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ISOLATION AND IDENTIFICATION OF INFECTIOUS BURSAL DISEASE VIRUS (IBDV) FROM INTERNAL ORGANS OF VACCINATED CHICKENS IN DAKAHLIA GOVERNORATE, EGYPT

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ABSTRACT

In the present study, a total of 35 samples including bursa of Fabricius, thymus, spleen,kidney and liver (7samples for each organ) were collected from vaccinated chickens flocks at Dakahlia Governorate, Egypt during the period from March 2015 to May 2015. Each sample was pooled randomly from three to five birds (25 to 44 days old). Trials for isolation of the suspected virus from the collected samples were carried out via chorioallantoic membranes (CAMs) of 9 days embryonated chicken eggs (ECEs), collected from hens free from infectious bursal disease virus (IBDV). Three egg passages were carried out for each sample. Hyperimmune serum was prepared against standard IBDV. IBDV in both field and egg passaged samples (after 3rd passage) was identified by agar gel precipitation test (AGPT) and indirect fluorescence antibody test (IFAT). The results revealed that 30 samples (85%) including 7 (70%) samples from bursa of Fabricius and 6 (60%) samples from thymus, spleen and liver and 5(50%) samples from kidney, showed positive results in virus isolation. The positive results percentage by AGPT and IFAT for field samples were 88.5% (31 out of 35samples), while after 3rd passage in ECEs were 70% (22 out of 35 samples) and 88.5%(31 out of 35samples) respectively. The results indicate that the bursa of Fabricius is the best organ for IBDV isolation and AGPT and IFAT could be used for IBDV diagnosis.

Keywords: *Infectious bursal disease virus(IBDV), Virus isolation, agar gel precipitation test (AGPT), indirect fluorescence antibody test (IFAT).*

INTRODUCTION

Infectious bursal disease (IBD) also known as Gumboro disease is a highly contagious and acute immunosuppressive viral disease of young chickens characterized by destruction of actively dividing immature IgM-bearing B cells in the bursa of Fabricius **(Sharma et al., 2000)**. Chickens are the only bird species known to develop clinical disease **(Van den Berg et al., 2000)**. The chickens are more susceptible to the disease when they are 2–6 weeks of age when the bursa of Fabricius is highly developed.

Infectious bursal disease virus (IBDV), the causative agent of IBD, is non-enveloped icosahedral symmetry, bisegmented virus belongs to genus Avibirnavirus of family Birnaviridae **(Mertens et al., 2015)**. Two antigenically distinct serotypes, designated as serotypes 1 and 2 have been identified **(McFerran 1980)**. Pathogenic strains are

grouped in serotype 1 viruses while serotype 2 strains are non-pathogenic **(Van den berg et al., 2004)**. Since the disease was recognized in the first outbreak in 1962 **(Cosgrove 1962)**, it continues to pose a threat to the poultry industry all over the world. In Egypt, the first occurrence of IBD was recorded in 1974 **(El-Sergany et al., 1974)** while the first isolation and identification of IBDV was done in 1976 **(Ayoub and Malek 1976).** Recently, evidence of circulating very virulent IBDV strains (vvIBDV) were isolated from Egyptian vaccinated flocks was reported **(Abdel-Mawgod et al., 2014, El-Bagoury et al., 2015)**.

IBD causes sharp economic losses in poultry industry represented by high morbidity and mortalities up to 30% or more and also a severe prolonged immunosuppression; so attention has been given to the early diagnosis of IBDV. Diagnosis of IBDV is based on clinical signs, virus isolation, and histopathology, serological and molecular methods **(OIE 2008)**.

