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Characterization of Infectious Bursal Disease Virus Isolated from Broilers in Egypt

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Abstract: An outbreak, during July and October 2015, had hit broilers population at two different localities within Sharkia governorate. Subjected birds showed diarrhea, mortalities and pathognomonic lesion of bursa of fabricius. Infectious bursal disease (IBD) virus was isolated and confirmed by precipitated antibody where the rate varied according to the locality with an average of 39.1%; multiplex polymerase chain reaction (MPCR) analysis categorized the two representative isolates based on amplified bands into genotype 1 and 2. Pathogenicity of each type was assessed by challenging of vaccinated and unvaccinated chicken groups against IBD. The identified genotypes differed in its mortality rate without significant differences of body weights and bursal lesions. Vaccinated birds had neither mortalities nor significant variant body weight than the control birds. Histopathology reflected severe lymphoid depletion of bursae and spleen in challenged group versus vaccinated group which showed mild and moderate lesions. The study concluded that multiplex PCR determined the diversity of circulating infectious bursitis virus at certain area which may lead to different pathogenic effect. Vaccination program is advisable to be applied specially in endemic localities but further researches needed on phylogenic diversity and virulence of the virus.

Key words: IBD · AGPT · Multiplex PCR · Pathogenicity · Histopathology

INTRODUCTION

Infectious bursal disease (IBD) is an acute and contagious poultry disease that caused by non-enveloped double stranded RNA virus family *Birnaviridae* of genus *Avibirnavirus*. It has socio-economic importance worldwide because its outbreak could result in 10-75% mortality of the birds [1].

The disease is also called Gumboro which is the worst and the major problem of poultry industry due to high mortality in spite of regular vaccination. At Ethiopia, a report of IBD outbreak in large-scale poultry farms in broiler and layer chickens indicated that the mortality rate of the disease ranges from 45 to 50%. However, another report of overall 49.89 and 93.30% were recorded for mortality rate and seroprevalence of IBD antibody, respectively [2]. Therefore the seroprevalence of IBD and the associated risk factors with seropositivity was studied in Ethiopia [3]. Although the major losses to the local farmers and the food industry in Nigeria 1988 due to the disease, only limited study of closely related to IBD isolation had been published [4]. Most infections detected in India have been associated with subclinical disease. Although these outbreaks cause no mortality, they result in impaired weight gain and immunosuppression which adversely influences infection with other common pathogens [5]. Despite of IBD threats to chicken industry at Egypt, the available data about it is scare.

There are two distinct serotypes of IBD virus, 1 and 2. Serotype 1 viruses are pathogenic to chickens and classified as classical virulent, antigenic variant and very virulent (VV) IBDVs based on their difference in virulence. Serotype 2 viruses are non-pathogenic to chickens [6].

In young chickens isolates of serotype 1 of IBD virus infect the bursa of Fabricius as the prime target organ cause hemorrhagic necrosis in different organs and infection results in immunosuppression in surviving birds [7].

Aiming at a rapid, sensitive and specific assay that may be used under standard laboratory conditions, two-step multiplex RT-PCR assays were developed [8]. The

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primers used in these assays were designed for detection of classical virulent strain F52/70, very virulent strain DK01, intermediate vaccine strains D78 and Bursine 2, and the "hot" vaccine strain E228 [9].

This study focused on characterization of circulating IBD isolates and its pathogenicity on performance and histopathologial changes of broiler chickens in certain locality at Egypt.

MATERIAL AND METHODS

Sampled birds: One hundred and twenty birds aged between 16-28 days old were collected from different broiler flocks at Belbis and Menia El-Kameh, Sharkia governorate during summer season of 2014 (Fig. 1). Sampled flocks were suffered from high sudden mortalities and survivals showed general signs of illness, diarrhea and vent pasting. Post mortem investigation revealed hemorrhagic streaks or patch on breast and thigh muscle, enlarged congest or pale kidneys with ureter filled with urates and edematous bursa with hemorrhagic spots. Sampled birds had no history of vaccination against IBD.

Virus Isolation: Bursal homogenates were suspended in treated phosphate buffer saline (PBS) 10% with Penicillin 10000 IU/ml, Streptomycin 1mg/ml and Gentamicin 0.4mg/ml. 0.2ml of the suspension were inoculated in chorio-allantoic membrane (CAM) of embryonated chicken eggs (ECE) at 11days old and incubated at 37°C with daily candling for 4 successive days [10].

