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Isolation and Pathotyping of Infectious Bursal Disease Virus (IBDV) from Field Outbreaks among Chickens in Egypt

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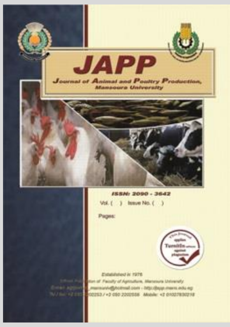

ABSTRACT

Infectious bursal disease (IBD) is a main health problem causing considerable economic losses in poultry flocks in Egypt. Throughout 2018-2019, bursae samples were assembled from different chicken's farms localized in four Egyptian governorates (Giza, Bani-Suif, Fayoum and Qalubia). The samples collected from farms recorded history of Infectious Bursal Disease **(**IBD) signs such as: sudden mortalities; depression; diarrhea; and hemorrhages on bursal tissues, leg and breast muscles. For virus isolation, the collected samples were cultivated in Specific Pathogen Free Embryonated Chickens Eggs (SPF–ECEs) via Chorio-Allantoic-Membrane (CAM).To confirm obtained data of virus isolation, both Agar gel precipitation test (AGPT) and the Reverse Transcription-Polymerase Chain Reaction (RT-PCR) were applied. In AGPT results, the four IBDV isolates gave positive reaction by reference IBDV antisera. RT-PCR was done for amplification of VP2 gene of IBDV isolates. The four viral isolates introduced specific band at size of 620 bp. Pathogenicity test for isolates of IBDV represented that the IBDV isolate no. 1, 2, 4 belong to classical virulent IBDV (cvIBDV) serotype, while IBDV isolate no. 3 belong to very virulent IBDV (vvIBDV) serotype that caused high mortality rates than cvIBDV isolates. The presented results emphasize the determined screened of the IBD field state, as well as apply of supplementary studies to found successful policies to obstruct the viral infection in chicken flocks in Egypt.

***Keywords*:** Infectious bursal disease (IBDV), virus isolation, Embryonated chicken egg inoculation, Agar gel precipitation test (AGPT), RT-PCR- pathogenicity test.

INTRODUCTION

Infectious Bursal Disease Virus (IBDV) is a very contagious and severe viral disease that differentiated by damage in the bursa of fabricius especially in lymphoid cells. The disease is a reason of an acute immunosuppression (Banda and Villegas, 2003 & Lukert and Saif, 2003). This viral disease was firstly documented in: Delaware State in USA in 1957, and it is named ''Gumboro''. Even as; it was firstly established in Egypt by El-Sergany in 1974 (Cosgrove, 1962 & El-Sergany *et al*., 1974) and both very virulent Infectious Bursal Disease Virus (vvIBDV) strains and variant IBDV strains were recorded. IBDV is a severe viral disease of chicks aged from 3 to 6 weeks. Characterization of IB viral disease was done based on some ruffled signs such as diarrhea that associated with high mortalities rates (up to 30%) (Eterradossi and Saif, 2013). Gumboro virus is classified to the family: Birnaviridae and Genus: Avibirnavirus (ICTV, 2017). Conversely, vaccines of classical Infectious Bursal Disease were introduced to a large number of chicken's flocks. Harsh incidences were recorded with massive mortalities rates. Gumboro virus is forming a timeless solemn problem in chicken's sectors; in Egypt (Helal *et al*., 2012; Mohamed *et al*., 2014; Abdel Mawgod *et al*., 2014). Viral replication of IBDV happens in differentiated bursa of fabricius lymphocytes; follow-on huge damage and disorders of developing B-lymphocytes cells, and obstructing the maturation of immune system (Wang *et al*., 2010; Biro *et al*., 2011; Liang *et al*., 2015). This conducts to an acute immunosuppression; in adding up amplified susceptibility of infected chickens to extra contagious diseases (Schat and Skinner, 2013). Gumboro virus has two serotypes: serotypes 1 and serotypes 2. Consequently; serotype one belongs to classical virulent Infectious Bursal Disease Virus (cvIBDV); very virulent Infectious Bursal Disease Virus (vvIBDV); antigenic variant Infectious Bursal Disease Virus (avIBDV) and attenuated gumboro virus (van den Berg *et al*., 2004; Li *et al*., 2009). Gumboro virus detection is chiefly depended on: etranal & postmortems signs and some serological methods such as; ELISA and agar gel precipitation test (AGPT) (Eterradossi and Saif, 2013). Gumboro virus genome consists of two segments of dsRNA; A and B. The segment-A is larger and encodes of about 110 kDa polyprotein; that is cleaved by viral protease (VP4) on the road to form the viral proteins (VP2, VP3, and VP4) and 4 structural peptides. The second segment partially overlapping, the polyprotein gene encodes viral protease (VP5); that has been identified both in infected chicken's embryos cells and in bursal tissues of chickens infected by gumboro virus (Murphy *et al*., 1999). Molecular techniques have been used to identify gumboro virus, and their uses increased in current years. RT-PCR method has been used to amplify sections regions of the genome of gumboro virus. VP2 is considered one of the important viral protease genes. It encodes for the most essential defensive epitopes that contains determinants for pathogenicity; and it is highly changeable in viral isolates (Abdel-Alem *et al*., 2003; Jackwood and Sommer-Wagner, 2007). So, molecular techniques are considering as a helpful tool for detection and identification of viral infection (Abdel-Alem *et al*., 2003;

