Genetic Characterization of Infectious Bursal Disease Viruses Associated with Gumboro Outbreaks in Chicken Flocks from El-Sharkia Province, Egypt.

Hashish, A.A. [2], Abdullah A. Selim [2], Mohamed F. Mandour [1], Mohamed M. Abd-Eldaim[1], Shahira A. M. Abdelwahab[1], El_Tarabili, M. M [1].

[1] Virology Department, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt
[2] National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute

Abstract

Re-emergence of very virulent forms of IBDV has been the cause of significant economic losses in poultry industry in Egypt. In present study, 15 bursal samples were collected from flocks showing typical clinical signs and post mortem lesions of IBDV outbreaks. These bursal samples were tested using reverse transcriptase-polymerase chain reaction (RT-PCR) targeting VP2 gene of IBDV. Out of the tested samples 10 (66.67%) were positives. Sequence analysis of PCR products of 4 selected samples according to strength of the bands on gel electrophoresis was carried out. All of the sequenced samples were characterized as very virulent (vvIBDV). The deduced amino acid sequence revealed that the four samples ***(Sharkia1,2,3,4)*** have amino acid sequence identity between each other (99.3 -100%) and between Egyptian vvIBDV (Giza 2008, Giza 2000) strains (98.6 – 100%). Genotyping of the four Egyptian vvIBDVs indicated that they were closely related to previous isolated strains from Egypt. So we aimed to find the homology between these collected IBDV samples and other field and vaccinal strains sequences.

Introduction

Infectious bursal disease (IBD) is an acute, highly contagious viral disease of chickens that characterized by degenerative lesions in the bursa of Fabricious result in fatal conditions and immune-suppression ***(Eterradossi and Saif, 2008)***. Infectious bursal disease virus (IBDV) is a non-enveloped virus belongs to the family Birnaviridae. IBDV has two serotypes; Serotype 1 IBDV strains ***(Van den berg et al, 2004),*** and serotype 2 strains ***(Mcferran et al, 1980).*** Serotype 1 viruses can be further categorized into 4 groups on

the basis of their pathogenicity: Classical strains, variants, attenuated strains and very virulent strains ***(Lim et al, 1999)***. vvIBDV strains were reported to break through high levels of maternal antibodies, lead to lesions typical of IBDV and 100% mortality rates in susceptible chickens ***(Xiumiao et al, 2012).*** IBDV genome is divided into segments A (3.4 kb) and B (2.7 kb). The larger segment A encodes 4 viral proteins, the two capsid proteins VP2 (48 kDa) and VP3 (32–35 kDa), the viral protease VP4 (24 kDa), and a non-structural protein VP5 (17–21 kDa). The smaller segment B encodes VP1 (90 kDa), an RNA-dependent RNA polymerase ***(Jackwood et al, 2008).***

Amino acid position from 206 to 350 called hyper variable region (HVR) on VP2 were shown to represent a major neutralizing antigenic domain by expression/deletion studies ***(Van den Berg, 2000,*** that includes the most variable region that is important for pathogenic variation. Despite of chicken flocks are vaccinated against IBD, severe outbreaks of IBD were reported in chicken flocks in Egypt, caused high mortalities, and have become a serious problem ***(Bekhit, 1998; Metwally et al, 2009)***. To increase our understanding of the molecular epidemiology of IBDV in El-Sharkia province, Egypt, the amino acid sequences of HVR of VP2 from Position 201 to 338 of 4 IBVs were analyzed and compared with other reference classical, variant and very virulent viral isolates from Egypt.

Material and Methods

1. Clinical signs and post mortem findings of chickens infected with IBDV:

Chickens from the affected flocks showed decrease feed intake, watery greenish diarrhea, ruffled feathers. Post mortem examination revealed that the affected chickens had enlarged hemorrhagic bursa, petical hemorrhage on thigh muscle and breast muscle and dehydrated carcass with degenerative changes in the kidney.

