Molecular Genotyping of Infectious Bursal Disease virus (IBDV) Isolated from layer Flocks in Iraq

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Abstract

Recently highly pathogenic strains of IBDV have been appeared causing great economic losses. In this research IBDV has been isolated in Iraq successfully from seven samples from different Layer flocks (32 field) in different Iraqi provinces. Bursa of fabricia samples were assessed using reverse transcriptase-polymerase chain reaction (RT-PCR) for the presence of IBDV adoption VP2 gene. All seven tested samples were positive. Rapid test was conducted for the all samples to identify positive cases of IBD. Seven positive samples were selected to isolate IBDV showed 350bp by the use of conventional RT-PCR technique as assurance to the presence of IBDV. Histological sections of the bursa of fabricia showed the presence of necrosis, depletion of the follicles and the some hetrophil infiltration, these features were consider pathognomic for IBDV infection. The virus has been isolated by injected bursal suspension in embryonated chicken eggs. CAM has been collected and examined by AGPT to confirm the existence of IBDV. After confirming the presence of the virus by RT-PCR sequence analysis by application of PCR products for the seven samples. All seven selected samples very virulent vvIBDV. Genotyping of Iraqi vvIBDV strains showed a gradual evolution of the IBDV in Iraq, where they were not closely linked to the previous isolated Iraqi strains.

Key word: Infectious bursal disease virus (IBDV); VP2 gene; Molecular characterization of IBDV.
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التنميط الجيني الجزيئي لفايروس التهاب الجراب المعدي المعزولة من قطعان الدجاج البياض في العراق

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الخلاصة

ظهرت سلالات شديدة العدوى في الفترة الأخيرة من IBDV تسببت في خسائر اقتصادية كبيرة. في هذا البحث تم عزل IBDV في العراق بنجاح من سبع عينات من حقول بياض مختلفة 32 حقل في المحافظات العراقية المختلفة. تم تقييم جراب فابريشيا للعينات باستخدام (RT–PCR) لوجود IBDV باعتماد جين VP2. وكانت جميع العينات السبعة المختبرة إيجابية. وأجري الاختبار السريع لجميع العينات لتحديد الحالات الإيجابية لمرض التهاب جراب فابريشيا المعدي. أظهرت العينات السبعة الموجبة 350bp عن طريق استخدام تقنية RT–PCR التقليدية للتأكد من وجود IBDV. وأظهرت المقاطع النسيجية من الجراب فابريشيا وجود نخر، ونضوب بصيلات وارتشاح بعض خلايا الهتروفيل، هذه الميزات ذكرت آنفا للإصابة IBDV. وقد تم عزل الفيروس عن طريق حقن عالق جراب فابريشيا في أجنة بيض الدجاج. وقد تم جمع CAM وفحصها من بواسطة AGPT لتأكيد من وجود IBDV. بعد التأكد من وجود الفايروس بواسطة RT–PCR اجري التحليل التسلسلي PCR products للعينات السبعة. تميزت جميع العينات السبعة ضارية جدا vvIBDV. اكد التنميط الجيني للعترات vvIBDV العراقية وجود تطور تدريجي لفايروس التهاب جراب فابريشيا العدي في العراق، حيث أنها لم ترتبط ارتباط وثيق بالعترات العراقية المعزولة سابقا.

الكلمات المفتاحية: التنميط الجيني الجزيئي، فايروس التهاب الجراب المعدي، الدجاج البياض.