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Jackwood and Sommer-Wagner, 2007). The present study was aimed to isolate and pathotyping of IBDV isolates from infected chicken in four governorates in Egypt and confirmed the virus isolation results by AGPT and RT-PCR technique.

MATERIALS AND METHODS

Field samples collection

During 2018–2019, sixty bursae were collected from chicken farms ranged between 15-32 days old from four Egyptian Governorates (Giza, Bani-Suif, Fayoum and Qalubia) showed depression, diarrhea, sudden mortality associated with hemorrhages on bursal tissues. Samples of bursae were obtained from morbid and recently dead chickens. The bursal samples from each governorate (15 bursae/ farm) were pooled and treated as single sample.

Samples preparations:

Bursae pooled samples were homogenized in: sterile phosphate buffer saline (PBS). Mixture of antibiotics: streptomycin sulphate (1mg/ml); gentamicin sulphate (0.4 mg/ml) and penicillin (1000 IU/ml) dissolved in 0.9% NaCl (SIGMA) was added to the homogenized samples to prepare 10% tissue suspensions. Followed by, centrifugation at 6000 rpm for 20 min. at 4^0C to clarify the suspension. The supernatant was filtered using syringe filters (0.45µm); then kept at -80^0C until use.

Virus isolation and titration

0.2 ml of filtrated samples were cultivated in 9 day old specific pathogen free (SPF) embryonated chicken's eggs (ECEs) achieved from the SPF production farm; Koum Oshiem; Fayoum; Egypt by chorio-allantoic-membrane (CAM) method. Then incubated at 37ºC and candling daily. CAMs and allantoic fluids were collected after 72-96 hrs of eggs inoculation according to the method described by Hitchner (1970). Followed by homogenization and centrifugation of the CAMs as mentioned above; then stored at –80ºC until using. The 50% egg infectious dose (EID_{50}) per ml was determined for viral titrations as earlier mentioned by Jackwood *et al*., (2009). The infectivity titers EID_{50}/ml were calculated according to method of Reed and Muench (1938).

Confirmation of IBDV isolates by AGPT:

Propagated of gumboro virus isolates were confirmed by Agar Gel Precipitation test by reference antisera at: Animal Health Research Institute (AHRI); Dokki, and Giza; Egypt according to Hirai *et al*., (1972).

RNA Extraction:

The Extraction of the viral RNA isolates was done by RNeasy® (QIAGEN GmbH; Hilden: Germany) according to the kit handbook instructions. After determining concentrations of viral RNA using the NanoDrop ND-1000; the viral nucleic acids were used for RT-PCR according to protocol mentioned by OIE (2016).

Design of Primers:

For amplification fragment of VP2 gene with respected size of 620 bp in viral isolates; set of primers were used. The forward PCR primer was: 5'-TCACCGTCCTCAGCT TACCCACATC-3'. The reverse PCR primer was: 5'-GGATTTGGGATCAGCTCGAAG TTGC-3'. (Metwally *et al*., 2009).