2. Sample collection

A total of 15 bursal tissues were collected from different chicken flocks showing typical clinical signs and gross lesions of IBD from different regions of El-Sharkia Province. The Chickens were vaccinated with a live attenuated IBDV vaccine (Bursavac®) at day 11 and 22. The specimens were kept frozen at −80℃ until processing.

3. IBDV detection by RT-PCR

IBDV RNAs were extracted from the Bursal homogenates [one part of each bursa sample disrupting in sterile saline (1:1)] using QiAmp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. A set of primers were used for the RT-PCR reaction and for the subsequent sequence analysis using forward and reverse

PCR primers for amplification of a 620 bp fragment within IBDV on VP2: Forward primer (F): [AUS GU: 50-TCA CCG TCC TCA GCT TAC CCA CAT C-3].Reverse primer (R): [AUS GL: 50-GGA TTT GGG ATC AGC TCG AAG TTG C-3] **(Metwally *et al.*, 2009)**. Briefly, the 50 µl reaction mixture contained 10 µl of 5×RT-PCR buffer, 2 µl primer F, 2 µl primer R, 2 µl dNTP mix containing 400 µM each dATP, dGTP, dCTP, dTTP, 2 µl of Qiagen One Step Enzyme Mix and 10 µl of extracted RNA. A fragment of 620 bp was amplified by PCR thermo cycling using (T3 Biometra-Germany) as follows: 20 min at 50 °C (RT reaction); 95 °C for 15 min (initial PCR activation); 40 three-step cycles of 94 °C for 30 s (denaturation), 59 ° C for 40s (annealing) and 72 °C for 1 min; then 72 ° C for 10 min (final extension). After amplification, 13 µl of PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide with final concentration of 0.5 µg/ml at 95 V for 30 min in 1× TBE buffer, against GeneRuler ™ 100 bp Plus DNA Ladder (Fermentas). Images of the gels were photographed on BioDoc Analyze Digital Systems (Biometra, Germany) **(Metwally *et al.*, 2009)**.

4. Sequence analysis of VP2 of IBDV

Gel containing DNA band of the expected size (620 bp) was excised and purified with the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer instruction. The purified PCR products were sequenced directly using the ABI PRISM BigDyeTM Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 3130 genetic analyzer (Applied Biosystems) with 80 cm capillaries. The sequences were edited with SeqScape Software Version 2.5 (Applied Biosystems), alignment trimming was performed With **Bioedit** software. The phylogram was drawn using also **MEGA 5** software with a bootstrap resampling method (1000 bootstraps). Egyptian viruses and other international reference strains from the Genbank were available from the National Center for Biotechnology Information (NCBI) infectious bursal disease viruses resource (http://www.ncbi). The identity percent and divergence between all viruses was carried out using **MegAlign** software.

Results

1. Identification of Virus Using RT-PCR

Out of 15 bursal samples tested with RT-PCR, 10 samples **(66.7%)** were positive. All RT-PCR positive samples showed specific bands at 620 bp on agarose gel (1.5%) as expected **(Figure 1).**

2. Sequence analysis and phylogenetic tree of IBDV:

Sequence analysis of the 414 nucleotides which express 138 amino acid sequences (Position 201

to 338) from the four selected samples were carried out.

The deduced amino acid sequence revealed that the four samples **(Sharkia1,2,3,4)** have amino acid sequence identity between each other (99.3 -100%) and between Egyptian vvIBDV (Giza 2000, Giza 2008) strains (98.6 – 100%). As shown in **Table (1)**

Phylogenetic tree **(Figure 2)** indicated that the four IBDV in this study showed close relationships with previously isolated Egyptian IBDVs (Giza 2000 and Giza 2008) and they are clustered together.

Sequence analysis of the four IBDV samples revealed the highly virulent nature of those viruses. It was found that none of the examined samples were of attenuated or vaccinal origin due to absence of 253-Histidine and 284-Threonine substitutions that typically found in attenuated vaccine strains. **Figure (3).**

All of the examined isolates showed the characteristics of vvIBDV amino acid substitutions at residues **222 (A), 242 (I), 253 (Q), 256 (I), 279 (D), 284 (A), 299 (S)** and **330 (S)**. Also the serine-rich heptapeptide **SWSASGS** that found next to the second hydrophilic region **326–332** was confirmed in all examined samples.

