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Immunopathological Cell Tropism Detection of Infectious Bursal Disease in Broiler Chickens

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Abstract: Infectious bursal disease virus (IBDV) is a threatening immunosuppressive disease facing poultry industry in Egypt. In this study, detection of very virulent IBDV of twenty broiler flock was shown with high mortalities and bursal lesions from different provinces in Egypt during 2014 (Giza, Dakahleya, Kaliobia and Sharkia). IBDV induces lymphoid cell depletion which can be diagnosed by Immunohistochemistry (IHC). A hyperimmune serum (HS) produced in rabbits through injection of live IBDV vaccine was used as primary antibody in the IHC diagnostic tool. Clinicopathological examination revealed varying bursal lesions from swelling severely inflamed bursal folds filled with whitish exudate to atrophied bursal size. Histopathologically, depletion of bursal lymphoid follicles with hemorrhages and hypercellularity of interfollicular connective tissue were observed. Positive IBDV antigen staining of lymphoid cells population in spleen and bursa of Fabricius was observed by using HS. The obtained HS was validated against standard IBDV antigen by Agar gel immunodiffusion test (AGIDT). The addition of Freund's adjuvant was yielded higher serum protein measured by Nanodrop spectrophotometer. Reverse transcriptase-polymerase chain reaction (RT-PCR) was applied for IBD targeting hypervariable region (HVR) of VP2 gene. The present study concluded to successful validation of produced HS against IBDV antigen and positive intranuclear staining of the lymphoid cell population. Better induction of immune serum total protein produced was obtained by adding adjuvant. RT-PCR for a specific target in VP2 gene was confirmed IBDV infection.

Key words: IBDV · Hyperimmune Serum · Immunohistochemistry · RT-PCR

INTRODUCTION

Infectious bursal disease (IBD) is an acute, highly contagious disease of young chickens caused by IBD Virus (IBDV) belongs to the family *Birnaviridae*, characterized by immunosuppression and high mortality generally at 3-6 weeks of age. It has contributed significantly to an overall loss to poultry industry because of increased mortality due to IBD and other diseases occurring because of vaccination failures due to the immunosuppressive effect of the disease [1].

IBDV replicates in the lymphocytes of the bursa of Fabricius, leading to an immunosuppressive disease that may cause death or impaired growth in young chicken. IBDV consists of four structural proteins, among which VP2 has been identified as the main host-protective antigen that carries major neutralizing epitopes and strain specificity [2, 3].

IBDV serotype 1 is pathogenic for the domestic chicken [4]. Numerous serotype 1 strains, differing in antigenicity have been identified and classified as classic and variant. Chickens are highly susceptible to disease when they are 2–6 weeks of age and symptoms are nonspecific, including depression, whitish diarrhea, anorexia, prostration and death [5]. Younger or older chickens may show milder disease symptoms, but all age groups subsequently experience a transient immunosuppression [6, 7]. Pathologically, infections were

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characterized by gross lesions on most parts of the bursa of Fabricius and other lymphoid tissues including spleen, thymus, harderian gland and cloacal tonsils [7, 9]. Although turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens. The virus does not affect human and has no public significance.

Classical strains cause bursal inflammation associated with severe lymphoid necrosis in infected chicken, resulting in immunodeficiency and moderate mortality from 20-30% in specific pathogen free (SPF) chicken [8]. Very virulent infectious bursal disease virus (vvIBDV) was isolated in the United States in 2008 and the virus had 80% sequence homology with classical IBDV strains [9,10]. These strains were antigenically different from classic strains and cause rapid and severe bursal lymphoid depletion [11]. Recent studies in Egypt through gene sequencing of vvIBDV circulating during 2013 and 2014 revealed clear differentiating pathological lesions between classical and vvIBD [12]. The IBDV infects and destroys actively dividing IgM-bearing B cells in the bursa of Fabricius [13, 14]. Infection with IBDV compromises both humoral and cellular immunity [15].