Introduction

Infectious bursal is acute highly contagious disease causing severe immunosuppression as a result of damage lymphocyte in bursa of Fabricius (1). IBDV belongs to the Birnaviridae family genus Avibirnavirus. Two different serotypes of the virus have been recognized. The pathogenic IBDV to chickens are serotype 1 while nonpathogenic are serotype 2. Divided Serotype 1 viruses is divided into different groups: (classical, variant, and very virulent) strains (2), (3). Lymphoid necrosis and bursal damage as a result of infection with classic IBDV strains resulting in 20‑30% mortality (4). In the mid-1980s emerged caused highly outbreaks in layers and broiler very virulent (vvIBDV) causing 60% and 30% mortality in respectively, then spread to Asia, Africa, South America and Middle East, (4), (5). In Iraq, Hassan (6), reported all poultry sectors; layers, broiler breeders, broilers, and grand parent (GP) stocks were infected with variants and vvIBDV isolates resulting in high mortalities about 20-58%. Currently, commercial chicken flocks suffer from high mortality rate and the presence of many of the causes are responsible for these outbreaks, but the IBDV is one of these causes. Therefore the need for many research on the local isolates of IBDV. Proof that the Spread of variant strain in vaccinated flocks with classic IBDV vaccines (7), (8). IBDV is a serious and persistent pathological problems in Iraq. The objective of this study was to define the molecular characterization of IBDV isolated from a Layer chicken flocks in Iraq using RT-PCR, and then isolating and analyzing the molecular HVR part of the VP2 gene. In present study, 32 layer outbreaks over the period from April 2015 to January 2016 were studied in an attempt to fix the molecular characterization of IBDV using RT-PCR, isolation, sequence analysis of HVR part of the VP2 gene, and histopathological study.

Materials and Methods

- **Field samples:** During April 2015 to January 2016, layer farms in different Iraqi Governorates showed sudden onset of high mortality. Necropsy showed gelatinous exudates and haemorrhages in Bursa of Fabricus. Using RT-PCR for viral isolation and detection in fifty-two collected bursa. High mortalities (15-21%) in chickens from 12 flocks revealed severe hemorrhages on thigh and breast muscles, severely hemorrhagic and edematous bursa. Also high mortalities with mild edematous bursa recorded in chickens of 5 flocks. While slight enlarged bursa and slightly muscular congestion with dehydration were recorded in rest flocks.
- **Histopathological examination:** Dehydrated histopathology specimens with varying degrees of alcohol, paraffin embedded and cut off a thickness of 4 µl, then H& E dye used to stain bursa samples (9).
- **IBDV detection by RT-PCR:** Bursal homogenates is extracted RNAs (disrupting one part of the bursal sample with sterile saline (1: 1)) according to the manufacturer's instructions by using QiAmp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany). In RT-PCR reaction and subsequent sequence analysis used forward primers while in amplification of a 620 bp fragment of VP2 within IBDV used reverse PCR primers:

Forward primer: [P1: 5'-CAGGTGGGGTAACAACAATCA-3'].

Reverse primer: [P2: 5'-CGGCAGGTGGAACAATG-3'] [14].

Briefly, 50 µl reaction mixture containing 10 µl of 5× RT-PCR buffer, 10 µl of extracted RNA, 2 µl primer R, 2 µl primer F, 2 µl dNTP mix contained 400 µM each dTTP, dGTP, dCTP, dATP, and 2 µl of Qiagen. Enzyme Mix is one step. using PCR thermo cycling amplified the fragment of 620 bp of the 5′-noncoding region (T3 Biometra-Germany) as follows: RT reaction 50 °C for 20 min; initial PCR activation for 15 min at 95 °C; (39 three-step cycles) (denaturation 30 s at 94 °C, annealing 40 s at

59 °C and 1 min at 72 °C; then final extension 10 min at 72 °C). Then analyzed 5 µl of PCR products after amplification via electrophoresis on a 1.5% agarose gel comprised final concentration of 0.5 µg/ml with ethidium bromide for 30 min at 95 V in 1× TBE buffer, against DNA Ladder Plus GeneRuler™ 100 bp (Fermentas). Using BioDoc Analyze Digital Systems photographed the images of the gels (Biometra, Germany).