The present study aimed to isolate IBDV from vaccinated chicken flocks in Egypt using ECEs and identification of the virus using agar gel precipitation test (AGPT) and indirect fluorescence antibody test (IFAT) in both field tissues and in egg passaged samples after three passages**.**

MATERIAL AND METHODS

Clinical specimens:

A total of 35 pooled samples including bursa of Fabricius, thymus, kidney, spleen, and liver (7 samples for each organ) were collected from vaccinated chicken's flocks. Each sample was pooled randomly from three to five birds (25 to 44 days old). Diseased chicken exhibited white diarrhea, anorexia, haemorrhage in the thigh muscles , leg and breast muscles, the bursa of Fabricius may be hypertrophic, haemorrhagic and oedematous.

Samples of bursa of Fabricius, thymus, spleen, kidney and liver from healthy and non-vaccinated chickens (4 weeks old age) were included as negative controls. Each collected sample was divided into two parts; part was taken rapidly to the freezing chamber of a cryostat for IFAT and another part was put in a sterile plastic bottle containing phosphate buffer saline (PBS) with antibiotics (1000 U/ml penicillin, 1000µg/ml streptomycin and 500 µg/ml gentamycin) then transported to laboratory in an ice box and stored at – 20 °C till used for virus isolation and identification.

Standard IBDV vaccine:

Live IBD vaccine (Bursine 2) having a titer of $10^{4.3}EID_{50}$ /ml was purchased from Pfizer animal health company in a lyophilized form and reconstituted by addition of 4 ml sterile PBS (according to the manufacturer).

Isolation of the suspected virus on ECEs:

Samples were prepared for virus isolation according to **Rodriguez-Chavez et al. (2002)** as following: Each Sample was minced using sterile scissors and forceps then homogenized using sterile mortar and pestle. A volume of 9 ml of sterile PBS (pH 7.2) containing 1000 U/ml penicillin, 1000µg/ml streptomycin and 500 µg/ml gentamycin was added to 1 g samples making 10% w/v suspension. The sample suspension was followed by three

cycles of slow freezing and rapid thawing and centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and stored at - 20 ℃ till used.

Inoculation of the prepared samples on CAM of ECEs:

It was done according to **(OIE, 2015)**. 0.2 ml from prepared field tissues (bursa of Fabricius, thymus, spleen, kidney and liver) was inoculated by dropped membrane method onto the CAM of 9 days old ECEs obtained from hens free from IBDV. The dead embryos at 3-5 days post inoculation and all living embryos at seven days were chilled for 6 hours then were collected aseptically and examined for IBDV specific lesions and the observed lesions were recorded. Three egg serial passages were performed for each sample, at each passage embryos head and limbs were cut off and removed and the main body is minced together with CAMs in sterile PBS containing antibiotics making 10% w/v suspension. The homogenates were centrifuged at 3000 rpm for 10 minutes at room temperature and the virus-containing supernatant was collected, aliquoted, and stored at –20°C until used for subsequent passaging.

Titration of isolated virus in ECEs:

It was done according to **Rodriguez-Chavez et al. (2002)**. 3rd egg passage samples from bursa of Fabricius that gave clear lesions in embryo and on CAMs of ECEs were selected. The virus was titrated according to the formula of **Reed and Muench 1938**. The titer was expressed as $\log_{10}$ EID_{50}/1ml.

Preparation of hyperimmune rabbit sera:

It was done according to **Tanimura et al. (1995)** as following: Four NewZealand white rabbits were inoculated by three intramuscular injections of 1 ml reconstituted live IBD vaccine emulsified in1 ml Freund complete adjuvant (first injection) and 1 ml Freund incomplete adjuvant (second and third booster injections) at 3weeks intervals. The rabbits bled out 21 days after the last injection and the hyperimmune serum was separated by centrifugation at 3000rpm for 10 minutes and kept at -20°C till used. The fifth rabbit was not injected and housed separately from injected rabbits for collection of a negative control serum sample. The concentration of total proteins was detected by spectrophotometer using readymade kits (provided from Stanbio laboratory- USA) according to **Young (2001)** then compared with non-inoculated control rabbit and the reactivity of prepared hyperimmune serum was tested by AGPT against standard IBDV and examined later for observation of precipitation lines which indicate the positive results.