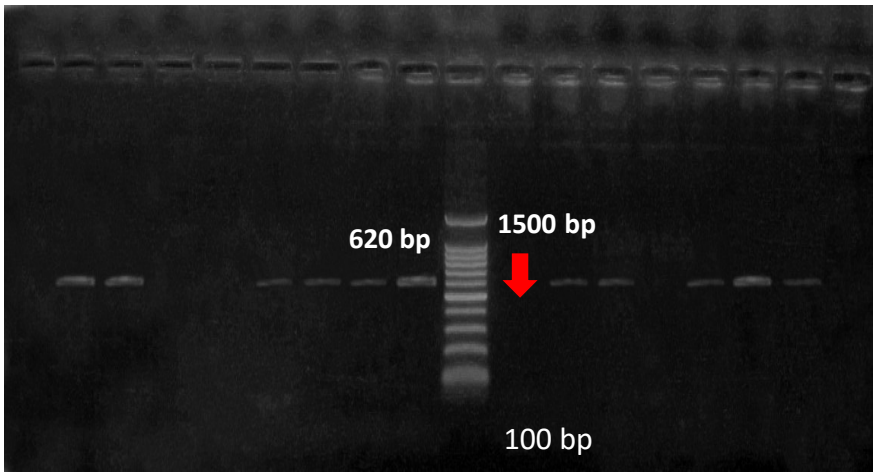

Figure 1: *showed RT-PCR results of 15 examined samples, M: 100 bp marker, NC: negative control, PC: positive control, Lane 1,2,3,5,6,8,9,10,13&14 positive samples, Lane 4,7,11,12&15 negative samples.*

Table 1: *amino acid similarity (%) of the four sequenced IBDV samples with reference classical, very virulent, variant and vaccinal IBDV strains*

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
1		92.0	93.5	90.6	91.3	92.8	92.8	92.8	92.8	94.9	95.7	95.7	92.0	98.6	91.3	91.3	90.6	91.3	1	kal2001
2	8.4		97.8	90.6	91.3	91.3	91.3	91.3	91.3	91.3	92.0	92.0	89.9	92.0	91.3	91.3	90.6	91.3	2	variant
3	6.8	2.2		92.0	92.8	92.8	92.8	92.8	92.8	92.8	93.5	93.5	91.3	93.5	92.8	92.8	92.0	92.8	3	variant
4	10.1	10.1	8.4		99.3	97.8	97.8	97.8	97.8	91.3	93.5	92.0	89.9	90.6	99.3	99.3	98.6	99.3	4	Giza
5	9.3	9.3	7.6	0.7		98.6	98.6	98.6	98.6	92.0	94.2	92.8	90.6	91.3	100.0	100.0	99.3	100.0	5	Giza
6	7.6	9.3	7.6	2.2	1.5		100.0	100.0	100.0	93.5	95.7	94.2	92.0	92.8	98.6	98.6	97.8	98.6	6	k357-88
7	7.6	9.3	7.6	2.2	1.5	0.0		100.0	100.0	93.5	95.7	94.2	92.0	92.8	98.6	98.6	97.8	98.6	7	NIE
8	7.6	9.3	7.6	2.2	1.5	0.0	0.0		100.0	93.5	95.7	94.2	92.0	92.8	98.6	98.6	97.8	98.6	8	D6948
9	7.6	9.3	7.6	2.2	1.5	0.0	0.0	0.0		93.5	95.7	94.2	92.0	92.8	98.6	98.6	97.8	98.6	9	KS
10	5.3	9.3	7.6	9.3	8.4	6.8	6.8	6.8	6.8		96.4	99.3	91.3	93.5	92.0	92.0	91.3	92.0	10	BursaVac
11	4.5	8.4	6.8	6.8	6.0	4.5	4.5	4.5	4.5	3.7		97.1	92.0	94.2	94.2	94.2	93.5	94.2	11	CEVAC
12	4.5	8.4	6.8	8.4	7.6	6.0	6.0	6.0	6.0	0.7	3.0		92.0	94.2	92.8	92.8	92.0	92.8	12	Univax
13	8.4	10.9	9.3	10.9	10.1	8.4	8.4	8.4	8.4	9.3	8.4	8.4		90.6	90.6	90.6	89.9	90.6	13	Bursine
14	1.5	8.4	6.8	10.1	9.3	7.6	7.6	7.6	7.6	6.8	6.0	6.0	10.1		91.3	91.3	90.6	91.3	14	D78
15	9.3	9.3	7.6	0.7	0.0	1.5	1.5	1.5	1.5	8.4	6.0	7.6	10.1	9.3		100.0	99.3	100.0	15	Sharkia
16	9.3	9.3	7.6	0.7	0.0	1.5	1.5	1.5	1.5	8.4	6.0	7.6	10.1	9.3	0.0		99.3	100.0	16	Sharkia
17	10.1	10.1	8.4	1.5	0.7	2.2	2.2	2.2	2.2	9.3	6.8	8.4	10.9	10.1	0.7	0.7		99.3	17	Sharkia
18	9.3	9.3	7.6	0.7	0.0	1.5	1.5	1.5	1.5	8.4	6.0	7.6	10.1	9.3	0.0	0.0	0.7		18	Sharkia
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		