- **Virus isolation:** For virus isolation, prepared 10% suspension contained 0.4 mg/mL of gentamicin sulphate, 1000 I.U/mL of penicillin and 1 mg/mL of streptomycin sulphate in PBS (phosphate buffer saline). Inoculated 0.2 mL suspension in SPF embryonated chicken eggs (9-10 day-old) through chorio-allantoic-membrane (CAM). Then incubated at 37 ° C with daily observation. CAM was harvested 96 hr post inoculation (10).

After virus isolation, lesions score was estimated (Mild, Severe and Negative), where Mild lesion refer to low embryos mortalities with mild hemorrhage on the CAM, sever lesion refer to high embryos mortalities, greenish liver and sever hemorrhage on the CAM and negative define no embryo mortalities and no lesions on the CAM (11).

- **Confirmation of the isolated virus by AGPT:** Using reference antisera against IBDV standard known positive and negative precipitating obtained from Animal Health Research Institute, the Netherlands (12).
- **Sequence analysis of VP2 of IBDV:** The expected size (350 bp) of DNA band in gel excised and purified with the Gel Extraction QIA quick Kit (Qiagen) according to the manufacturer instruction. Using the ABI PRISM® BigDyeTM sequenced directly the purified PCR products Terminators the ABI PRISM® 3130 genetic analyzer (Applied Biosystems) and v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with 80 cm capillaries. Using SeqScape® Software Version 2.5 (Applied Biosystems) edited The sequences, by the Lasergene DNASTAR group of programs (DNASTAR Inc., Madison, WI) nd Clustal V method assembly of the consensus sequences and alignment trimming, Using also MEGA 5 software to draw the phylogram (13). In the study using DNAstar-MegAlign software to alignment the viruses. infectious bursal disease viruses resource (http://www.ncbi) were available from the National Center for Biotechnology Information (NCBI) and Genbank are sources for Iraqi viruses and other international reference strains. Finally implementation the congruence and divergence percentage between all viruses.

Results

- **Histopathological analysis:** Atrophy and depleted lymphoid follicles and heterophils invasion with manifestation of vacuoles in the medullar and cortical portion these typical lesions were registered in infected Bursa. (Fig. 2).
- **IBDV detection by RT-PCR:** With application of RT-PCR; 32 bursal samples were tested; 25 samples (78%) were negative (Table 1). Seven positive samples (21%) by RT-PCR exposed specific bands at 350 bp on agarose gel (Fig. 1).

Table (1) Results of detection of IBDV using RT-PCR

Provinces	No. of cases	No. of positives	No. of negatives
Al-Qadisiyah	5	-	5
Dialya	5	2	3
Kirkuk	6	1	5
Babel	3	1	2
Baghdad	6	2	4
Wasit	2	-	2
Karbala	5	1	4
Total No.	32	7	25

Describes the tested 32 bursal sample with RT-PCR where 7 samples were positives and 25 samples were negatives with positive percent 21%.