Identification of the isolated virus:

Agar gel precipitation test (AGPT):

The detection of suspected IBDV in field tissues and 3rd egg passaged samples using AGPT was done according to **OIE (2008)** as following: 10 ml of 1.5% agarose dissolve in PBS (pH 7.2) was poured in petri dish . In each dish ,6 peripheral wells and one central well were performed. The hyperimmune serum is put into the central well, while the peripheral surrounding wells are filled with the

supernatant fluid of field tissues (bursa of Fabricius, thymus, spleen, kidney and liver) and 3rd egg passage samples (embryo together with the CAM) , control positive IBDV(standard vaccine) and control negative IBDV(normal tissues and CAM) . Dishes are incubated at 37°C in a humidified chamber for 48 hours and examined later for observation of precipitation lines which indicate the positive results.

Indirect fluorescence antibody test (IFAT) for detection of IBDV in field samples and 3rd egg passages :

It was carried out according to **Allan et al. (1984)** as following: Prepared cryostat slides were fixed with cold acetone for 10 minutes then incubated for 1hour at 37°C with a few drops of 1:100 dilution of the prepared rabbit hyperimmune serum in a humidified chamber, then washing the slides with PBS pH 7.2 for 30 minutes 3 times (10 minutes each). Slides were incubated for 30 minutes at 37°C in the dark humidified chamber with a few drops of 1:200 dilution of antirabbit FITC conjugate (Sigma Aldrich Company) then, the slides were thoroughly washed with PBS three times for 15 minutes (5 minutes each), then mounted with glycerol, covered with a cover slip and examined under a fluorescent microscope to show yellowish green color which indicate a positive result.

RESULTS

As illustrated in **table (1)**, out of the 35 collected samples, the virus was successfully isolated on CAM of ECEs from 30 samples (85%) including 7(100%) samples from bursa of Fabricius and 6 (85.7%) samples from thymus, spleen and liver and 5(71.4%) samples from kidney. In positive cases the embryos were showed oedematous distention of the abdominal region, stunted growth **(Fig. 1)**, mottled appearance and ecchymotic hemorrhages or swollen greenish pale liver as well as pale appearance of the heart and the harvested CAM were congested and thickened. These lesions became more pronounced from the 2nd passage. The results indicate that the bursa of Fabricius is the best organ for IBDV isolation.

Titration of the isolated virus from bursa of Fabricius samples after 3rd passages on CAMs of ECEs showed that the virus titer was ($10^{5.5}$, $10^{4.2}$ and $10^{4.6}$ EID_{50}/1 ml) for samples number 2, 5, 7 respectively.

White line of precipitation was observed within 24-48 hours between prepared hyperimmune serum and 31(88.5%) field samples including 7 (100%) samples from bursa of Fabricius **(Fig. 2)** and 6 (85.7%) samples from thymus, spleen and liver and kidney.

Also, white line of precipitation was observed within 24-48 hours between prepared hyperimmune serum and 22 (70%) egg passage samples after 3rd passage in ECEs including 7 (100%) 3rd samples from bursa of Fabricius and 4(57.4%) 3rd samples from thymus, liver

and kidney and 3(42.8) 3rd samples from spleen. The obtained results showed that the bursa of Fabricius is the best organ for identification of IBDV by AGPT.

Regarding IFAT, yellowish green color as positive result appeared in 31(88.5%) field including 7 (100%) samples from bursa of Fabricius **(Fig. 2)** and 6 (85.7%) samples from thymus, spleen and liver and kidney samples **(Fig. 3-4)** and frozen CAMs prepared sections (after 3rd passage) including 7 (100%) 3rd samples from bursa of Fabricius **(Fig. 2)** and 6 (85.7%) 3rd samples from thymus, spleen and liver and kidney samples.