Agar Gel Precipitation test (AGPT): For identification of IBD virus using reference known antigens and antisera were supplied by courtesy from Veterinary Serum and Vaccine Research Institute, Egypt [11].

Multiplex Real Time Polymerase Chain Reaction (MPCR): Was carried out according to Kabell *et al*., [9] using denaturized RNA extracts with 2 set of primers, polymerase enzyme for amplification at thermal cycles. The denaturation cycling began at 95°C for 5 min then continued 30 cycles for 30 sec, annealing at 60°C for 1 min with extension for 1 min at 72°C. Forward primer (1) was 5'-AGAGACCTCGACCTACAATTTGACTG-3' and its reverse was 5'-CATCTGTCAGTTCACTCAGGCTTCC-3' while forward primer (2) was 5'-AGTGGCTCCTCTTCTTGATGATTCTAC-3' and its reversewas5'-TGTGTTGGGTAGTATTTAGGGAAGTAC-3' (Lena- Christ-Strasse, Germany). Finally electrophoretic analysis in agarose gel was acted.

Experimental Design: One hundred apparently healthy day old chicks, Hubbard sourced from Cairo Poultry Company, reared on floor pens and fed on un-medicated commercial ration. Chicks were divided at 14 days old into 3 groups. Group A (40 chicks) vaccinated at 14 days old then at 21 days old subdivided equally into subgroups A1 and A2 and challenged orally with isolate 1 and 2, respectively. Group B (40 chicks) was subdivided at the same age to be challenged in the same manner without vaccination while group C (20 chicks) was served as

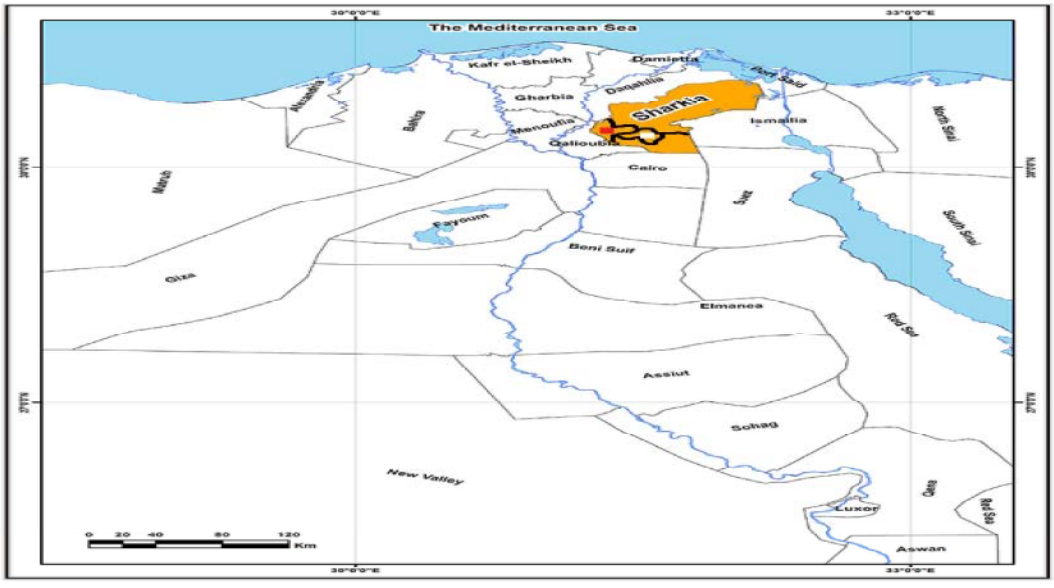

Fig. 1: Sharkia governorate of Egypt Map
Red square shows Menia El-Kameh White square shows Belbis

control neither vaccinated nor challenged. All groups were weighed, observed for clinical signs, autopsy and histopathological study.

Challenge: The isolated IBD virus was titrated in ECE according to Reed and Muench [12] for calculation of embryo infected dose (EID) where EID_{50} was $0.2mlx10^{4.2}$. Birds were observed for morbidity and mortality for 7 days post oral infection.

Vaccine: Commercial IBD live attenuated intermediate D78 strain (Boehringer Ingelheim Vetmedica) each dose contains $10^{3.5}$ $TCID_{50}$. Vaccination was carried out according manufacturer instruction using eye dropper at 14 days old.

Histopathology: Bursae and spleens of dead or birds showed clinical signs were collected and fixed in 10% buffered formalin for 24 hr. The tissues were then processed and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin. Bursal lesions were scored on the basis of lymphoid depletion according to the following scores: 0 = no lesions, 1 = mild, 2 = moderate or multifocal, 3 = severe and extensively spread [13].