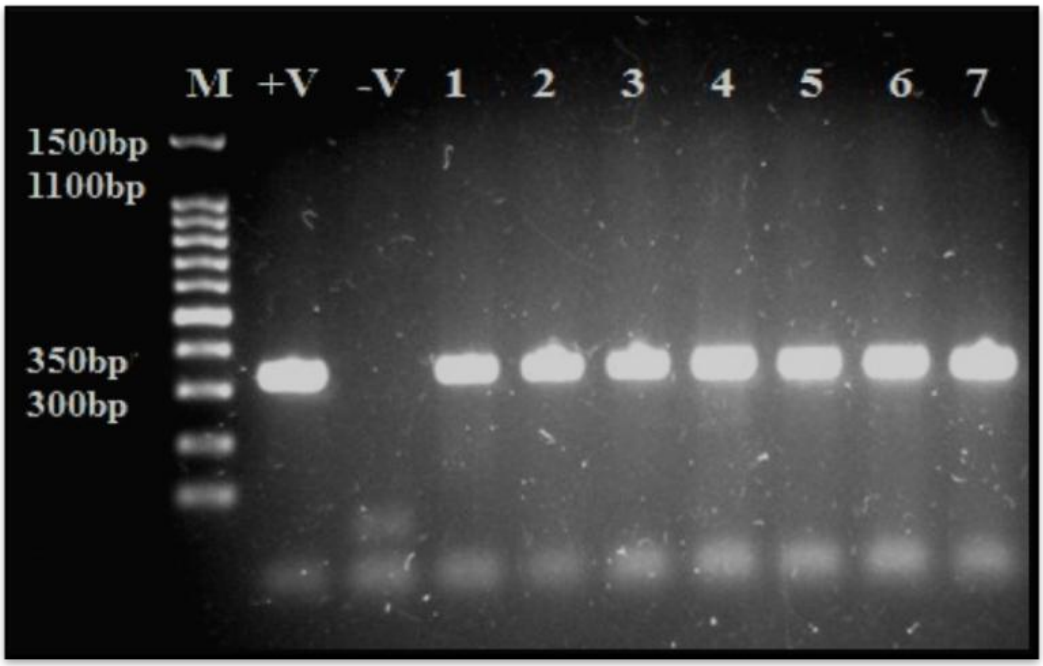

Fig. (1) Gel electrophoresis of RT-PCR showing 350 bp band in positive samples and no band was observed in negative samples.

