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EPIDEMIOLOGICAL AND MOLECULAR STUDIES ON INFECTIOUS BURSAL DISEASE VIRUS INFECTION IN LUXOR PROVINCE

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ABSTRACT

The infectious bursal disease (IBD) is a continuing serious problem facing poultry industry in Egypt. In this study, 500 bursae samples were collected from different broiler flocks in different localities of Luxor Governorate. The flocks were suffering from mortality and bursal lesions during Dec.2014 to Jan. 2016. The samples were tested by AGPT and the result was 12 flocks only were positive for IBD and then the positive samples were isolated in SPF embryonated chicken eggs. All the inoculated embryos died within 2-3 days. The embryos were smaller than normal, congested with haemorrhagic head, and necrotic foci of liver. Then the presence of virus in embryonated eggs was confirmed by convential PCR. Furthermore, molecular characterization was performed by direct sequencing of a 620-bp cDNA corresponding to the VP2 variable domain of the polyprotein gene synthesized by PCR. With deduced amino acid analysis found that all examined isolates are very virulent strains. four local strains used for nucleotide sequence, percent identity and phylogenetic tree analysis revealed that four isolates (F21,F23,F24,F26) were very close to very virulent old Egyptian strains Giza 2008.

***Key Words**: IBDV; PCR; VP2; Pathotyping*

INTRODUCTION

Infectious bursal disease (IBD) is an acute highly contagious viral disease of young birds characterized mainly by sever lesions in bursa of fabricious and immunosuppression in chickens (Eterradossi and Saif, 2008).

Infectious bursal disease has a great concern in Egyptian poultry industry for a long time but particularly for the past decade. Infectious bursal disease virus is a member of the genus Birnavirus family Birnaviridae which has the potential of immunizing the chicks even in the presence of moderately higher levels of maternally derived antibodies (MDA) (Delmas *et al.,* 2011). Firstly reported as severe kidney lesions; later it was termed as Infectious Bursal Disease virus (IBDv) referring to the specific lesions caused by the disease in the bursa of fabricious, and severe renal damages (Eterradossi and Saif, 2008).

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The IBDV genome has two segments designated A and B segments A (3.4 kb) and B (2.8kb). The segment A encodes 4 viral proteins, the two capsid proteins VP2 (48 kDa) and VP3 (32 -35 kDa) and viral protease VP4 (24 kDa) and nonstructural protein VP5 (17-21 kDa). The smaller segment B encodes RNA-dependent RNA polymerase VP1 (90 kDa) (Xu *et al.,* 2011).

Infectious bursal disease virus (IBDV) infection destroys the bursa of Fabricius, causing immunosuppression and rendering chickens susceptible to secondary bacterial or viral infections (Lee *et al.,* 2015).

Sever acute disease of 3-6 week-old birds is associated with high mortality, but a less acute or subclinical disease is common in 0-3-week-old birds. This can cause secondary problems due to its effect on the bursa of Fabricius. IBD virus (IBDV) causes lymphoid depletion of the bursa, and if this occurs in the first 2 weeks of life, significant depression of the humeral antibody response may result. Two distinct serotypes of infectious bursal disease virus (IBDV) are known to exist. Serotype 1 virus causes clinical disease in chickens younger than 10 weeks. Older chickens usually show no clinical signs. Antibodies

are sometimes found in other avian species, but no signs of infection are seen. Serotype 2 antibodies are very widespread in turkeys and are sometimes found in chickens and ducks. There are no reports of clinical disease caused by infection with serotype 2 virus (Lasher and Shane, 1994).

In spite of the most commercial broiler chicken flocks that are vaccinated against IBD, severe outbreaks were reported in Egypt, caused high mortalities, and have become a priority problem (El-Batrawi, 1990 and Bekhit, 1998).