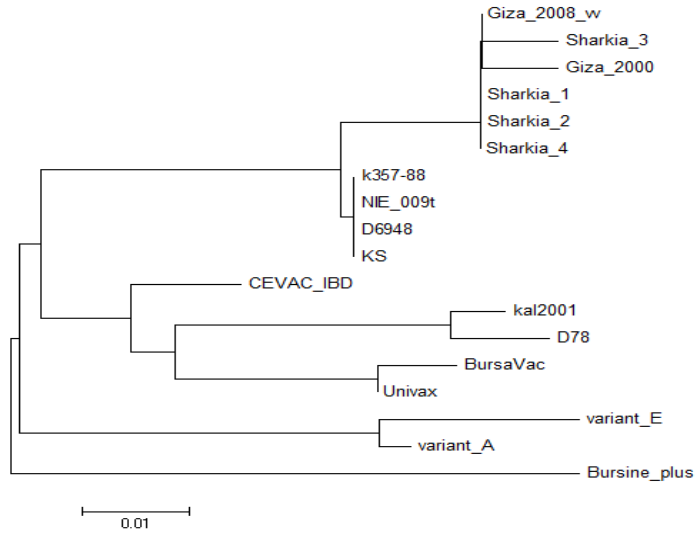

Figure 2: *Phylogenetic tree of deduced amino acids sequences of the four IBDV samples and other reference classical, very virulent, variant and vaccinal strains of IBDV.*