RT-PCR amplification:

The RT-PCR reaction was presented in a total volume of 50 µl per viral sample containing: extracted template RNA (10 µl), 5x RT-PCR buffer (10 µl), forward primer (2 µl), reverse primer (2 µl), dNTPs mix (2 µl) (400 µM of each dATP, dGTP, dCTP, dTTP, 2 µl of Qiagen One Step Enzyme Mix). Then, the final volume was completed to 500 µl by water free of RNase. Amplification of fragment of VP2 region was done by T3 thermal-cycler(Biometra-Germany); as follows: 20 min at 50°C; 95°C for 15 min followed by 39 three-step cycles of 94°C for 30 s, 59°C for 40s and 72°C for 1 min; finally 72°C for 10 min.

PCR Products Analysis:

Analyzes of PCR products (5 µl) after amplification were done by electrophoresis on a 1.5% agarose gel containing ethidium bromide dye with final (0.5 µg/ml) at 95 V for 30 min in 1x TBE buffer; aligned with GeneRulerTM100 bp Plus DNA Ladder. The gel was visualized by a gel documentation system according to method of Sambrook *et al*., (1989).

Determination of Pathotypes of IBDV isolates:

Seventy-five, chickens (4-weeks old) were separated into five equal groups (each group containing of 15 chickens). In first to forth group; chickens were treated by of viral isolates (10^5 EID_{50}/dose) via the intraocular route according to OIE (2016). The fifth group was kept as negative control group (inoculated via the intraocular route with Phosphate buffer saline). Each treatment was housed separately at virology unit, Dept. of Microbiolgy, Fac. of Agric., Ain Shams Univ. For ten days; chickens have been given water and feed *ad libitum*. Daily, chickens were examined for mortalities and morbidity. All surviving birds after 10 days post challenge were humanely euthanized. Bursa and body weights were recorded and the bursa–to–body weight (B: BW) ratio was calculated as: (bursal weight (g)/body weight (g)) × 1000. (OIE, 2016).

RESULTS AND DISCUSSION

Prevalence of IBDV and Post mortem findings:

Gumboro virus is being a harsh trouble in chicken′s flocks in Egypt. A protective program is critical to pass up virus infection (Hussein *et al*., 2003; Metwally *et al*., 2003). Immunosuppression significantly decreases the capability of young chickens to respond efficiently to vaccines and disposes them to infection by other pathogens. Detection of immunosuppression involves isolation and identification of pathogens using diagnostic tests (Frederic and Hoer, 2010). For this reason, this study was approved to isolate one of the most dangerous of the causative agent responsible for high mortality rates in chicken farms in Egypt. Sixty bursae were collected from different breeds of ages 15-35 days localized in four different Egyptian governorates. Diagnosis of the disease starts from surveillance of the clinical and post-mortem signs. In this study, IBDV was isolated from commercial chicken farms during 2018-2019 from four different governorates in Egypt. Chickens flocks showed depression, diarrhea, sudden high mortalities rate (20–50%) associated with and/or severe hemorrhages on bursal tissues as show in Fig.1. The severity of viral signs associated with the virulence of viral isolates, maternal immunity, age of chickens and the presence or absence of passive immunity (Hassan, 2004; Rauw *et al.,* 2007). Van den Berg *et al*., (2000) noticed that highly virulence viral isolates encourage more noticeable viral replication and pathogenesis than low virulent and moderate strains.

Fig. 1. Different clinical and postmortems signs appear on the native IBDV infected chickens such as diarrhea (A), depression (B) and hemorrhages on bursal tissues (C)

Cultivation and titration of IBDV in ECEs:

The four prepared samples were cultivated in the 9 day Embryonated Chicken Eggs (ECEs) with the rate of five ECEs as replicates per sample. Data on table (1) observed that, the samples caused death of 5 from 5 ECEs after 72-96 hrs. On the other hand, the control caused no death of the inoculated ECEs. The concentrations of the four IBDV isolates were determined and the dilution of inoculums producing 50 percent infection of eggs was calculated by Read Muench formula. The 50% egg infectious dose (EID50) was $10^{-6.7}$, $10^{-8.3}$, $10^{-9.7}$ and $10^{-6.5}$ EID_{50} per ml for isolates no. 1, 2, 3 and 4, respectively.