MATERIALS AND METHODS

1. Field samples

Five hundred (n=500) samples were collected from fifty commercial poultry flocks (3-6 weeks of age) in different localities of Luxor governorate during the period (December 2014 to January 2016) suspected of having IBD. Post mortem examination was performed on a variable number of freshly dead birds which succumbed to the disease after onset of mortalities on the examined farm, Gross lesions were recorded from birds with typical IBD lesions.

2. Detection of IBDV antigen in bursal homogenates by AGPT

Pooled bursal homogenates were prepared from the affected chicken farm and were checked by the AGPT, using reference IBD serotype 1 antiserum, and known reference positive and negative bursal homogenates antigen as control were performed. Reaction appeared as 2-3 precipitation lines within 48 hours after setting the test.

3. Isolation of IBDV using egg inoculation by CAM route

All positive samples from AGPT were isolated in SPF ECE via CAM route. The inoculated embryos showed 100% mortalities within 3 days.

4. Confirmation of positive Isolates from egg inoculation by Convential PCR:

All isolated positive samples were confirmed for presence of IBDV using convential PCR. RNAs were extracted from the bursal homogenates using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif. USA) according to the manufacturer's instruction.

A set of primers were used for RT-PCR reaction using forward and reverse PCR primer

Forward primer AUS GU:5’TCA CCG TCC TCA GCT TAC CCA CAT C-3'

Reverse primer AUS GL5’GGA TTT GGG ATC AGC TCG AAG TTG C-3;

- Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR): using Qiagen one step RT-PCR Kit. (According to the manufacturer's instruction).

- Agarose Gel Electrophoresis

The PCR products were inoculated in 1.5 % agarose gel placed in the electrophoresis chamber and covered with electrolyte solution in I X TBE buffer with allowing running the PCR product in the gel at constant volt for 40 minute, to determine the base pairs of the PCR product which could be visualized by the presence of marker (Qiagen) Gene ruler 100 bp plus DNA ladder and using Gel documentation system.

5. Sequence for the purified PCR product:

Gel containing DNA band of the expected size (620 bp) was excised and purified with the QIA quick Gel Extraction Kit (Qiagen) according to the manufacturer instruction. The purified PCR products were sequenced directly using the ABI PRISM. Big Dye TM Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, CA,USA), The products of the sequencing reactions were cleaned – up using Centrisep purification kit Analyzer (Applied Biosystems, Foster city, CA,USA) and the purified products were sequenced directly using the ABI (Applied Biosystems, Foster city, CA,USA) and the ABI PRISM 3130 genetic analyzer (Applied Biosystems).

The phylogram was drawn using MEGA 5 software. The alignment of the viruses in the study was done using DNA star – Meg Align software. Egyptian viruses and other international reference strains from the Genbank were available from the National Center for Biotechnology Information (NCBI). Finally the identity percent and divergence between all viruses was carried out.

RESULTS

1. Detection of IBDV antigen in bursal homogenates by AGPT: The result recorded twelve farms out of fifty farms were positive for IBD by AGPT.

2. Isolation of IBDV using egg inoculation by CAM route: The result of isolation revealed ten farms were positive for virus isolation.

3. Confirmation of positive samples from inoculation by convential PCR:

The presence of virus in embryonating eggs was confirmed by convential PCR. All PCR positive samples showed specific bands at 620 bp on agarose gel. **Fig 1.**

