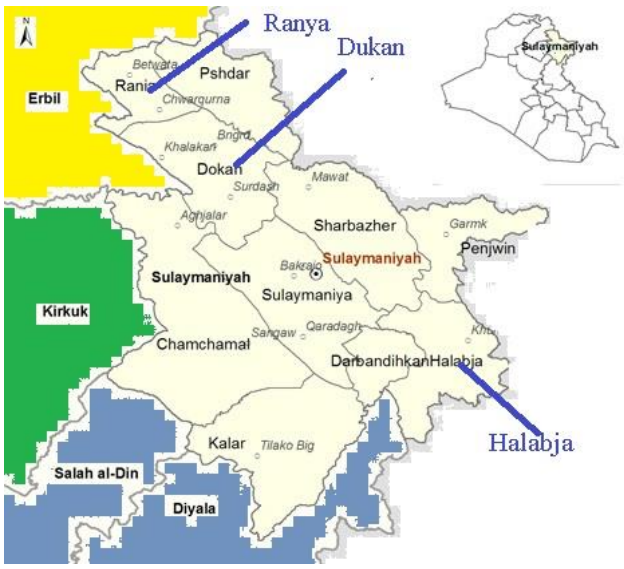

Figure 1: The districts of Ranya, Dukan and Halabja were chosen for the current study, and the blue arrows represent the geographic regions where the samples have been obtained.

RNA Extraction

Following the manufacturer's instructions, the total IBDV RNA was isolated from the homogenized liver and bursa of chickens in each flock using the tissue extraction kit (Genaid mini kit, Republic of Korea).

Reverse Transcriptase-Polymerase Chain Reaction

The RNA was used to amplify IBDV gene fragments from segment A and segment B. A previously reported primer set f-P2 (TCACCG TCC TCA GCT TAC), and TCA R-P2 (TCA GGA TTT GGG ATC AGC) was Utilized to amplify a 643-bp segment

from the highly variable region (nt 587–1229) of the VP2 gene in segment A (Thompson *et al*., 1994), however, to amplify a 1051-bp segment (nucleotides 319–1369) from the VP1 gene in segment B, we employed the primer set B-Univ-F: 5´-AAT GAG GAG TAT GAG ACC GA-3´and B-Univ-R: 5´-CCT TCT CTA GGT CAA TTG AGT ACC-3) (Kumar *et al*., 2018).

The RT-PCR reaction was performed in a 0.2 mL PCR tube, and included: 10 µL of SuprimeScript Pre-mix (2x) (Genetbio, Republic Korea), 5 µL of total RNA, a forward primer of 1 µL (10 pmol), a reverse primer of 1 µL (10 pmol), and 5 µL of ultra-pure water, and the total volume of the reaction was 20 µL. The thermal cycler employed for amplification was from (Hercuvan Lab Systems, Cambridge, UK). The PCR procedure consisted of the following steps: cDNA synthesis at 50 °C for 30 minutes, followed by an initial denaturation step at 95 °C for 10 minutes. This was succeeded by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 57 °C for 30 seconds, extension at 72 °C for 45 seconds, and a final extension at 72 °C for 5 minutes. The PCR program for amplifying both target fragments is similar, differing only in the extension step duration: 45 seconds for primer VP2 and 1 minute for primer VP1.

Agarose Gel Electrophoresis

Trisacetate-EDTA (TAE) 1x buffer was used for preparing 1% agarose gel and stain with 4 µl of safe dyes (EURx, Banino, Poland). 10 µl PCR products loading gel and run in electrophoresis at 60 minutes in 120 volts. The result was visualized by a UV transilluminator and photographed (UVETIC, UK). The amplicon sizes were estimated according to the migration pattern of a 100 bp DNA ladder.

Nucleotides Sequencing and Registration

In this research, three field partial nucleotide sequences of the *VP2* gene and one sequence of VP1 were determined by (Macrogen co, Republic Korea) Then, these sequences were registered under certain accession numbers within the GenBank databases, which belong to the National Center for Biotechnology Information (NCBI). Field strains and representative strains of GenBank's *VP2* and VP1 genes were used for genetic analysis to assess and verify the evolutionary lineage of field samples.

