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Detection and isolation of a recent infectious bursal disease virus from chicken farms in Egypt during 2021

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ABSTRACT

Infectious bursal disease virus (IBDV) is considered as an economic challenge to the poultry industry. Monitoring of circulated recent IBDV is very significant in controlling the spreading of disease in Egypt. In this study, we are targeting VP2 gene of IBDV in bursal samples from 15 different infected chicken commercial farms in seven Egyptian governorates. Reverse transcriptase polymerase chain reaction (RT-PCR) was used for virus detection, 7 out of 15 examined farms from four governorates involving Qaluobia, Dakahlia, Sharkia, Gharbiya were IBDV positive. Isolation of IBDV was carried out by inoculation on chorioallantoic membrane of specific pathogen free embryonated chicken eggs (SPF-ECEs). The infectivity titration of the third passage of IBDV strains was 6.4, 5.6, 5.5 and 5.4 Log^{10} EID_{50}/0.1ml respectively for Dakahlia, Sharkia, Qaluobia and Gharbiya, then identified by RT-PCR and sequenced. The Neutralization index of Qaluobia isolate was 3. In conclusion, a recent Egyptian very virulent Infectious bursal disease virus (vvIBDV) strains antigenically different serotype 1 was circulated and further molecular characterization is required.

1. INTRODUCTION

Infectious bursal disease virus (IBDV), an icosahedral non-enveloped bi-segmented double stranded RNA virus, is classified under the genus Avibirnavirus in the family Birnaviridae (Delmas et al., 2019). The enormous economic damages in the poultry industry worldwide are according to IBDV infection (Jackwood 2017). IBDV firstly attacked the developing B cells in the bursa and caused enormous loss of B cells, which resulted in a lower immune response and higher chance of secondary infection and vaccination failure (Fan et al., 2020).

The IBDV genome encodes five viral proteins, VP1, VP2, VP3, VP4 and VP5. These proteins have functions in virus composition, antigenicity, pathogenesis, and replication. There are two serotypes of IBDV; serotype 1 is pathogenic for chickens and serotype 2 is nonpathogenic. Serotype 1 viruses differ in their pathogenicity and are commonly known such as classical virulent, antigenic variant and very virulent strains (Zhang, 2017). The viral structural protein VP2 (441 aa) is characteristic of IBDV serotype 1 and responsible for formation of the primary IBDV capsid protein. The IBDV viral proteins are composed of 51% of capsid protein which is the only IBDV protein recorded by host neutralizing antibody (Jayasunrya et al., 2017).

The most susceptible age of infection are 3 to 6 weeks of age, during topmost of bursal development occurs (Jayasunrya et al., 2017). Variant strains induce early bursal degeneration without the hypertrophic stage which characterizes the standard virus type, while the more acute disease and higher mortality rates with turgid, edematous, and sometimes hemorrhagic bursa caused by very virulent IBDV (Igrao et al., 2013).

IBDV firstly recorded in Egyptian chicken farms in early seventies (El-Sergany et al., 1974). In Egypt, the first identification and isolation of IBDV were in 1976 (Ayoub and Malek, 1976). Many records classified the Egyptian IBDV isolates as classical IBDV (El Senousi, et al., 1994; Bekhit, 1996). Recent research has confirmed with suggestion of the persistence of antigenically variant and very virulent IBDV strains in Egyptian rearing (Hassan et al., 2002; Metwally et al., 2009; El-Bagoury et al., 2015; El-Samadony et al., 2019)

Additionally, to clinical and gross observations, laboratory confirmation of disease is required for isolation and identification of IBDV in suspected samples using serological and molecular techniques (OIE, 2015). During 2021, based on the historical data for suspicion of IBDV infection in 15 different chicken commercial farms in seven Egyptian governorates including Qaluobia, Dakahlia, Sharkia, Gharbiya, Fayoum, Minya and Damietta were monitored for laboratory confirmation of IBDV. In order to follow up the circulating strains, overcome vaccination failure and development of the vaccine used against IBDV in commercial chicken Egyptian farms.

So, in this study, we detected IBDV in vaccinated and unvaccinated chicken commercial farms using reverse transcriptase-polymerase chain reaction RT-PCR beside a

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trial for IBDV isolation and identification in four Egyptian governorates.