Table 1. Results of Virus isolation of bursal samples in ECEs:

Samples	Incubation period (hours)	No. of died embryos	Egg Infectious Dose (EID50)
Control	Zero	0/5	ND**
1*	96	5/5	$10^{-6.7}$
2	96	5/5	$10^{-8.3}$
3	72	5/5	$10^{-9.7}$
4	96	5/5	$10^{-6.5}$

***Samples no.1, 2, 3 and 4 collected from Giza, Bani-Suif, Fayoum and Qalubia, respectively. **ND = Not Determined.**

In this study data obtained from virus isolation process were confirmed by AGPT. Data presented in Fig. (2) confirm the isolation process of four IBDV isolates and all viral isolates gave positive reaction by pecific reference antisera of gumboro virus.

This data was arranged with that exposed by Islam *et al.,* (2005); Ibrahim (2011); Abdel Mawgod *et al*., (2014); El-Bagoury *et al*., (2015); Zohair *et al.*, (2017)

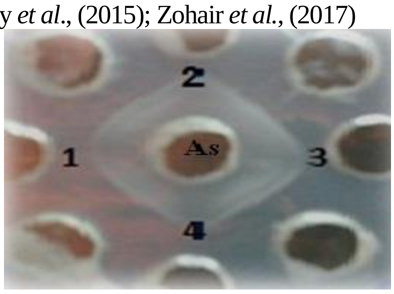

Fig. 2. Agar gel precipitation test results indicating the occurrence of IBDV in bursal samples using hyperimmune serum. 1, 2, 3 and 4, samples of IBDV collected from Giza, Bani-Suif, Fayoum and Qalubia, respectively. As: Hyperimmune serum against IBDV.

RT-PCR is another one of the commonly used technique using for virus confirmation that recognized as sensitive procedure to viral detection (van den Berg, 2000). In current years; molecular techniques for amplification of VP2 gene gave more susceptible and definite results than serological methods such as AGPT (van den Berg, 2000). In this investigation RNA was extracted from four IBDV isolates. Subsequent RNA extraction, the VP2 gene was amplified using RT-PCR by the above mentioned primers. The size of the PCR products amplified from all IBDV isolates was expected after running in 1.5 % agarose gel electrophorasis by comparing its electrophorasis mobility with those of the standard DNA marker as shown in Fig. (3). Data in Fig.3 revealed that all samples gave represented specific bands at 620 bp. no band was observed in negative control sample. In a comparable to study carried out by (Abdel-Alem *et al*., 2001).

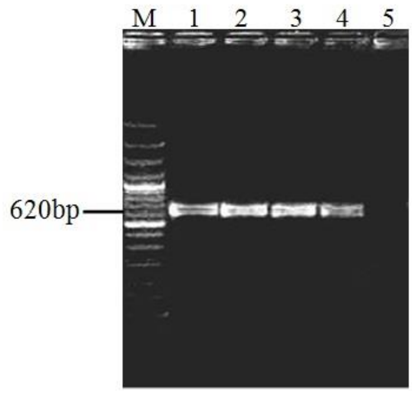

Fig. 3. 1.5 %Gel electrophoresis showing 620 bp. band in viral samples. Lan 1, 2, 3, 4 for IBDV isolate no.1, 2, 3 and 4 respectively, M : DNA molecular weight marker. Lan 5: negative control sample.

To determine the pathotypes of gumboro virus isolates, chickens were inoculated with IBDV isolates via the intraocular route. Data in table.2 and fig.4 exposed that the isolate no.3 of IBDV caused higher morbidity and mortalities rates 100% respectively. But isolates no.1, 2 and 4.caused lower morbidity and mortality rates (20, 26.66 and 33.33%) respectively. The virus isolate no.3 belongs to serotype 1 that agreed to very virulent IBDV (vvIBDV) but virus isolate no. 1, 2, 4 belong to classical virulent IBDV (cvIBDV). The clinical and Postmortems signs noticed on both sacrificed and dead chickens such as; watery diarrhea, hemorrhages and/or enlargement on bursa of fabricius linked with hemorrhages on leg and breast muscles. On the other hand, negative control group showed neither signs nor mortalities. As concluded from the data of the bursa– to–body weight (B: BW) ratio, challenged groups with four IBDV isolates were comparable but it was higher than the results of negative control group.