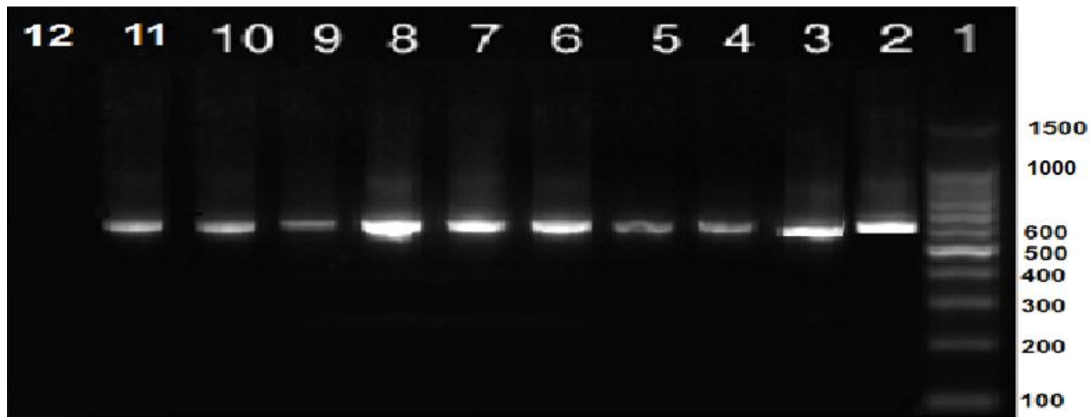

Figure 1: Gel electrophoresis of RT-PCR showing 620 bpbands in the positive samples.

4. Phylogenetic characterization

Phylogenetic tree of Nucleotide sequences of VP2 gene: There were four new Egyptian isolates (F21, F23, F24, F26) characterized as very virulent and were very close to old Egyptian viruses Giza 2008. **Fig 2.**

Four samples were selected from positive isolates for sequencing (F 21, F 23, F 24, F 26) .And a sequence of 33 nucleotides were used for nucleotides analysis and deduced amino acid analysis. Sequence analysis and comparison of 620 bp of the hypervarible region of VP2 for the four local isolates with various reference IBD strains revealed that F 21,F 23, F 24, F26 are very virulent and more closer to The Egyptian VVIBDV strain (Giza 2008). F21 had a highest nucleotide identity to VVIBDV strain Giza 2008 (91.1 %). F 23 had a highest nucleotide identity to VVIBDV strain Giza 2008 (92 %). F 24 had a highest nucleotide identity to VVIBDV strain Giza 2008 with Percentage (85.7%). F 26 had a highest nucleotide identity to VVIBDV strain Giza 2008 with percentage (89.3%). **Fig 3.**