2. MATERIAL AND METHODS

2.1. Collection of bursal samples

During 2021, bursal samples were aseptically collected from 15 different infected chicken commercial farms in seven Egyptian governorates including Qaluobia, Dakahlia, Sharkia, Gharbiya, Fayoum, Minya and Damietta. The historical data for each farm suspected to be infected by IBDV was summarized in table (1). From each farm, 5 bursal samples were collected and pooled as a single tested sample. The fifteen bursal tested samples of 15 examined Egyptian farms were preserved at -80 °C.

Table 1 Historical data of 15 farms sampled from seven Egyptian governorates for IBDV detection and isolation during the year 2021.

Location	Number of Farms sampled	Sample Code	Type	Flock Age (days)	Flock No.	IBDV Vaccine	Signs &P/M lesion	Morbidity rate	Mortality rate
Qaluobia	3	O1	Broiler	21	7000	Intermediate at 7 days Bursa vacc. at 13 days	Dehydration and depression	40%	10%
		O 6	Broiler	27	18000	Intermediate at 7 days Bursa vacc. at 12 and 21 days	White Diarrhea, depression	60%	20%
		O 12	Breeder	63	9000	Vaccinated	White Diarrhea, depression & bursitis	65%	25%
Dakahlia	3	D1	Layer	20	5000	Bursa vacc. at 12 days	Pericoloacal feather stained with urates	60%	20%
		D6	Broiler	21	12000	Bursa vacc. at 12 days	Peck at vent &enlargement of bursa	50%	15%
		D7	Broiler	25	12000	Bursa vacc. at 12 days	White Diarrhea, depression & bursitis	60%	20%
El-Gharbia	3	G1	Broiler	25	8000	Bursa vacc. at 12 days	Peck at vent &enlargement of bursa	40%	10%
		G2	Broiler	21	3500	Not vaccinated	Peck at vent & enlargement of bursa	60%	25%
		G3	Broiler	20	5000	Not vaccinated	Pericoloacal feather stained with urates	65%	25%
Sharkia	1	Sh1	Layer	33	24.000	Bursa vacc. at 12 days	White Diarrhea & depression	60%	20%
Minia	2	M1	Layer	21	6000	intermediate	Swelled bursa with creamy like exudate	50%	15%
		M2	Broiler	21	6500	intermediate	White Diarrhea, depression & bursitis	40%	10%
Domitta	1	E2	Layer	25	5000	Vaccinated	White Diarrhea, depression &bursitis	40%	10%
El-Fayoum	2	F1	Broiler	25	3000	Vaccinated	Dehydration, depression and proventriculitis	50%	15%
		F2	Baladi	39	6000	intermediate	White Diarrhea, depression & bursitis	40%	10%

Each sampled farm represented by 5 collected bursal samples that pooled as one bursal sample.

2.2. Processing of bursal samples

Processing of bursal samples was carried out according to Rosenberger et al. (2008), bursal samples for each farm were chopped and emulsified in sterile phosphate buffer saline (PBS, pH 7.4) with 1 mg/ml of streptomycin, 0.4 mg/ml of gentamicin and 1000 IU/ml of penicillin in 0.9% NaCl (SIGMA) for processing of 10% tissue suspension. The suspension was vortexed followed by three cycles of freezing and thawing, centrifugation at 10000 rpm for 10 minutes at 4 °C was performed. The supernatant was harvested and filtered by 0.45 µm syringe filter and used for IBDV RNA extraction and CAM inoculation of SPF-ECE.

2.3. Viral RNA extraction

Supernatants from the prepared bursal samples and the homogenized 3rd passage CAMs were used for the extraction of viral RNA by PathoGene-spin™ DNA/RNA Extraction Kit (INTRON Biotechnology, Seongnam, Korea) as recommended by the company directions and kept frozen at –80°C.

2.4. One step RT-PCR

For virus detection in the prepared bursal samples and chorioallantoic membrane (CAM) homogenates, RT-PCR was done by a set of primers that were formerly designed for the amplification of a 620 bp fragment the IBDV VP2 gene (Metwally et al., 2009). The VP2 primer sequences (purchased from Macrogen Europe) were:

Forward [AUS GU 5′- TCA CCG TCC TCA GCT TAC CCA CAT C -3′]
Reverse [AUS GL 5′- GGA TTT GGG ATC AGC TCG AAG TTG C 3′].