Sequences analysis

Clustal W was utilized to align nucleotide sequences, modify them, and predict amino acids of the small portion of the *VP2* and *VP1* protein (Kumar *et al*., 2018). The partially amplified *VP2 and VP1* genes sequences of three IBDV and isolates were used to predict and compare their amino acids. The neighbor-joining approach was employed to create the phylogenetic tree in the MEGA-X software framework, with 1000 bootstrapping replications (Islam *et al*., 2021).

RESULTS

RT-PCR Results

Three out of the six specimens examined by RT-PCR were IBDV positive, where the agarose gel showed bands of approximately 643 bp that corresponded to the partially amplified IBDV *VP2* gene (Figure 2).

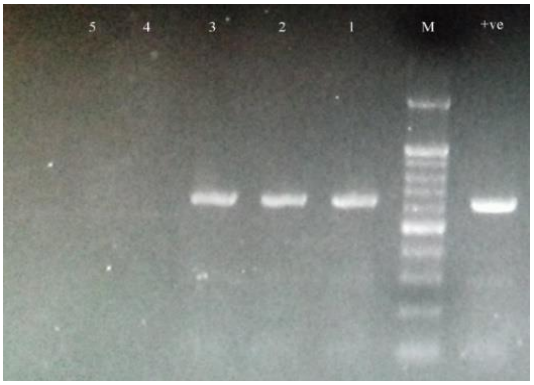

Figure 2: Agarose gel showing amplification of the partial IBDV VP-2 gene (643 bp). Lane M = DNA marker 100 bp, Lane 1, 2, and 3 positive sample. Lane 4, and 5 negative samples. Lane +ve Positive control.

Sequencing Results

The nucleotide sequences of three hvVP2 samples and two VP1 samples were established. The GenBank accession numbers for segment A are IBD/SK/18 (MH676059), IBD/SK2/18 (MH676060), and IBB/SK3/18 (MH676061). For segment B, the accession numbers are IBD/SK/VP1 (OR678938) and IBD/SK2/VP1 (OR678937). These sequences were compared to IBDV references in GenBank to assess their genetic similarity (Table 1).

Table 1: Amino acid and DNA sequence similarity of HVR of the VP2-2 gene of the one field isolate of IBDV (IBD/SK1/2018) with vaccine strains available in GenBank.

Accession No.	Strain name	Country	Amino acid Identity	DNA Identity	Geno group
EF208038	UPM 0081T	Malaysia	99.32	97.5	Geno group3
AJ632141	CEVAC IBD L	Hungary	96.49	93.19	Geno group1
EU162087	D78	Netherlands	94.19	92.44	Geno group1
DQ355819	USA/Atten	USA	96.48	93.30	Geno group1
AJ878894	Bursin	France	94.15	92.61	Geno group2
AJ878893	Tadforte	France	94.15	92.61	Geno group1
AJ878882	V877	France	92.40	86.96	Geno group7
AJ878892	228E	France	93.57	92.80	Geno group1
AF416620	Atten/BK912	Singapore	93.04	92.63	Geno group1
KY930929	IBD/Italy	Italy	90.45	92.96	Geno group6
DQ355820	Atten/Blue	Vietnam	96.98	93.47	Geno group1
KX759584	Isol 40/00	Poland	99.90	98.32	Geno group3
AF006700	HK46	Hong Kong	99.90	98.32	Geno group3
KC352668	IRAQ26-743	Iraq	100	98.76	Geno group3
JX974583	Slemani\IBDV\02	Iraq	100	99.16	Geno group3
Ay870343	Iran/vp2	Iran	100	98.02	Geno group3
MH137952	TR_B607/17	Turkey	100	95.76	Geno group3
DQ916110	Mexico04M101	Mexico	87.76	89.87	Geno group5
MH981945	URY/1302/16	Uruguay	90.85	91.95	Geno group4
KY930929	IBD/Italy	Italy	90.45	92.96	Geno group6
AJ878882	V877	France	92.40	86.96	Geno group7