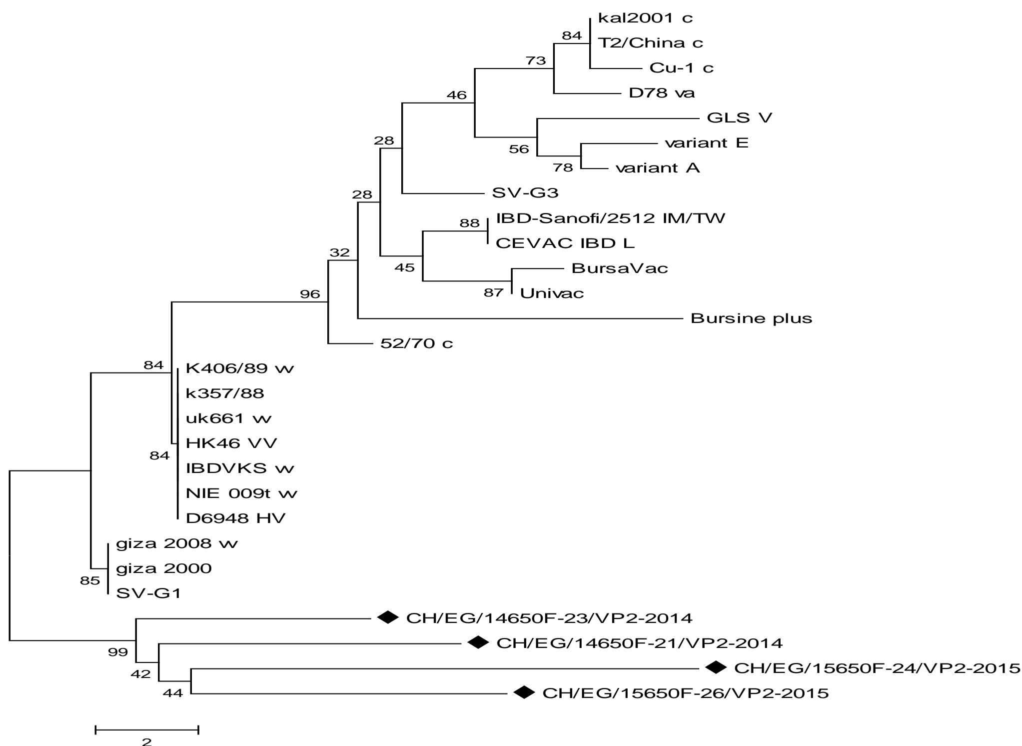

Figure 2: Phylogenetic analysis tree of the studied IBDV strains (F 21, F23, F24, F26) and other references classical, very virulent, variant and vaccine strains of IBDV.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
1		100.0	92.9	100.0	89.3	91.1	92.0	89.3	90.2	90.2	91.1	92.0	92.9	92.9	91.1	92.0	85.7	89.3	1	giza-2008-w
2	0.0		92.9	100.0	89.3	91.1	92.0	89.3	90.2	90.2	91.1	92.0	92.9	92.9	91.1	92.0	85.7	89.3	2	giza-2000
3	7.5	7.5		92.9	91.1	96.4	97.3	94.6	93.8	95.5	92.0	92.9	100.0	95.5	83.9	85.7	80.4	83.9	3	IBD-Sanofi/2512-IM/TW
4	0.0	0.0	7.5		89.3	91.1	92.0	89.3	90.2	90.2	91.1	92.0	92.9	92.9	91.1	92.0	85.7	89.3	4	SV-G1
5	11.6	11.6	9.5	11.6		91.1	92.0	91.1	90.2	92.0	90.2	91.1	91.1	92.0	80.4	82.1	76.8	80.4	5	Bursine-plus
6	9.5	9.5	3.7	9.5	9.5		99.1	94.6	93.8	95.5	92.0	92.9	96.4	95.5	82.1	83.9	78.6	82.1	6	BursaVac
7	8.5	8.5	2.7	8.5	8.5	0.9		95.5	94.6	96.4	92.9	93.8	97.3	96.4	83.0	84.8	79.5	83.0	7	Univac
8	11.6	11.6	5.6	11.6	9.5	5.6	4.6		97.3	99.1	92.0	92.9	94.6	95.5	80.4	82.1	76.8	80.4	8	Cu-1-c
9	10.6	10.6	6.5	10.6	10.6	6.5	5.6	2.7		98.2	92.9	93.8	93.8	94.6	81.2	83.0	78.6	81.2	9	D78-va
10	10.6	10.6	4.6	10.6	8.5	4.6	3.7	0.9	1.8		92.9	93.8	95.5	96.4	81.2	83.0	77.7	81.2	10	kal2001-c
11	9.5	9.5	8.5	9.5	10.6	8.5	7.5	8.5	7.5	7.5		98.2	92.0	93.8	82.1	83.0	77.7	82.1	11	variant-E
12	8.5	8.5	7.5	8.5	9.5	7.5	6.5	7.5	6.5	6.5	1.8		92.9	94.6	83.0	83.9	78.6	83.0	12	variant-A
13	7.5	7.5	0.0	7.5	9.5	3.7	2.7	5.6	6.5	4.6	8.5	7.5		95.5	83.9	85.7	80.4	83.9	13	CEVAC-IBD-L-
14	7.5	7.5	4.6	7.5	8.5	4.6	3.7	4.6	5.6	3.7	6.5	5.6	4.6		83.9	85.7	79.5	83.0	14	SV-G3
15	9.5	9.5	18.1	9.5	22.8	20.5	19.3	22.8	21.6	21.6	20.5	19.3	18.1	18.1		90.2	85.7	88.4	15	CH/EG/14650F-21/VP2-2014
16	8.5	8.5	15.9	8.5	20.5	18.1	17.0	20.5	19.3	19.3	19.3	18.1	15.9	15.9	10.6		86.6	89.3	16	CH/EG/14650F-23/VP2-2014
17	15.9	15.9	22.8	15.9	27.8	25.3	24.1	27.8	25.3	26.6	26.6	25.3	22.8	24.1	15.9	14.8		85.7	17	CH/EG/15650F-24/VP2-2015
18	11.6	11.6	18.1	11.6	22.8	20.5	19.3	22.8	21.6	21.6	20.5	19.3	18.1	19.3	12.6	11.6	15.9		18	CH/EG/15650F-26/VP2-2015
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		