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                    204       214       224       234       244
                    ...|....|....|....|....|....|....|....|....|
kal2001             DRPRVYTITAADDYQFSSQYQPGGVTITLFSANIDAITSLSVGG
variant E           ............N........T.....................
variant A           ....................Q.....................
Giza 2000           ..................F.A...................I..
Giza 2008 vv        ..................F.A...................I..
k357-88             ....................A...................I..
NIE 009t            ....................A...................I..
D6948               ....................A...................I..
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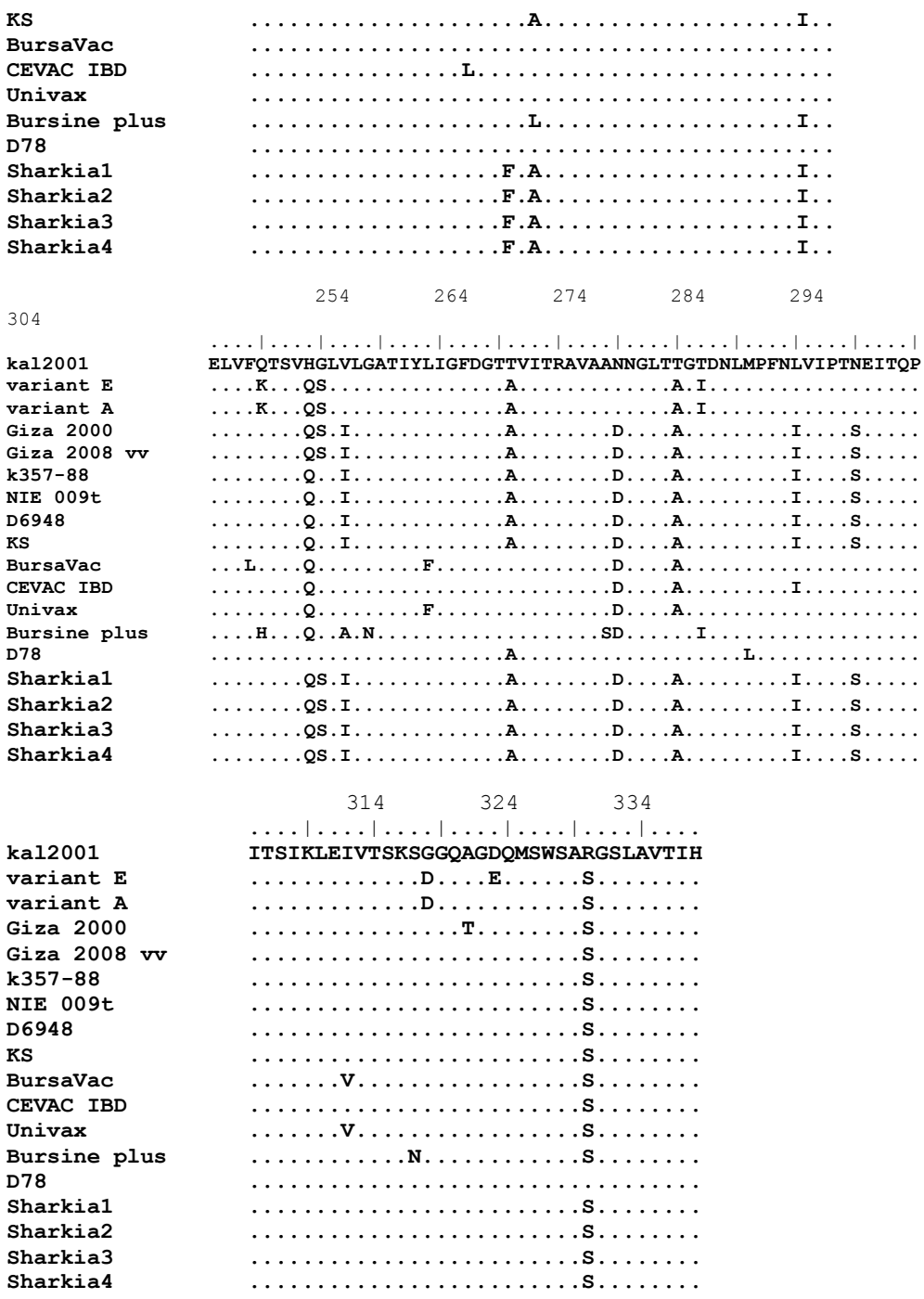

Figure 3: *Alignment of deduced amino acid sequence of the VP2 variable domain from aa positions 201 to 338 (numbering according to* ***Bayliss et al (1990****) of the four selected samples.*

Discussion

Infectious bursal disease virus continuous to be a serious threat in Egypt as it does in other poultry producing countries worldwide. A preventive program is necessary to avoid virus spread and disease appearance ***(Hussein et al, 2003).*** The clinical diagnosis of the acute form of IBD depends on the observation of the symptoms and gross lesions examination. Clinical signs depend on the virulence of the virus, age of bird and immune status of the bird ***(Rauw et al, 2007)***.