Analyzing the sequence of nucleotides and amino acids

The nucleotide sequences of the three IBDV's VP2 genes had a limited diversity and were closely related to one another, with 99–99.5% DNA sequence identity and 100% amino acid homology. The chicken IBDV isolates and Poland and Hon Kong strains with accession numbers (KX759584 and AF006700, respectively) had the largest identities 98.32% (Table 3). When field virus amino acid sequences were compared to vaccine strains in GenBank, the Malaysian (UPM 0081T) strain was found to be the most related, with a 99.32% similarity. In addition, there were no big differences between the current study (IBD/SK/2018) sequences and those of an early Sulaimani strain (Slemani/IBDV02) (Table 3).

Based on the phylogenetic analysis, it was determined that the three field strains belong to Genogroup 3 (Figure 3). Additionally, subgenotype analysis showed that all field strains, along with other very virulent IBD strains from Poland, Hong Kong, and Iran, were grouped within the vvIBD genotype. Field virus sequences were found to be homologous to the UPM 0081T strain, according to the phylogenetic tree, but heterologous with the current commercial vaccination strains (e.g., D78, bursin, and cevac) (Figure 3).

By analysis, amino acid sequences derived, the presence of VP2 vvIBD markers, such as 222A, 256I, 294I, 242I, and 299S was

identified. The field strains might have a very virulent IBD genotype (Figure 4).

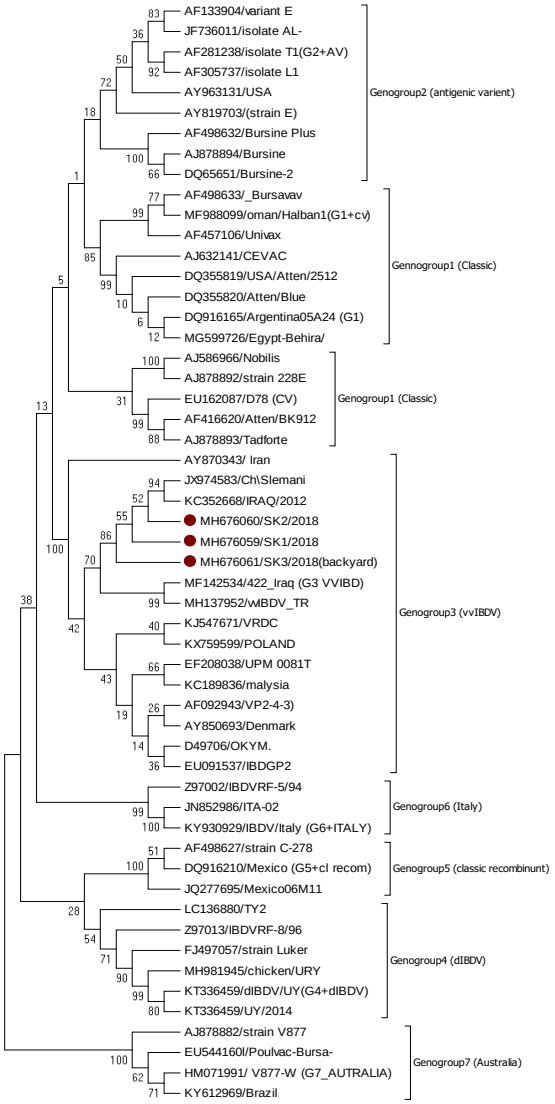

Figure 3: Neighbor-joining (NJ) phylogenetic tree generated using the nucleotide sequences for the partial VP2 coding regions of IBDV Genogroup, the red circle indicator for field isolates.