Figure 3: The similarity between IBDV isolates and other Egyptian and representative reference strains.

DISCUSSION

The clinical signs include sudden onset, ruffled feathers, prostration, diarrhea, and death. Disseminated muscular hemorrhages, the kidneys were swollen and enlargement with edema in BF, sometimes with bloody exudates were observed at postmortem examination. Our results were agreed with (Paula *et al.,* 2004; Richard and Miles*,* 2004; Okoyo and Uzonkwu*,* 2005 and Rajaonarison *et al.,* 2006).

Ten farms were positive from fifty farms with percentage 20%, these similar tothe results of Roussan *et al.* (2012) that used 80 bursa fabricius samples were examined from 20 commercial broiler chicken flocks in Jordan with clinical symptoms of IBD. However, 20%of local IBDV strains were positive using a specific primer for vvIBDV. While Jackwood and Nielsen. (1997) revealed only 48 bursal samples out of 151 collected from the United States, Mexico, and Puetro Rico were detected to contain IBDV using RT-PCR assay.

In the present investigated outbreak, IBD was laboratory diagnosed, based on the demonstration of IBDV antigens (s) by the AGPT in bursal homogenates from fresh, acutely affected birds, which appeared as precipitation line. The successful use of the AGPT as a rapid diagnostic means for IBDV antigen detection in the bursa of acutely affected birds have been previously reported by Rosenberger *et al.* (2008).

Trial to isolate IBDV from AGPT- positive samples of bursal homogenates by chicken embryo inoculation via CAM in 10 days SPF embryonated chicken eggs resulted in embryo deaths during -5 days pi, associated with typical gross embryonic lesions recorded by Rosenberger *et al.* (2008).

In present study all ten positive homogenate samples from AGPT inoculated in chorioallantoic membrane (CAM) of 10 days SPF embryonated chicken eggs showed embryo lesions, they were typical characteristic to IBDV infection (edema, distention of abdominal region, cutaneous congestion, mottled necrosis and ecchymotic hemorrhages on the liver).Similar lesions were recorded by (Lukert*,* 1992 and Amer *et al.,* 2007).

All positive samples from isolation were confirmed by convential PCR given amplicon size 620bp in the hyper variable region of the VP2 gene. (Fig1). These similar to result by Naglaa *et al.* (2015) that isolated IBDV from chicken broiler farms in different Egyptian Governorates and all isolated IBDV subjected to molecular detection by RT-PCR which showed amplification of a 620 bp fragment. The similar result were obtained by Mohammed (2013) who isolated IBDV from south valley area and the result by RT-PCR showed positive reaction and giving amplicon size at 620bp. These similar result were obtained by Metwally *et al.* (2009) who reported that bursa and proventriculus samples were RT-PCR tested using novel primers flanking VP2 region coding the two major and two minor hydrophilic peaks produced amplicons size 620bp.

Nucleic acid-based methods are useful tools for direct detection and sub typing without isolation and propagation (Stram *et al.,* 1994). Reverse transcription polymerase chain reaction (RT- PCR) techniques on selected fragments of the genome, essentially the variable domain of VP2 followed by sequencing and phylogenetic comparison represents

a valuable molecular alternative for the classification of IBDV strains (Van Den Berg., 2000). The significance of VP2 molecular analysis stems from that VP2 is responsible for virulence and pathogenicity (Garriga *et al*., 2006) as well as antigenic variations (Letzel *et al.,* 2007). The VP2 gene is commonly studied because it encodes for the major protective epitopes, contains determinants for pathogenicity, and is highly variable among strains (Abdel-Alim, 2003; Jackwood and Sommer-Wanger, 2006).