None of the negative control samples showed any positive reaction**.**

Table (1): Comparative results of the virus isolation and identification in 35 prepared field samples and after 3rd passage using AGPTand IFAT:

Samples type	No. of samples collected	Virus isolation		AGPT				IFAT			
		3rd passage possitive samples		Field tissues positive samples		3rd passage possitive samples		Field tissues positive samples		3rd passage possitive samples	
		No.	%	No.	%	No.	%	No.	%	No.	%
Bursa	7	7	100	7	100	7	100	7	100	7	100
Thymus	7	6	85.7	6	85.7	4	57.4	6	85.7	6	85.7
spleen	7	6	85.7	6	85.7	3	42.8	6	85.7	6	85.7
kidney	7	5	71.4	6	85.7	4	57.4	6	85.7	6	85.7
liver	7	6	85.7	6	85.7	4	57.7	6	85.7	6	85.7
total	35	30	85%	31	88.5	22	70	31	88.5	31	88.5

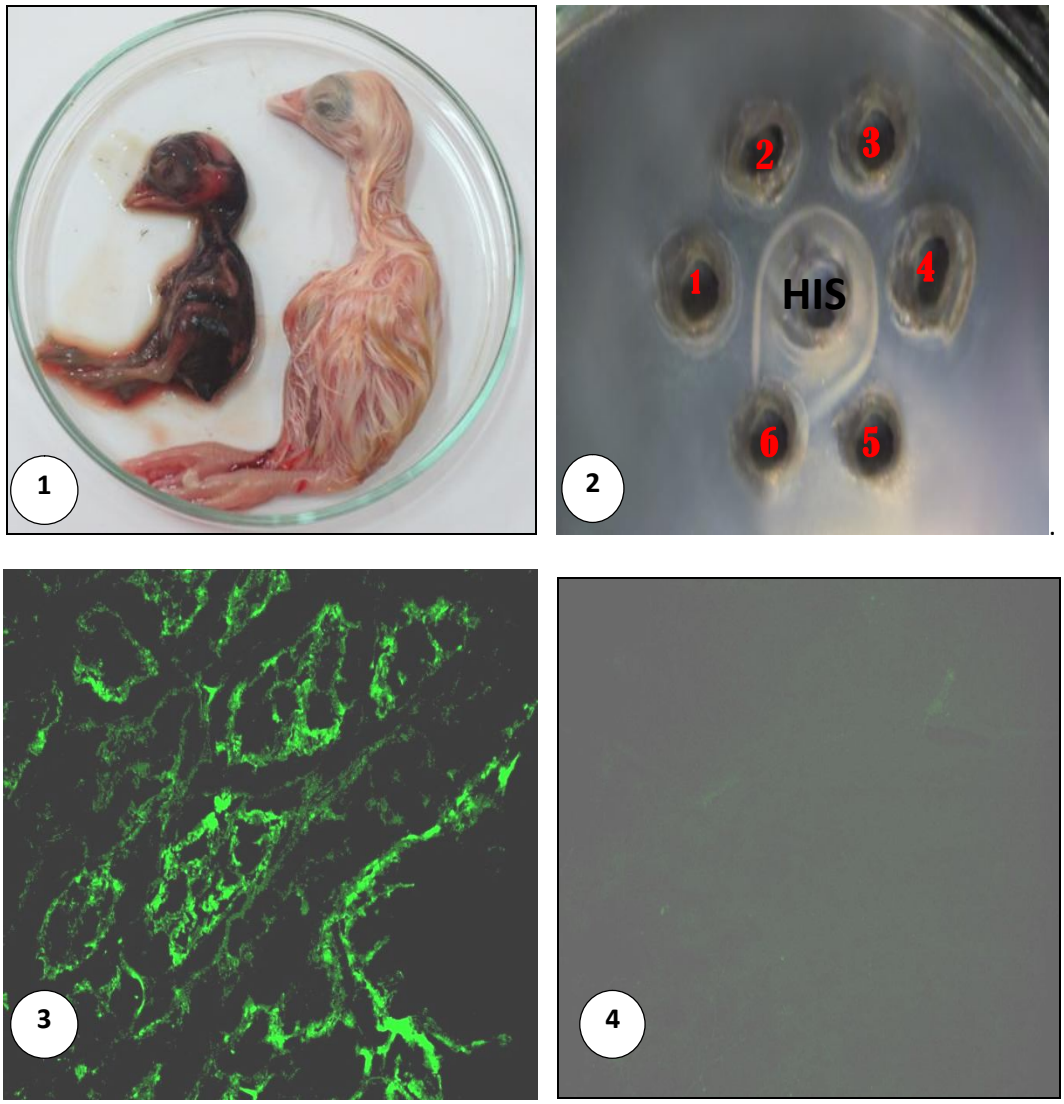

Fig. 1- 16 day old harvested egg embryo was infected with inoculated virus after 1st passage showed stunted growth comparing with normal embryo.

Fig. 2 - White clear precipitin lines appeared between bursa virus samples (1-4) and control +ve(5) and the prepared IBDV hyperimmune serum; No(6) indicate –ve result.

Fig. 3 – Positive immunofluorescence reaction shows yellowish green colour, in bursa of Fabricius section X40.

Fig. 4 - Negative immunofluorescence reaction,no yellowish green colour in cryostat section of bursa of Fabricius section X40.

DISCUSSION

In this study, a trial for isolation and identification of IBDV from the 7 vaccinated different poultry farms at Dakahlia Governorate was done. A total of 35 samples including bursa of Fabricius, thymus and spleen, kidney and liver (7samples for each organ) were collected as aseptically as possible from morbid and freshly dead chick's between 25 to 44 days old. Isolation of suspected IBDV on CAMs of ECEs was done for three passages. The lesions appeared on the inoculated embryo were showed oedematous distention of the abdominal region, stunted