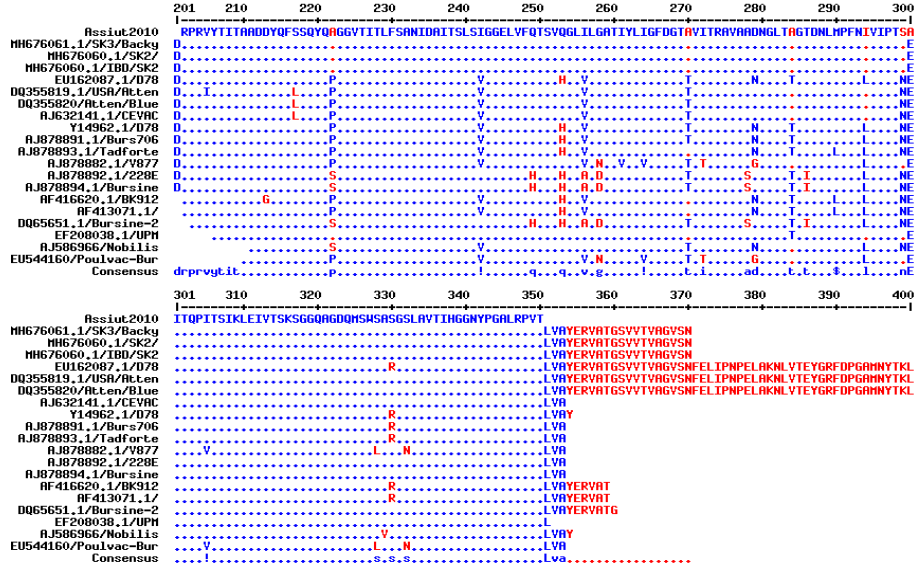

Figure 4: Multiple sequences alignment of the amino acid residue of hyper variable region of VP2 region the isolates (IBD/SK/18) with reference strains including commercial vaccine.

Sequence and phylogenic analysis of VP1

In the analysis of sequence homology for the selected portion of VP1, two IBDV strains exhibited a shared identity ranging from 94.27% at the nucleotide level and 97.42% at the amino acid level among themselves. The nucleotide coding sequence of the VP1 gene from the vvIIBDV strain (IBD/SK/VP1) exhibited a 95.24% and 95.45% identity with the DQ679813

-Turkey and MF142501-Malaysia variants, respectively. Similarly, the variant strain non-vvIBDV isolated (IBD/SK2/VP1) shared 95% nucleotide identity with the BD14/2026-Bangladesh strain.

The selection region of the VP1 gene of both sequences from Sulaimani/Iraq was aligned and compared with the reference strains, indicating that one of them (IBD/SK/VP1) had the typical vvIBDV TDN amino acid triplet, whereas the other isolates (IBD/SK2/VP1) had a new variant belong non-vvIBDV HEG amino acid triplet at the 145/146/147 position respectively (Figure 6).

We constructed a phylogenetic tree using the aligned nucleotide sequences of the partial VP1 from both the study strains and the reference IBDV strains obtained from GenBank. The resulting tree demonstrated the sequences grouping into two primary clusters, with the isolated IBD/SK/VP1 forming a cluster along with vvIBDV strains from various geographical regions, however, the isolated IBD/SK2/VP1 formed a separate cluster along with non-vvIBDV groups, which also included antigenic variant viruses (Figure 7). This result suggests the existence of a new variant, which may potentially involve genetic material re-assortment.