RT-PCR was done in a total volume of 50 µl per sample. The RT reaction for 20 minutes at 50°C; initial denaturation at 95°C for 15 minutes; after those 39 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 40 sec and extension at 72°C for 1 minute; and final extension at 72°C for 10 minutes. The amplified products checked for 620 bp for VP2 gene by electrophoresing in agarose gel 1.5% and ladder 100 bp (NEW ENGLAND BioLabs) and visualized by a gel documentation system.

2.5. Inoculation of SPF-ECEs for IBDV isolation

Inoculation of SPF-ECEs, 9-11 days with 0.2 ml from positive RT-PCR bursal supernatant, which inoculated through CAM (OIE, 2015). The sample was grown at least three passages in CAM. The eggs were daily examined and the embryos that died within the first 24 h were discarded. The embryo proper and CAMs were harvested 96-h post-inoculation to confirm IBDV existence by RT-PCR. The recorded lesions after virus isolation calculated as Mild (mild hemorrhage on the CAM and low embryos mortalities), Severe (sever hemorrhage on the CAM, greenish liver, and high embryos mortalities) and Negative (no lesions on the CAM and no embryo mortalities).

2.6. Titration of IBDV isolates

The third egg passage of each IBDV isolate was titrated on 10 days old SPF-ECE via CAM (Rodriguez-Chavez et al., 2002). Each isolate was serially diluted 10^{-1} to 10^{-6} in PBS with antibiotics, pH 7.4. From each dilution 0.1 ml was inoculated / 5 egg / dilution via CAM, incubated at 37 °C and examined daily by Candler. Six days post inoculation, all eggs euthanized overnight on refrigeration at 4 °C. The embryos were evaluated for gross lesions and virus titers were calculated (Reed and Muench, 1938) expressed as EID50/ml.

2.7. Serum neutralization index (NI)

A neutralization test was carried out according to Rodriguez-Chavez et al. (2002) on Qaluobia isolate, using 10-day old, SPF-ECE via CAM inoculation. A known reference polyclonal antisera prepared against IBDV-D78 strain (Intervet, Holland) used in the SNT which was supplied by virological unit, poultry diseases and research department, AHRI, ARC, Giza, Egypt. The neutralization index is the difference between the log titer of the virus in the negative serum and the test serum. Standard virus with different dilutions in PBS was used with constant serum dilution. The Qaluobia IBDV isolate was serially ten-fold diluted, antiserum was combined in a 1:1 ratio with each virus dilution and at 37 °C incubated for 45 min. The combined serum-virus mixture was inoculated in SPF-ECE via CAM inoculation and titrated (Reed and Muench 1938). Virus neutralization indexes were determined through subtracting a virus neutralization assay titer achieved from assessing embryos from eggs inoculated with combined antiserum-virus mixture from the estimated IBDV titer. An

index of NI ≥ 2.0 log units was recognized as significantly different (Cunningham, 1967).

3. RESULTS

3.1. Detection of IBDV by RT-PCR in bursal samples of examined Egyptian farms.

From total 15 farms examined at seven Egyptian governorates only 7 farms were positive for IBDV by RT-PCR at four Egyptian governorates including 2 and 3 farms at Qaluobia and Dakahlia governorates respectively and only one farm in both Sharkia and Gharbiya governorates (table 2)

Table 2 Numbers of positive IBDV farms in different examined Egyptian governorates by RT-PCR

Egyptian governorates	Number of examined farms	Number of Positive IBDV farms
Qaluobia	3	2
Dakahlia	3	3
Sharkia	1	1
Gharbiya	3	1
Fayoum	2	0
Minya	2	0
Damietta	1	0
Total	15	7

3.2. IBDV isolation on CAM of SPF-ECE

The four positive IBDV samples by RT-PCR representative for Qaluobia, Dakahlia, Sharkia and Gharbiya governorates were isolated on CAM of SPF-ECE. The examined harvested egg embryos revealed hemorrhage, head edema, hepatic necrosis, meanwhile the harvested CAM were thickened and congested (Fig. 1). These lesions were more apparent from the 2nd passage and were characteristic for very virulent IBDV.