growth, mottled and ecchymotic hemorrhages or swollen greenish pale liver as well as pale appearance of the heart while the harvested CAMs were congested and thickened. These lesions became more pronounced from the 2nd passage. The similar results was reported by **Ibrahim (2011) and El-Bagoury et al. (2015)** who observed an increase in infectivity titer EID50 of IBDV inoculated ECEs from the first to the third passage and the positive lesions obtained were edematous and haemorrhagic embryos with liver necrosis and death while CAMs were edematous and haemorrhagic.

The bursa of Fabricius give better results than other organs including thymus, spleen, kidney and liver in virus isolation, these findings were in aggrement with **Rodriguez-Chavez et al. (2002)** who indicate that the bursa of Fabricius is the best organ for IBDV isolation.

IBDV was detected in field samples and after 3rd passages by AGPT. The obtained results for field samples revealed that, presence of IBDV in all 7 pooled bursal suspension and 6 samples from (thymus, spleen, kidney and liver), these results were in concurrence with the findings of **Mahmood and Siddique (2006)** who diagnosed the IBD in vaccinated broiler farms of 17 to 35 days old birds using AGPT in pooled bursa ,spleen, kidneys , liver and thymus suspension and homologous antiserum but disagree with **Faragher (1972)** who found that precipitating antigen being detected only in the bursa of the infected chickens. The result of virus identification by AGPT on 3rd egg passage agreed with results recorded by **Okoye and Uzoukwe(1990)** who confirmed isolated IBDV from thymus and spleen on CAMs of inoculated 10 day old ECES by AGPT and also with **Abdel Mawgod et al. (2014)** and **El-Bagoury et al. (2015)** who observed white line of precipitin between isolated IBDV on CAMs of inoculated SPF-ECEs from bursa samples and reference antiserum.