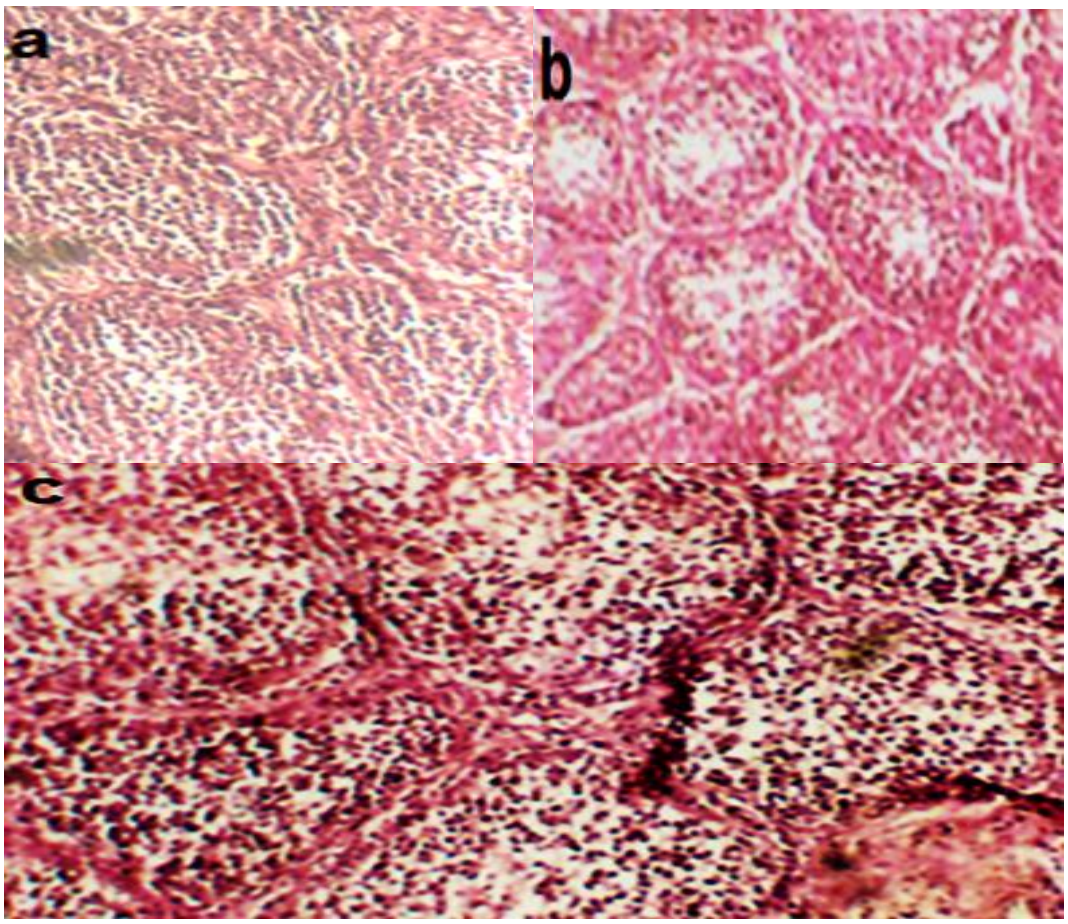

Fig. (2) Histopathological findings of bursa showed from infected birds with IBDV. (a and b) Show atrophied lymphoid follicles and depleted, thickness of bursal wall and presence of vacuoles cystic in the medullar and cortical portion (10X), (c) show heterophils inflitration and necrosis in bursa with infectious bursitis (40X).

- **Virus isolation and confirmation:** Table 2 summarizes the obtained results of virus isolation. greenish liver and hemorrhages on the CAM in varying degree of severity were observed in gross lesions. Five IBDV viruses were confirmed the isolation by AGPT employed on the harvested CAMs

Table (2) Results of virus isolation and confirmation by AGPT

Samples code	Lesions in inoculated eggs			Results of AGPT
	Passage (1)	Passage (2)	Passage (3)	
Amjad_Suhayl (Dialya)	+++	Nd	Nd	Pos
Mohamed_Najm (Dialya)	+++	++	Nd	Pos
Alwatamyah_Farm_1_Hs_7(Baghdad)	+++	+	Nd	Pos
Alwatamyah_Farm_7_Hs_6 (Babel)	+++	+	+	Pos
Alwatamyah_Farm_3_Hs_8 (Baghdad)	+++	Nd	Nd	Pos
DelE (Kirkuk)	+++	++	+	Pos
STC (Karbala)	+++	Nd	Neg	Pos

The results of virus isolation and confirmation by AGPT. +, Mild lesions; ++, moderate lesion +++, severe lesions; CAM, chorio-allantoic-membrane; AGPT, agar gel perciptation test; Pos, positive; Neg, negative; Nd, not done.

- **Results of sequence analysis and phylogenetic tree:** Sequence analysis of the 350 nucleotides for 7 isolates which express 119 amino acid sequence (position: 183–302 bp).

The results of the amino acid sequence showed (89%) identity between the isolates (Amjad_Suhayl and Mohamed_Najm) and between Uk661 vvIBDV strains. while, isolates (Alwatamyah_Farm_1_Hs_7 and Alwatamyah_Farm_7_Hs_6) have amino acid sequence identity between each (70%) and (Alwatamyah_Farm_3_Hs_8 and STC) isolates have amino acid sequence identity between each (67%), while DelE strain have different amino acid sequence with UK661. as shown in (Table 3).