Statistical Analysis: Data were collected, organized and analyzed using one-way analysis of variance (ANOVA) through the general linear models (GLM) procedure of the Statistical Package for Social Sciences version 22.0 (SPSS for Windows 22.0, Inc., Chicago, IL, USA). Duncan multiple range test was used to separate means at $P < 0.05$.

RESULTS

Clinical findings of studied flocks presented high mortalities where-as survivals showed depression, ruffled feathers and white-yellowish diarrhea. Macroscopic examination of sampled birds revealed hemorrhagic spots or streaks on thigh and breast muscle, petechial hemorrhage on proventriculus and gizzard, enlarged pale or congested kidneys and swollen bursa of fabricius with gelatinous exudate. Homogenized bursae of each locality were inoculated in ECE for isolation of IBD virus that identified by AGPT (Table 1). Molecular assay confirmed IBD isolates of both localities according to the presence or absence of the amplified bands at 620bp (Fig. 3). Experimental infection of chicken groups revealed significant differences ($P < 0.05$) in mean body weight between the unvaccinated-challenged group and the other groups at 28 and 35 days old (Fig 2) (Table 2).

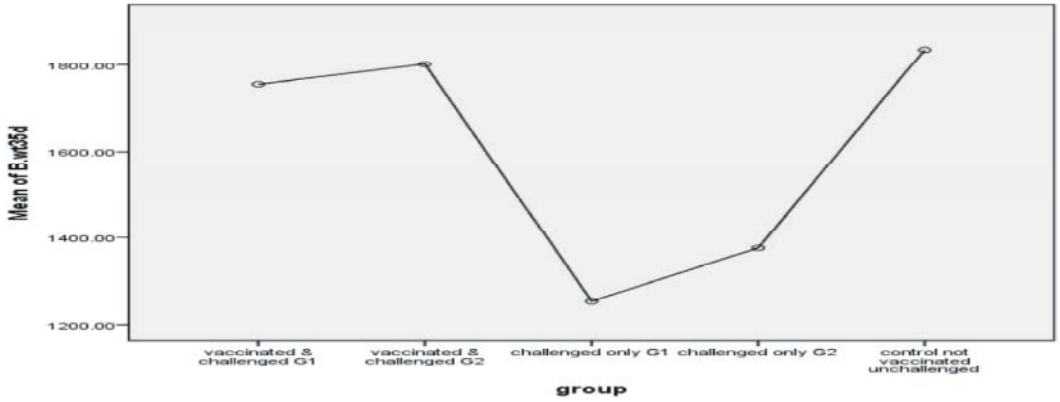

Fig. 2: Effect of isolated genotypes on body weights at 35 days old

Table 1: Results of virus isolation and identification

Locality	Breed	Capacity	Age	Specimen No.	AGPT(+ve)	Percent
Menia El-Kameh	Cobb	5000	20 d.o.	10	5	46.6%
	Cobb	6000	22 d.o.	10	6	
	Cobb	5000	18 d.o.	10	4	
	Hubbard	8000	24 d.o.	10	5	
	Hubbard	4000	18 d.o	10	3	
	Arbor +	5000	21 d.o.	10	5	
Belbis	Hubbard	3000	16 d.o.	10	4	31.6%
	Hubbard	5000	20 d.o.	10	3	
	Cobb	4000	18 d.o.	10	3	
	Cobb	5000	17 d.o	10	2	
	Cobb	5000	21 d.o.	10	5	
	Cobb	5000	16 d.o.	10	2	

Table 2: Results of IBD pathogenicity on body weight

Age/ Grp	Mean body weight in gm (± Sd) 21 days	28 days	35 days.	Mortality post challenge 1st	2nd	3rd	4th	5th	6th	Mortality%
A1	891.75±47.89[a]	1249.45±95.36[a]	1754.70±94.36[a]	No						
A2	900.50±24.77[a]	1307.20±76.91[a]	1801.50±87.81[a]	No						
B1	888.10±79.72[a]	984.40±426.76[b]	1253.50±647.42[b]	0	0	1	2	1	0	20%
B2	885.00±48.64[a]	1064.10±368.63[b]	1375.90±603.65[b]	0	0	0	2	1	0	15%
C	905.40±59.65[a]	1344.10±61.34[a]	1835.25±70.65[a]	No						

A1: Vaccinated group at 14 days old and challenged with genotype1 at 21 days old.
A2: Vaccinated group at 14 days old and challenged with genotype2 at 21 days old.
B1: Challenged group with genotype1 at 21 days old.
B2: Challenged group with genotype2 at 21 days old.
C: Control group (not vaccinated unchallenged)
(± Standard deviation)