Observed clinical signs in this study were anorexia, watery diarrhea, ruffled feathers and general weakness. Post mortem examination revealed that the affected chickens had enlarged hemorrhagic bursa, poetical hemorrhage on thigh and breast muscle in addition to dehydrated carcass. These clinical signs and gross lesions observed in the affected birds were all suggestive of IBD. More or less similar types of signs and/or gross lesions have been recorded earlier by various researchers ***(El-Khayat, 2003; Sara et al, 2014).*** All these symptoms may be attributed to the harmful effect of the virus on the kidney.

The definite identification of IBDV was obtained using RT-PCR which was known to be a sensitive test to detect the IBDV ***(Abdel-Alem et al, 2003).*** In this study 10 out of 15 field samples were positive by RT-PCR. Similar results were obtained by ***Moemen et al (2014).***

In this study amino acid sequencing, comparative alignment, and phylogenetic analysis of the (hVP2) was done for selected four field samples.

The deduced amino acid sequences for each tested IBDV samples from position **(201 to 338)** numbering according to **(Bayliss *et al.*, 1990)** were analyzed.

The virulence marker aa residues 222(A), 242(I), 253(Q), 256(I), 279(D), 284(A), 299(S), 330(S) and 451 (L) are conserved in the VP2 of vvIBDVs (Van Den Berg *et al.,* 2004).

In our study all of the examined samples had 8 of the virulence marker aa residues (except 451 L not involved in the sequenced fragment in this study). The presence of these aa residues in the four samples proved the very virulence nature of these samples.

The hydrophilic regions A (from aa 212 to 224), B (from aa 312 to 324), 1 (from aa 248 to 254) and 2 (from aa 279 to 290) have been shown to be important in the binding of neutralizing monoclonal antibodies and are presumed to be the dominant parts of the neutralizing domain ***(Schnitzler et al, 1993).***

Sequencing results in this study revealed changes in amino acid substitutions at major hydrophilic peak A at position (220 and 222).

Substitution occur at position 220 revealed that the four examined samples had F220 that was similar to vv strain Giza 2000 and Giza 2008.

Presence of Alanine (A) at position 222 instead of Proline (P) in the four sequenced samples was similar to very virulent Giza 2008. Amino acid residue at the position 222 is very critical as various publications reported a change in the aa residue at position 222 in some vvIBDV strains (P to A) and in some variant strains such as variant A (P to Q) ***(Heine et al, 1991)***. Similar results were predicted by ***Brown et al (1994)***.

In minor hydrophilic peak 1, regarding to aa residue at position 253 (Q) our results agreed with ***Islam et al (2012)*** who reported that presence of Glutamine (Q) residue at position 253 was found in the two isolates examined in his study as well as the previously isolated Bangladeshi IBDV. This residue thought to be responsible for pathogenicity and is unique to highly virulent IBDV. This result also supported by ***Negash et al (2012)*** who reported Glutamine (Q) at position 253.

Other amino acid substitution occur at position 254, that four examined samples had Serine **(S)** at position 254 that agreed with ***Negash et al (2012)*** who reported that all Ethiopian strains have amino acid residues characteristic of variant strains at the minor hydrophilic peak 1 position (254S). Exchanges of amino acid in this position may likely result in antigenic changes ***(Durairaj et al, 2011)***, and lead to a modified structure of the neutralizing epitopes ***(Martin et al, 2007)***, which was verified recently in an in *vivo* study ***(Jackwood and Sommer Wanger, 2011).***

These results agreed with ***Brandt et al (2001)*** who reported that reverse genetic experiments have shown that the amino acid at position 253 in VP2 affects the virulence of IBDV.

In minor hydrophilic peak 2, other aa substitution at position 279 and 284 were shown. Four examined samples had Aspartic acid (D) at position 279 and Alanine (A) at position 284 that were similar to vvIBDV Giza 2000 and Giza 2008. This is in agreement with ***Islam et al (2012)*** who reported that presence of Alanine (A) at position 284 was found in the two isolates examined in his study.