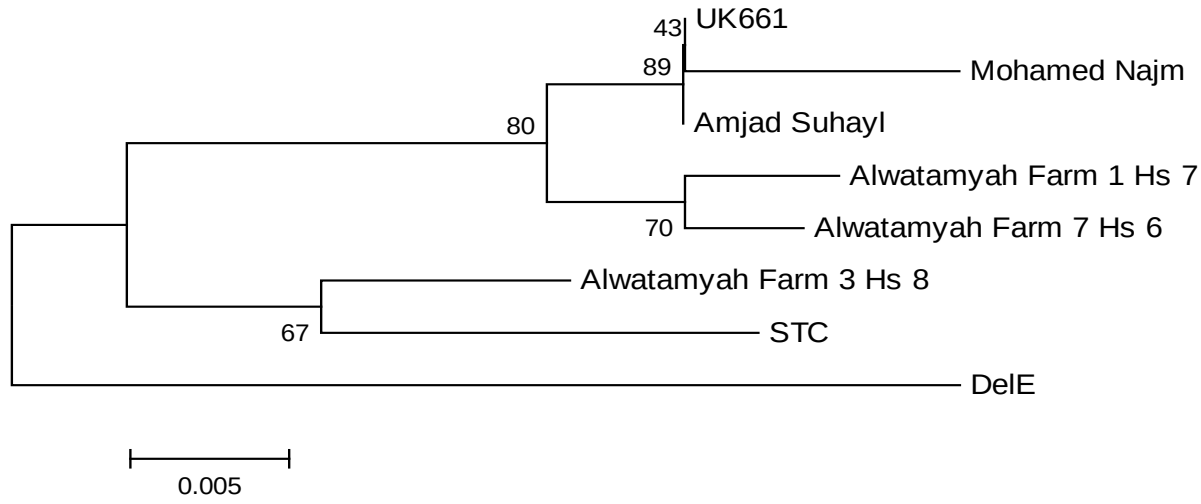

Fig. (3) Phylogenetic tree of sequences deduced amino acid of the seven IBDV isolates and other classical reference, very virulent, and variant and vaccine strains of IBDV.

Table 3. Identity and diversity of IBDV.

Boehringer Ingelheim **April 24, 2016**
Infectious Bursal Disease Virus VP2 sequences
Iraq

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                        210        220        230        240        250        260        270        280
UK661                     AADDYQFSSQ YQAGGVTITL FSANIDAITS LSIGGELVFQ TSVQGLILGA TIYLIGFDGT AVITRAVAAD NGLTAGTDNL
Amjad_Suhayl              .......... .......... .......... .......... .......... .......... .......... ..........
Mohamed_Najm              ..N....... .......... .......... .......... .......... .......... .......... ..........
Alwatamyah_Farm_1_Hs_7    .......... F....E.... .......... .......... ....S..... .......... .......... ..........
Alwatamyah_Farm_7_Hs_6    ...N...... F......... .......... .......... ....S..... .......... .......... ..........
Alwatamyah_Farm_3_Hs_8    .......L.. F.P....... .......... ..V....... ......V... .......... T......... ..........
DelE                      ...N...... ..T....... .......... ..V......K ....S.V... .......... .........N ......I...
STC                       .......... ..P....... .......... ..V....... ......V... ...F...... T......... ..........

                        290        300        310        320        330        340        350        360
UK661                     MPFNIVIPTS EITQPITSIK LEIVTSKSGG QAGDQMSWSA SGSLAVTIHG GNYPGALRPV TLVAYERVAT GSVVTVAGVS
Amjad_Suhayl              .......... .......... .......... .......... .......... .......... .......... ..........
Mohamed_Najm              .......... .......... .......... .......... .......... .......... .......... ..........
Alwatamyah_Farm_1_Hs_7    .......... .......... .......... .......... .......... .......... .......... ..........
Alwatamyah_Farm_7_Hs_6    .......... .......... .......... .......... .......... .......... .......... ..........
Alwatamyah_Farm_3_Hs_8    .........N .......... .......... .......... .......... .......... .......... ..........
DelE                      ....L....N .......... .........D. ...E...... .......... .......... .......... ..........
STC                       ....L....N .......... ..V....... .......... .......... .......... .......... ..........

                        370        380        390        400        410        420        430        440
UK661                     NFELIPNPEL AKNLVTEYGR FDPGAMNYTK LILSERDRLG IKTVWPTREY TDFREYFMEV ADLNSPLKIA GAFG
Amjad_Suhayl              .......... .......... .......... .......... .......... .......... .......... ....
Mohamed_Najm              .......... ....I..... .......... .......... .......... .......... .......... ....
Alwatamyah_Farm_1_Hs_7    .......... .......... .......... .......... .......... .......... .......... ....
Alwatamyah_Farm_7_Hs_6    .......... .......... .......... .......... .......... .......... .......... ....
Alwatamyah_Farm_3_Hs_8    .......... .......... .......... .......... .......... .......... .......... ....
DelE                      .......... .......... .......... .......... .......... .......... .......... ....
STC                       .......... .......... .......... .......... .......... .......... .......... ....
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Discussion