Previous Data reported that the vvIBDV induces high mortality range 50-100%, while the cvIBDV typically causes mortality ranged from 20-40%. (van den Berg *et al*., 1991; OIE, 2004; Jackwood *et al*., 2009). As regards to B: BW ratio; results of challenged groups with four viral isolates were similar but it was higher than control group. Like results were mentioned by Stoute *et al*., (2013).

Fig. 4. Different signs appeared on the infected chickens after challenged with IBDV isolates such as diarrhea (A), hemorrhages on leg and breast muscles (B) hemorrhages and enlargement on bursal tissues (C).

Table 2. Pathogenesis of viral isolates on 4-week-old chickens

No. of Treatments	No. of dead chickens/ 15	Mortality %	Mean B: BW Ratio	Viral Pathotype
1*.	3	20	3.57	cvIBDV
2.	4	26.66	3.97	cvIBDV
3.	15	100	4.30	vvIBDV
4.	5	33.33	3.86	cvIBDV
5.	Zero	Zero	3.00	ND**

***Treatments no.1, 2, 3 and 4 are challenges with viral isolates that were collected from Giza, Bani-Suif, Fayoum and Qalubia, respectively. Treatment no.5 is a negative control. **ND = Not Determined.**

CONCLUSION

Isolation and pathotyping of four gumboro virus isolates were done from field outbreaks of disease in Egypt during 2018–2019. The obtained data indicate perseverance of the vvIBDV in the Egyptian environment. This study presented highlight to search for effective explanations in order to control IBDV infection in chicken flocks.

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عزل وتحديد الأنماط الإمراضية لفيروس غدة البرسا المعدى من التفشى الحقلى للمرض بين الدجاج بجمهورية مصر العربية

هبة الله حسين جابر ، خالد عبد الفتاح الدجدج و سمر سيد المصرى*

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يعتبر مرض غدة البرسا (فرشيس) المعدي من أهم الأمراض التى تسبب خسائر اقتصادية فادحة في قطعان الدواجن في مصر. وخلال عامي 2018-2019 ، تم تجميع عينات من مزارع دواجن مختلفة من أربع محافظات مصرية (الجيزة ، بني سويف ، الفيوم ، القليوبية). العينات التي تم جمعها من المزارع كان لديها تاريخ مرضى من أعراض محتملة للمرض مثل الإسهال، النفوق المفاجئ والنزيف الحاد في أنسجة غدة البرسا وكذلك فى عضلات الفخذ والصدر. ولعزل الفيروس، تم زراعة العينات التي تم جمعها في أجنة دجاج مخصبة عبر الغشاء الكوريوالنتيوسى. ولتأكيد النتائج التي تم الحصول عليها من عملية عزل الفيروس تم إجراء اختبار الترسيب في الآجار وكذلك تكنيك تفاعل البلمرة الإنزيمي المتسلسل العكسى. نتائج اختبار الترسيب في الآجار أوضحت أن جميع المعزولات اعطت نتيجة ايجابية عند استخدام الأنتيسيرم المتخصص المرجعي لفيروس غدة البرسا المعدي. وعند إجراء تكنيك تفاعل البلمرة الإنزيمي المتسلسل العكسى لتضخيم جين VP2 للمعزولات الفيروسية أعطت كل المعزولات باندات عند الحجم المتوقع وهو 620 زوج قاعدة . وعند تحديد الأنماط الإمراضية للمعزولات الفيروسية وجد آن المعزولات رقم 1 و 2 و 4 تم تصنيفهم سيرولوجيا إلى النمط الكلاسيكى من المرض ، بينما المعزولة رقم 3 تم تصنيفها سيرولوجيا إلى النمط شديد الضراوة والذي تسبب في معدلات نفوق عالية بنسبة 100 % عن المعزولات من النمط الكلاسيكى والتي تراوح معدل النفوق بها من 20-33,3%. وتؤكد النتائج المتحصل عليها على ضرورة الرصد للحالة الميدانية لمرض غدة البرسا المعدي ، بالإضافة إلى تطبيق المزيد من الأبحاث التكميلية لإيجاد استراتيجيات فعالة من أجل محاولة السيطرة على المرض في قطعان الدجاج في مصر .