Hernandez et al (2006) noticed that four amino acid residues have been conserved in most vvIBDV strains (222A, 256I, 294I and 299S) and were detected in all Uruguayan sequences. All of these amino acid residues were detected in the four examined samples

The serine rich heptapeptide SWSASGS (326-332) located immediately downstream of the second hydrophilic region was believed to be involved in the virulence of IBDV ***(Heine et al, 1991)***. At this study presence of typical heptapeptide (SWSASGS)

in the four examined samples indicating that they were pathogenic strains. This was in agreement with ***Heine et al (1991)*** and ***Razmayr and peighambari (2008).***

These results indicate that our strains are highly related to vvIBDV strains belonging to serotype 1 isolates and also indicating that they are pathogenic strains and that vvIBDV belong to Egyptian strains succeeded in surviving in the Egyptian environment despite the intensive use of vaccination programs. This observation had the support of ***Eterradossi et al (2004)***.

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التوصيف الجينى لفيروسات مرض التهاب غدة فابريشيس المرتبط بتفشى مرض الجمبورو فى قطعان الدواجن فى محافظة الشرقيه، مصر

عمرو عبد العظيم حشيش[٢]، عبد الله عبد الظاهر سليم[٢]،محمد فوزى مندور[١]، محمد عبد الدايم[١]، شهيره عبدالوهاب[١]،مختار الطرابيلى[١]

[١]قسم الفيروسات، كلية الطب البيطري، جامعة قناة السويس، الاسماعيليه، جمهورية مصر العربية
[٢]المعمل المرجعى للرقابه البيطريه على الانتاج الداجنى ، معهد بحوث صحة الحيوان، جمهورية مصر العربية

اتخذ هذا العمل البحثى الحالى اهدافه فى التوصيف الجزيئى لعينات فيروس مرض التهاب غدة فايبريشيس. حيث تم عزل عترات من عدد ١٥ عينه من فيروس مرض التهاب غدة فايبريشيس من قطعان الدجاج المختلفه فى محافظة الشرقيه التى تظهر عليها اعراض الاصابه بالمرض. حيث تم تجميع العينات عشوائيا من مناطق مختلفه من محافظة الشرقيه.
بتشريح العينات تبين وجود جفاف تحت الجلد كذا وجود تغيرات تنكسيه فى الكليه اضافة الى وجود نزيف فى الغشاء المخاطى للاغشيه الالغديه بجانب وجود تضخم فى غدة فايبريشيس.
و قد تم تحضير معلق من ال ١٥ عينه المجمعه كل على حده فى محلول الفوسفات المعقم. ثم تم طرد المعلق مركزيا و استخدام الجزء الطاف فى استخراج الحامض النووى RNA للفيروس.
ثم تم اختبار العينات لوجود الفيروس باستخدام تفاعل البلمره المتسلسل التصاعدى العكسى عن طريق استخدام بادئين يحيطان المنطقتين العظمى و الصغرى لبروتين الفيروس 2. و قد نتج عن استخدام هذا البادىء منطقه مكبره بحجم 620 Bp. و كان عدد النتائج الايجابيه ١٠ عينه من اصل ١٥ عينه.
ثم تم اختيار عدد٤ عينه من اصل عدد١٠ عينات ايجابيه ليتم اجراء التحليل الجينى لها بناءا على أعلى نتيجه ايجابيه.
و قد اظهرت نتائج التحليل الجينى ان كل العينات المختاره اظهرت نسبة تشابه عاليه للمعزول المصرى vvIBDV Giza 2008 .
وقد اظهرت نتائج هذه الدراسه استمرار تحور الفيروس و مدى وبائية الفيروس فى قطعان الدواجن فى مصر على الرغم من برامج التحصينات المختلفه و المستخدمه لمقاومة المرض. لذلك ننصح بادماج هذه العترات فى أى لقاحات تحضيريه مستقبليه.