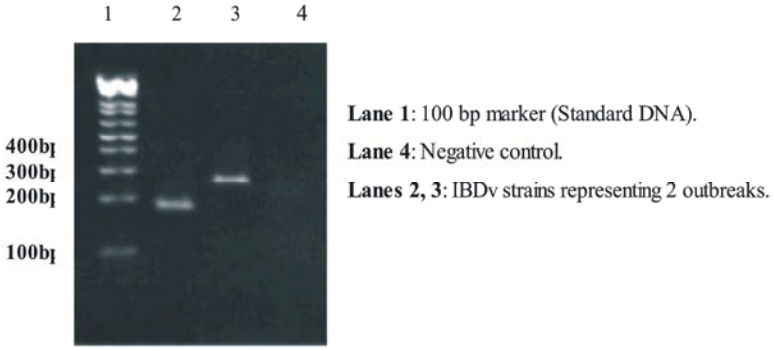

Fig. 3: Multiplex RT- PCR for IBD virus printing

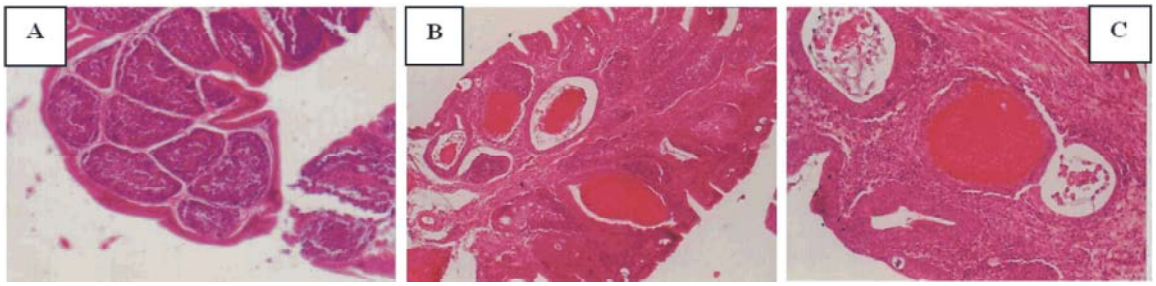

Fig. 4: Histopathological findings of Bursa of Fabricius.
A)Control group: Normal busal follicles and covering epithelium. H&E. ×120.
B)Challenged group: Lymphoid necrosis and replaced by necrotic debris and cystic lymphoid follicles. H&E. ×120.
C)Vaccinated group: Heterophilic infiltrations, fibro plasia and cystic spaces. H&E. ×150.

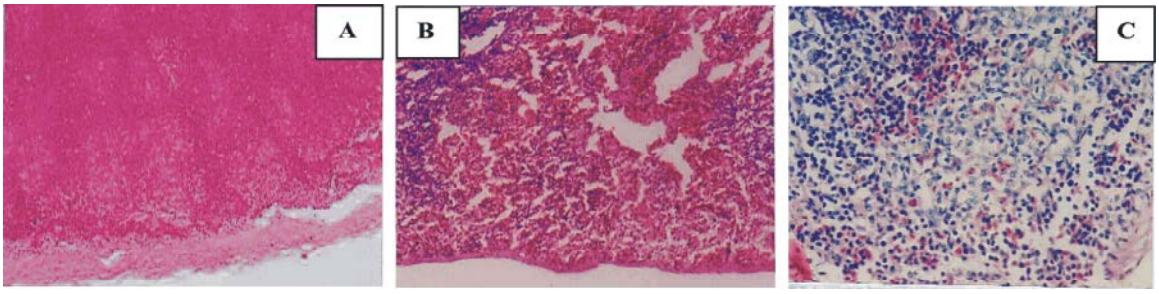

Fig. 5: Histopathological findings of Spleen.
A) Control group: Normal splenic structure. H&E. × 120
B) Challenged group: Lymphoid depletions and necrosis of the periarterial sheath. H&E. ×150.
C) Vaccinated group: Hyperplasia of reticuloendothelial cells around adenoid sheath arteries. H&E. × 150.