The results showed that not all samples positive on virus isolation showed positive results with AGPT. These results agreed with that of **Abdel Mawgod et al. (2014)** who failed to obtained white line of precipitin in AGPT from positive samples on isolation on ECE but disagree with **El-Bagoury et al. (2015)** who recorded that all samples positive on isolation on ECEs showed positive results with AGPT.

Concerning the results of indirect fluorescence antibody technique (IFAT), the results confirmed presence of IBDV as yellowish green colour in both 31 field tissues organs and after 3rd passages on CAMs. Our result in agreement with **Singh et al. (2015)** who found specific fluorescence reactions in bursa of Fabricius and spleen, thymus, kidney and liver but in contrast with **Nunoya et al. (1992)** who found specific fluorescence reactions in bursa of Fabricius and spleen but not in other organs including thymus, kidney and liver.

Further studies to investigate of IBDV at the molecular level using reverse transcriptase polymerase chain reaction (RT- PCR) depending on VP2 gene of IBDV and nucleotide and amino acid sequence analysis of amplified VP2 gene must be applied to identify and characterize of IBDV circulating in Dakahlia Governorate and formulating a suitable vaccine, used in protection against of local isolates recovered from field cases .

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الملخص العربي

عزل وتعريف فيروس غدة فابريشيس المعدي من الاعضاء الداخليه لدجاج محصن فى محافظه الدقهليه، مصر

ا.د/على على إبراهيم القناوى ، ط.ب امانى امين السيد و د/ محمد صلاح الثلث

قسم الفيروسات – كلية الطب البيطرى- جامعة المنصورة

فى هذه الدراسة، تم تجميع عدد ٣٥ عينه تتضمن ٧ عينات من غدة فابريشيس ، ٧ من الغدة الثيموسية ، ٧عينات من الطحال، ٧عينات من الكلى و٧ من الكبد و ذلك من قطيع دواجن محصنه بمحافظة الدقهلية خلال الفترة من مارس ٢٠١٥ حتى مايو ٢٠١٥ وقد تم تجميع كل عينه بصورة عشوائية من ثلاث إلى خمس طيور (عمر ٢٥-٤٤ يوما) يشتبه باصابتها بمرض غده فابريشيس المعدى . وقد تم عزل الفيروس المسبب للمرض في العينات التي تم جمعها من خلال حقنها على الغشاء اللقانقى المشيمى لبيض دجاج مخصب عمر ٩ أيام وقد تم اجراء ثلاثة تمريرات لكل عينة. ثم تم التعرف على الفيروس بأستخدام اختبار الترسيب فى الاجار واختبار الفلورسنتى المناعى غير المباشر فى الانسجه وكذلك فى الغشاء اللقانقى المشيمى المحقون (بعد التمريرة الثالثة).

وقد خلصت نتائج الدراسه الى انه قد تم عزل الفيروس من ٣٠ عينه من اصل ٣٥عينه مجمعه بنسبه ٨٥% هذه العينات تتضمن ٧(٧٠%) عينات من غدة فابريشيس و٦ (٦٠%) عينات من كلا من الغدة الثيموسية والطحال والكبد و٥ (٥٠%) عينات من الكلى . كما ظهرت النتائج الإيجابية لكلا من اختبار الترسيب فى الاجار واختبار الفلورسنتى المناعى غير المباشر فى ٣١ من العينات الحقليه بنسبه (٨٨,٥٪) في حين كانت بعد ثلاثه تمريرات على الغشاء اللقانقى المشيمى فى ٢٢بنسبه (٧٠٪) لاختبار الترسيب فى الاجار، و ٣١عينه بنسبه (٨٨,٥٪) لاختبار الفلورسنتى المناعى غير المباشر. قد تبين من النتائج افضلية غدة فابريشيس لعزل الفيروس بالمقارنة بباقى العينات . كما اشارت النتائج الى امكانية استخدام كلا من اختبار الترسيب فى الاجار واختبار الفلورسنتى المناعى غير المباشر في الكشف عن الفيروس .