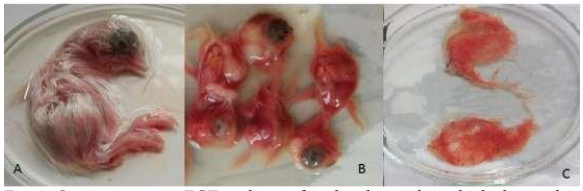

Fig 1. Signs appear in ECE embryo of isolated samples which detected in positive IBDV samples by RT-PCR. The isolated virus induces congestion and hemorrhage of embryos (B) compared with normal embryo (A) as well as congestion and hemorrhage of CAM (C) after the third passage.

3.3. Titration of IBDV isolates

It was revealed that IBDV isolates of Dakahlia had the highest infectivity titer on ECE that was about one log increase from other three isolates of Qaluobia, Sharkia and Gharbiya. The infectivity titer was 6.4, 5.6, 5.5 and 5.4 Log^{10} EID_{50}/0.1ml for Dakahlia, Sharkia, Qaluobia and Gharbiya IBDV isolates respectively by the third passage on SPF-ECE (fig 2)

3.4. RT–PCR Identification of IBDV isolates

Using one step RT–PCR, all four IBDV isolates produced the same amplified specific PCR products at the exact expected size of the VP2 encoding gene 620 bp without significant difference (fig3)

3.5. Neutralization index

It was revealed that using reference polyclonal antisera of IBDV-D78 strain against Qaluobia IBDV isolate induced NI to equal 3 that indicates significant antigenic different of our Qaluobia IBDV isolate from D78 strains.

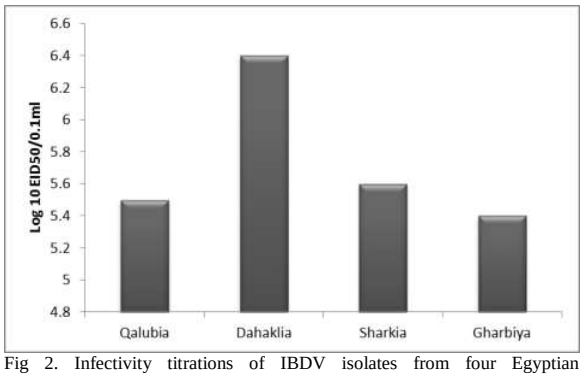

Fig 2. Infectivity titrations of IBDV isolates from four Egyptian governorates in SPF-ECE after the third passage.

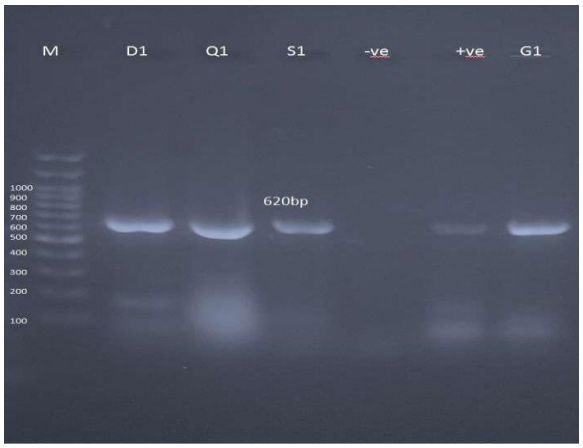

Fig 3. Electrophoresis of the amplified products 620 bp of VP2 for four IBDV isolates of Dakahlia, Qaluobia, Sharkia and Gharbiya (Lane D1, Q1, S1 and G1 respectively). Lane M: marker, Lane +ve: positive IBDV detected in bursal samples before isolation, Lane –ve: negative control.

4. DISCUSSION

IBDV infects the bursa of Fabricius a lymphoid organ for controlling B-cell maturation, it triggers secondary infection's susceptibility, causing great mortality and morbidity among infected chicken. Also, handling IBDV post-outbreaks involves significant time and budgets. The programs of vaccination and early detection of virus is the important in control strategy of outbreak (Syahruni et al., 2021).

Present study reports molecular detection, isolation, and identification of IBDVs in 15 different infected chicken commercial farms in seven Egyptian governorates including Qaluobia, Dakahlia, Sharkia, Gharbiya, Fayoum, Minya and Damietta during the year 2021. These broiler and layers farms had a history of variable degrees of IBDV symptoms and postmortem examination despite IBDV vaccination (Table 1). This difference in clinical signs depend on the maternal immunity, age of birds and virulence of causative agent reported that very virulent strains make more apparent pathogenesis and multiplication of the virus than milder strains (Hassan, 2004; Rauw et al., 2007).