Table 3: Histopathological lesion scores:

			Challenge with IBD			Lesion score post challenge				
Grp	No.	IBD vaccine	Age	Route	Dose	0	1	2	3	Spleen
A1	20	14 days old	21 days old	Orally	$0.2mlx10^{4.2}$			+		
A2	20						+			
B1	20	N/A							+	+
B2	20								+	+
C	20		-ve			+				

0: No lesion 1: Mild lesion of bursa 2: Moderate or multifocal lesion
3: Severe and extensively spread lesion +: recorded lesion

Moreover control and vaccinated-challenged groups showed neither morbidity nor mortality unlike challenged groups. Mortality rate of challenged group with genotype1 was 20% while it was 15% in challenged group with genotype2 and the course of mortality was summarized (Table 2). Clinical signs and postmortem lesions were related to the record of sampled birds. Histopathological investigation of bursa and spleen of vaccinated-challenged group showed cellular infiltration and fibroplasia with depletion of follicular cells and hyperplastic reticuloendothelial cells infiltration in the center of white pulps, respectively (Fig 4, 5 C). Challenged group displayed marked vacuoles in the cortical areas, severe lymphoid depletion with necrotic debris and cystic lymphoid follicles of bursae and lymphoid necrosis occurs in the white pulp of the spleen. (Fig 4, 5 B) on the contrary of control group which had normal dorsal and splenic structure (Fig 4, 5 A). Bursal lesions were scored and tabled (Table3).

DISCUSSIONS

IBD is a serious threat to poultry industry in Egypt with little data about the virus characterization. The study aimed to clarify the pathogenesis of the field isolates in broilers at certain area in Egypt. Two outbreaks were monitored at different localities of the same geographic area, where clinical signs and mortalities incriminated Gumboro. The isolation rate of the virus by using precipitating antibodies was 46.6% at Menia El-Kameh and 31.6% at Belbis with an average of 39.1%. Similar results 43.8% were recorded by Mutinda *et al*., [14] in Kenya while higher rates in broilers flocks were reported 51.61% by Singh and Dhawedkar [5] in India, 58.82% by Rakibul Hasan *et al.,* [15] and 91.4% by Islam *et al.,* [16] in Bangladesh. The variations of isolation/positive rate may attribute to the virulence of virus genotypes, identification technique, immune status, age and breed resistance. Multiplex PCR marked IBD isolates to genotype1 amplified at 199bp and genotype 2 amplified at 297bp. Likewise Bidin *et al*., [17] who differentiated IBDV strains by RT-PCR/RFLP analysis as two vaccine strains were amplified at fragments of 144 and 278 bp while two field strains where obtained at 422 bp. Meanwhile Fernandez *et al*., [18] stated that there are natural IBD reassortant viruses that combine genomic segments A and B from different strains and exhibit particular pathogenic characteristics. Moreover phylogenic analysis of isolated IBD in Tanzania from 2001 to 2004 was resulted in two genotypes [19]. Experimental infection with identified genotypes resulted in different mortality, performance and bursal lesions confirming the diversity where genotype 1 was more pathogenic than genotype 2. Commercial IBD vaccine used induced protection against challenge where no mortality and significantly higher mean body weights were recorded. Also Banda *et al*., [20] noted that irrespective the vaccination routes, vaccinated groups against IBD had higher body weight than unvaccinated groups post challenge, moreover vaccination prevented the development of clinical signs regardless the challenge dose and strain, no urate containing diarrhea was observed in any of the challenged groups. Statistically standard deviation (sd) of the mean body weight for challenged groups was higher than 30%, that may be justified by the mortality occurred within the experimental course post infection reflecting the pathogenic variety of the isolates.

Histological study of bursae and spleen evoked progressive changes among vaccinated and infected groups. The lesion score of challenged groups was equal despite varied mortality while genotype 1 induced more damage than genotype 2 among vaccinated groups which confirms the severity variation of isolates tolerated by the vaccine strain in relation to cellular damage. Likely Banda *et al.,* [20] recorded severe bursal atrophy in the unvaccinated-challenged birds and moderate to severe lymphoid depletion in challenged vaccinated birds regardless the vaccination method where the lesion scores were significantly ($P<0.05$) higher than in the unchallenged group. Moreover Metwally *et al.,* [21] spotted that the pathological changes of the tested bursae were similar with little difference of severity

degree. The main lesions in the bursa were congestion of blood vessels, edema and lymphocytic infiltrations in the interstitial tissues and connective tissues proliferation. Also glandular necrosis and atrophied lymphoid follicles with presence of vacuoles in the cortex and medulla were observed.

CONCLUSION

In conclusion the severity of circulating IBD virus differs according to its genotype features that may be diverted in the same environment and further study needed to understand its evolution. Adoption of vaccination programs in endemic areas is advisable to minimize economical loses.

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