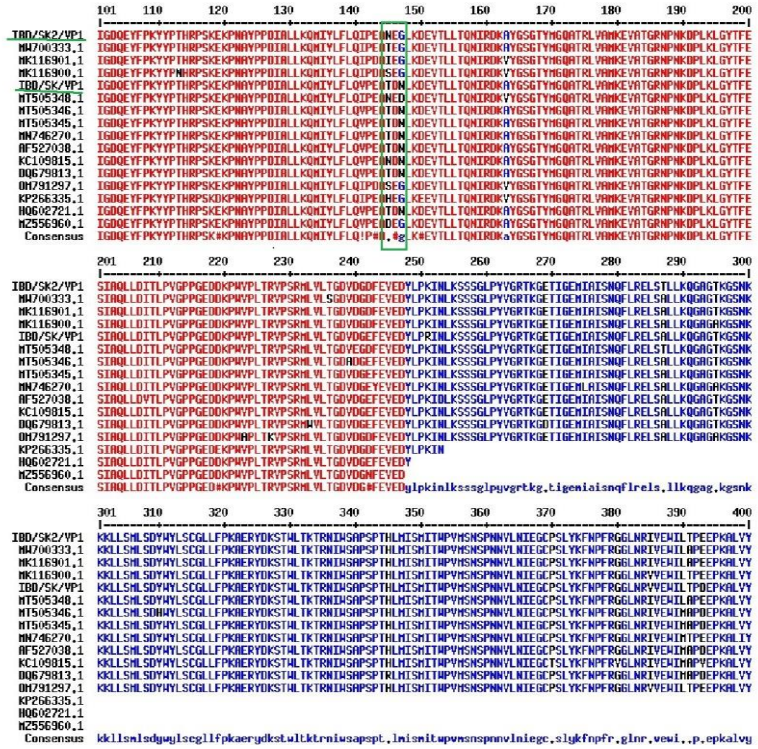

Figure 5: Amino acid alignment was performed between the field virus strains of IBDV, using the partial sequence of the VP1 gene, and reference genes. This alignment included the triplet amino acid motif at positions 145, 146, and 147, (highlighted within a green box).

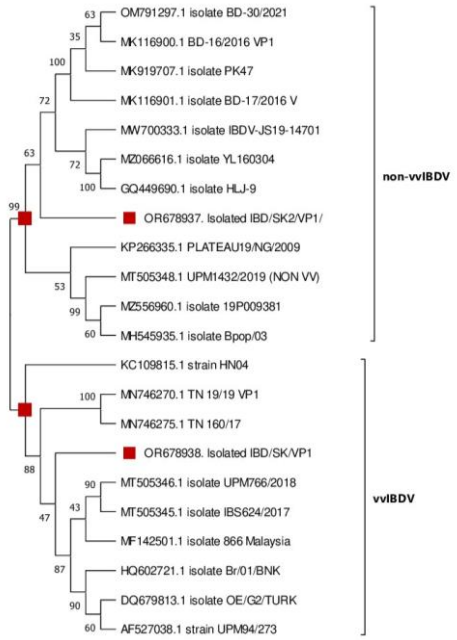

Figure 6: Neighbor-joining (NJ) phylogenetic tree generated using the nucleotide sequences for the partial VP1 coding regions of IBDV strain (segment B), red square indicator for field isolates

DISCUSSION

Variants of the IBD virus have the potential to seriously harm poultry health and productivity worldwide, causing significant financial losses. In 2016, researchers investigated the IBD virus and revealed the presence of the very virulent IBDV in the Sulaimani province (Nawzad *et al*., 2016). In a previous study in Iraq (Ulaiwi *et al*., 2015), the researchers stated that IBDV infection in commercial and non-commercial poultry flocks resulted in a high mortality rate and outbreaks. According to sequence analysis results, viruses from Genogroup 3 might be the cause of the highly virulent IBDV outbreaks in the region. A high level of sequence identity between backyard and broiler chickens reveals the presence of the same strain among both species. The fact that the field isolates of the present study and the previous Sulaimani isolate did not significantly change suggests that the strains were stable in the circulatory region during this period. The infectious bursal disease virus *VP2* gene's hypervariable region was utilized to evaluate the genetic evolution and alterations in virulence (Hussain *et al*., 2019; Chen *et al*., 2022; Thai *et al*., 2022). Based on some data (not shown), it seems that the optimal time for maternal antibodies associated with Infectious Bursal Disease (IBD) to decline to a level conducive for the vaccine to induce a strong immune response is approximately between 12 to 14 days of age. Consequently, administering the vaccine at the 16-day mark does not appear to interfere with the existing maternal antibodies. In some cases, the limited efficacy of vaccines in hosts could be attributed heterogeneity of a few amino acids at this particular location in the vaccine strains used for immunization. In the phylogenetic tree shown, the UPM 0081T vaccine strain homologs with field virus isolate share 99.32% amino acid identity with the field virus isolate. As a result, the safest option for a vaccination program appears to be this attenuated vaccine variant. Results from comparing the VP1 region of the one isolated virus (IBD/SK2/VP1) from Iraq with others in GenBank showed the presence of reassortants. Three of the isolates had vvIBDV hvVP2 sequences, but only one had non-vvIBDV VP1 amino acid sequences. The amino acid motifs observed at triplet positions (145, 146, and 147) of the Iraqi variant reassortant viruses were 'HEG,