Bursa was selected as the primary organ for viral detection and isolation (Rodriguez-Chavez et al., 2002; El-Kenawy and El-Tholoth, 2017) as the presence of IBDV in organs as the thymus, liver and bone marrow was significantly low affected (Lukert, and Saif, 2003). The bursae were taken from birds aged (20-63 days) to avoid isolation of IBDV viral vaccinal strains as these strains prolonged in the body of birds up to 14 days after vaccination (Igrao et al., 2013).

Recognition of IBDV in clinical samples is suitable to carried out serologically by AGPT and ELISA using hyperimmune antiserum, which besides being less sensitive, are frequently complicated to judged. The nucleic acid-based detection tests as RT-PCR and nucleic acid hybridization overcome these difficulties and so used for detection and differentiation of different IBDV. At the present time, RT-PCR is a molecular technique commonly useful for IBDV diagnosis (Cardoso et al., 2008).

In this study, the RT-PCR was successfully developed based on two sets of primers for VP2 gene sequence to detect IBDV in bursal samples and harvested CAM. Virus was detected in 7 farms at four Egyptian governorates Qaluobia, Dakahlia, Sharkia and Gharbiya. The visualization of amplifying fragments of 620 bp matching IBDV gene sequences encoding VP2 of structural protein was demonstrated in an optimized RT-PCR assay (Metwally et al., 2009; El-Bagoury et al., 2015; El-Samadony et al., 2019).

Four positive RT-PCR homogenates that representative to Qaluobia, Dakahlia, Sharkia and Gharbiya were subjected to trial for IBDV isolation on CAM of SPF ECE as most IBDV field isolates cannot be adapted to grow in primary cell culture (Sali, 2019).

Relatedness to the isolation of positive RT-PCR homogenate on the CAM of SPF-ECE, the homogenate presented lesions ideal and specific to vvIBDV infection (Rosenberger et al, 2008,). The observed gross lesions in embryos revealed hemorrhagic areas, head edema and hepatic necrosis. Hemorrhagic CAM was also recorded by (El-Bagoury et al, 2015; El-Samadony et al., 2019). RT-PCR done on the harvested embryo proper, and CAMs confirmed isolation of four IBDV from the four Egyptian governorates (Fig 3).

The third egg passage of each isolate was titrated on CAM of SPF-ECE. The four isolates had a minor difference between EID50 (≤1.0 logs/ml) and induced vvIBDV-like lesions in embryos. This supported the belief that the field isolates circulating in Egypt more closely like vvIBDV (El-Bagoury et al., 2015; El-Samadony et al., 2019)

Till now, several research works considered pathogenic strains of IBDV as serotype 1 and grouped them into classical virulent (cv) strains, antigenic variant strains and very virulent (vv) strains (Li et al., 2015; El-Bagoury et al., 2015; El-Samadony et al., 2019). Our isolates were very virulent pathogenic serotype 1 IBDV.

Virus neutralization assays have been mostly used as a proper tool to evaluate antigenic and immunogenic variations in IBDV (Lukert and Saif 2003). Significant antigenic difference of our Qaluobia IBDV isolate from IBDV-D78 strains was observed by cross neutralizations assay. The antigenic or immunogenic differences of the significant isolate supporting different antigenic subtypes of IBDV serotype 1 viruses.

Our study demonstrates the presence of recent vvIBDV strain in Egypt in vaccinated and unvaccinated chicken commercial farms. Since the beginning 1990, (vvIBDV) recorded in Egypt which caused high morbidity and mortality (El-Batrawi and El Kady 1990; Khafagy et al., 1991) and still exist among chicken flocks despite regular vaccination programs effort. The pathotypes have been described with different pathogenicity for chickens (Abdel-Alem et al., 2003) due to the bisegmented nature of IBDV, reassortment between serotype 1 and serotype 2 and between various pathotypes/genotypes of serotype 1 IBDVs (Pikula, and Smietanka, 2020) contributes to genetic and pathogenic diversity.

5. CONCLUSION

IBDV serotype I viruses causes many financial losses among the poultry industry in Egypt even with the intense vaccination programs. In the present study, a recent isolated Egyptian very virulent infectious bursal disease virus (vvIBDV) strains and antigenically different from serotype 1, however further molecular characterization is needed.

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