Gamboro is serious pathological problems in poultry producing countries, including Iraq and throughout the world. The clinical diagnosis of the acute IBD forms depend on the notice of the clinical signs and gross lesions of the pathognomonic bursal lesions. In this study, the gross lesions showed edematous hemorrhagic bursa and mild to severe haemorrhge, congestion on thigh muscle. Clinical observation depend on the absence or presence of passive immunity, age of birds and virulence (15). It was suggested that very virulent strains is more pronounced in viral multiplication and pathogenesis as compared with milder strains (16). In this study, the histopathological characteristic to IBDV infection showed different degrees of lymphoid follicles depletion, for vvIBDV it revealed more rapid and severe depletion of the lymphoid follicles in comparison with milder strains (17, 18, 19). Using RT-PCR was applied to obtain the definite identification of IBDV which was recognized to be a sensitive test to detect the IBDV (20, 21, 22). In this study, we used RT-PCR in which 7 positive IBDV was recognized for detection out of 32 field samples. This agree with (14). Gross lesions of SPF embryonated chicken eggs after inoculation with bursa suspension for virus isolation showed greenish liver in different degree of severity and hemorrhages on the CAM, similar finding have also been described (23). Isolation five IBDV viruses was confirmed with AGPT on the harvested CAMs (Table 2). AGPT results inconsistency was previously described by (24, 25). The most informative genetic data was recognized in (206-350 a.a.) hypervariable region, molecular characterizations of IBDV strains by sequence analysis to determine the variability in strains, changes in virulence and/or antigenicity occurred due to naturally happened of variations analysis or by attenuation in diverse strains (26, 27). In this study, the IBDV local isolates was grouped into at least two pathogenic different subgroups and geographical assess subtypes depend on phylogenetic analysis and comparative alignment of the VP2 hypervariable domain. Mutation in (DeIF) isolate was confirmed the different forms of the previously isolated UK661 IBDV strain that explained the IBDV outbreaks which occurred in Kirkuk as results the heavily use of IBD different vaccination programs also the extensive abuse of IBDV vaccines. Significant antigenic variation occurred a results of variation in the major Hydrophilic region (peak A 210–250) which is responsible for the presumed to be the dominant parts of the neutralizing dominan, and binding of neutralizing monoclonal antibodies (Mabs) (28, 29). Virus antigenicity changes was affect by substitution of amino acid which may has an important role in increasing virulence which causes infection in the existence of high maternal antibody. VP2 region have residues at position (N299S), (V256I), (P222A), (N279I), 294I. revealed to be exceptional for all vvIBDV strains in comparison to classical strains (30, 31, 32). In this study, the seven (very virulent) isolates revealed different amino acids in N 270 D position but the same at position 220 A, 250 I, 290 I, 300. Amino acids that are responsible for pathogenic of highly virulent IBDVs found at positions 250 and 280. (33, 34). (Q 250 H; A 280 T) are consider specific amino acids for cell tropism involved in cell culture adaptation (35). my isolates show (250 Q, 280 A) these results supposed the cell culture difficulty of these isolated strains. Strains which have histidine at position 250 reported to be less pathogenicity than those with a glutamine at position 250 which are much highly pathogenic. All seven isolates examined in the present study and previously isolated UK661 IBDV showed glutamine residue at position 250 indicating high pathogenicity. The possibility of emergence of mutants a results of intensive vaccination program with live attenuated viruses may lead to persistent change and their pathogenic potential that require evaluation the vaccination programs in Iraq. **Conclusion**, We concluded that IBDV posses the property of antigenic drift, this means that periodic evaluation for the vaccine should be done, and the vaccine used should be of the local strain.

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