The remaining isolated virus (IBD/SK/VP1) had VP1 sequences that closely resembled vvIBDV, containing the triplet position TDN. Important components in the prevention and management of IBDV infection are the immunizations as well as the vaccination technique used. In order to undertake efficient prevention and control approaches, the field IBDV strains and vaccine strains must remain under supervision. There is no thorough study on the IBDV strains that are now prevalent in broilers in Iraq. In order to provide additional safety precautions, a national effort should be established to administer an alternative vaccination strain in place of the current vaccine strain, which has less heterogeneity and better antigenic similarity with the field virus strain.

CONCLUSION

This study is the first in Iraq to report potential genetic re-assortment between segments A and B of IBDVs. Segment A is derived from vvIBDV, while segment B is from non-vvIBDV-like viruses. The VP2 protein sequence of the circulating vvIBDV genotype showed some heterogeneity with a few amino acid substitutions within the hypervariable region compared to the vaccine strains, which could be one of the reasons for vaccination failure. This study recommends collecting a substantial number of samples from poultry flocks in different regions of Iraq to accurately identify and assess genetic material re-assortment in IBDV.

AUTHORS CONTRIBUTION

MOB carried out the molecular genetic study and data analysis, the HSR wrote the manuscript, RRS collected the samples and participated in the study design, and ZHM participated in the data analysis. The final manuscript was read and approved by all authors.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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التحليل الجزيئي لمعزولات فيروس مرض الجراب المعدي في مدينة السليمانية / العراق

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مرض الجراب المعدي (IBD) ، المعروف أيضا باسم مرض Gumboro ، نسبة للمكان الذي تم فيه تحديد المرض في البداية. إنه فيروس مثبط للمناعة شديد الانتقال له تأثير اقتصادي كبير على قطاع الدواجن في جميع أنحاء العالم مما يؤدي إلى زيادة التعرض للعدوى المختلفة، تؤثر سلبا على الآثار على فعالية اللقاحات. ظهرت سلالات شديدة الضراوة وتسبب قدرا كبيرا من الوفيات في الدجاج في السنوات الأخيرة. تصاب الطيور المصابة بالفم والبراز، وبعد أسبوعين من الإصابة، تبدأ في طرد كميات كبيرة من العامل المعدي. يمكن أن ينتقل المرض بسرعة من الدجاج المريضة إلى الدجاج الصحي من خلال الطعام والماء والتلامس الجسدي. استخدمت الدراسة الحالية تفاعل البوليميرازالمتسلسل للنسخ العكسي (RT-PCR) وتليها تفسيرات تسلسلية تستخدم للكشف عن جين *VP2* ل IBDV وتوصيف جزيئي. تم تحديد عزلات IBDV من بولندا وهونغ كونغ وإيران لترتبط بالمجموعة الجينية ٣ من خلال تقييم التحليل التطوري لجين *VP2* غير المكتمل، مع هويات قوية تبلغ ٩٨,٣٢٪ و ٩٨٪, على التوالي. كشف النمط الجيني الخبيث عن عدم تجانس عدد قليل من طفرات الأحماض الأمينية داخل المنطقة شديدة التغير. أظهر تحليل الأحماض الأمينية ل VP1 في هاتين العزلتين أن إحداهما تحتوي على ثلاثي الأحماض الأمينية vvIBDV TDN المميز، بينما تحتوي العزلات الأخرى على ثلاثي حمض أميني غير vIBDV HEG في المواضع ١٤٥/١٤٦/١٤٧, على التوالي. يوثق التحقيق الحالي الكشف عن فيروس التهاب الأمعاء شديد الضراوة من المزارع المحلية خلال الفاشيات التي ظهرت في محافظة السليمانية. لدينا تشير هذه البيانات إلى أن فشل التطعيم الحالي قد يكون مرتبطا بالاختلافات في بروتين VP2 في HVR لسلالة IBDV المنتشرة إقليميا.

الكلمات المفتاحية: التطور الفيروسي ، IBD، *VP2* ، تحليل الحمض النووي