A 620bp product was generated from an infected bursa, using simplified RNA extraction procedure and RT-PCR. It was used as template for automated sequencing. Ncleotide sequences of the hypervariable region of the VP2 gene of isolates. The examined isolates showed similarity and identity ranged from 92% to 85.7% with other reference (very virulent, classical and variant) strains. These results agree with (Kataria *et al.,* 2001) who reported that none of the Indian isolates were 100% similar, But Kasanga *et al.* (2007) reported 17 nucleotides differences among 14 Tanzanian IBDVs sequences. Three of this sequence (KARS-53, KDSM-32 and KMRG-46) were identical even though they were isolated in different years and places and the other 11 were different from each other. The hyper variable region of the VP2 was chosen for our study because it has been shown to mutate frequently. Using more conserved regions of the genome for comparison of the isolates would not allow us adequate discrimination among the strains. Antigenic and molecular analysis of this part of the protein helped in understanding the nature and behavior of the isolated strains *(*Zierenberg *et al.,* 2000).

The nucleotide sequence of the VP2 hypervaraible region of our four examined strains was compared with different reference strains and revealed that (F 21, F 23, F 24, F 26) more identity to Egyptian very virulent IBDVs (Giza 2008). F 21 revealed highest identity (91.1%) to Giza 2008. F 23 had highest nucleotide identity (92%) to Egyptian very virulent IBDV (Giza 2008). Also F 24 and F 26 had identity to Giza 2008 (85.7 % - 89.3%).

In this study phylogenetic tree showed that our isolated field viruses (F 21, F23, F24, F26) were close to each other and also were close to old Egyptian virus Giza 2008.

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دراسات وبائية وجزيئية علي مرض التهاب كيس فبريس المعدي في محافظة الأقصر

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يعتبر مرض التهاب جراب فابريشيا مشكله خطيرة مستمرة تواجه صناعه الدواجن في مصر. وخلال هذه الدراسة تم فحص ٥٠٠ عينه جراب فابريشيا من أصل خمسين مزرعة تم جمعها من مناطق مختلفة في محافظه الأقصر كانت تعاني من وفيات وإصابة في جراب فابريشيا وذلك في الفترة من ديسمبر ٢٠١٤ إلي يناير ٢٠١٦ وتم اختبار العينات بواسطة اختبار الترسيب في الأجار وكانت النتيجة ١٢ مزرعة ايجابيه وتم عزل العينات الايجابية الناتجة من اختبار الترسيب في الأجار عن طريق الحقن في الاجنه وكانت الإصابات الملحوظة عبارة عن نزيف في الرأس ، ارتشاح مائي محيط بالجنين ، احتقان ونزف علي القدمين والمناطق الدماغية وعلي الكبد وكانت الوفيات بنسبه ١٠٠%. وتم تأكيد وجود الفيروس في أجنة البيض المحقونة بواسطة تفاعل إنزيم البلمره التسلسلي، علاوة علي ذلك تم إجراء التوصيف الجزيئي بواسطة التسلسل المباشر ٦٢٠ زوج من القاعدة في المنطقة شديدة التغير في جزيء البروتين الثاني للفيروس. وأشار تحليل تسلسل استخلاص الحمض الاميني من الجين في جزيء البروتين الثاني أن جميع المعزولات التي فحصها بين سلالات IBDV شديدة الضراوة. وكشفت الأربع سلالات المحلية المستخدمة لتسلسل النيوكليتيدات ونسبه التقارب وتحليل شجرة النشوء والتطور أن الأربع معزولات (F21,F23,F24,F26) كانت قريبه جدا من السلالة المصرية شديدة الضراوة جيزة ٢٠٠٨.