Routine diagnosis of IBD has been conducted by using immunodiffusion, immunohistochemistry, virus neutralization, enzyme-linked immunosorbent assay [16]. The specific hyperimmune serum is also very helpful in the diagnosis of IBD during an outbreak [17]. In-house sandwich ELISA was prepared using Hyperimmune serum against IBDV obtained in rabbits, given results was 90% of sensitivity and specificity [18].

Also, it is important for studying the distribution of antigen tropism. Locally, the diagnostic serum is not being produced commercially and the imported one is very expensive.

Therefore, the present study was aimed to prepare hyperimmune serum against IBDV in rabbits and evaluate the reactivity against standard IBDV antigen. As well as reactivity of hyperimmune serum in immunohistochemistry test. Understand the distribution of IBDV antigen in lymphoid organs by the immunohistochemical reaction. Also, study immunosuppression induced by IBDV through histopathological examination.

MATERIALS AND METHODS

Field Samples: Twenty (20) chicken broiler farms in (Giza, Dakahleya, Kaliobia and Sharkia) showed different mortality rates associated with bursal lesions. Necropsy was conducted on diseased birds.

Samples Preparation: Specimens from affected organs showed lesions were collected and subjected for molecular identification and for histopathology. Samples were obtained from the bursa of Fabricius of IBD suspected chickens. The collected samples were homogenated then suspended in an equal volume of sterile saline with antibiotic. The suspension was then centrifuged at 3000 rpm for 15 min. The supernatant was stored in screw-capped bottles at -65C until be used [16].

Production of Hyperimmune Serum Against IBDV in Rabbits: A total of 10 adults dewormed New Zealand white rabbits (4 months old) were divided into two groups (five rabbits each). The rabbits were bled prior to immunization and this pre-immune serum was assayed to ensure that the antibody activity detected in later bleeds is due to the immunization with IBDV. The first group (Group A) were inoculated with live IBDV vaccine (strain D78) diluted as instructions without the addition of adjuvant [19]. The second group (Group B) was inoculated with the viral antigen emulsified with an equal volume of complete Freund adjuvant (sigma Aldrich). Each rabbit was injected with 0.5 ml inoculum subcutaneously in multiple sites. After the priming immunization, 2 further booster immunizations were administered at 2-weeks intervals by the same route of injection. The rabbits were bled 14 days following the last immunization and the blood samples were collected for sera preparation. The serum was stored in aliquots in screw-top tubes at -20°C. The polyclonal antibody reactivity of the hyperimmune serum was detected and assayed by AGPT against standard IBD antigen [20].

Measurement of Protein Concentrationbynano Drop Spectro-Photometer: The NanoDrop2000 module is used to determine the concentration of total protein for the collected serum samples. The Beer-Lambert equation (A =E * b*c) is used for all protein calculations to correlate absorbance with concentration. A is the absorbance value (A), E is the wavelength-dependent molar absorptivity coefficient (or extinction coefficient) with units of liter/mol-cm, (b) is the path length in centimeters, (c) is the analyte concentration in moles/liter or molarity (M). The software offers the concentration of tested protein at wave length 280nm given by mg in µl. For detection the best serum titer measuring total protein of serum samples after each inoculation compared with serum samples of uninoculated rabbit [21].

Validation of Prepared Hyperimmune Serum Versus IBDV Antigen by Agar Gel Immunodiffusion Test (AGIDT): The reactivity of the sera samples raised in rabbit using live IBDV vaccine were tested using standard IBDV antigen (GD lab). AGID permits visualization of antigen- antibody reaction as precipitation line through diffusion in semisolid agar medium. A specific positive result is recorded when the precipitin line between the known positive control wells is continuous with the tested well and standard antigen. The test was performed following the procedures described by Wyeth [22].

Histopathology: Thymus, bursa of Fabricius, spleen, cecal tonsils, were harvested from euthanized birds and fixed in buffered formalin saline solution. The tissues were later transferred through several changes of xylol and ethanol, embedded in low-melting-point paraffin wax. Sections of 4ìm in thickness were cut and mounted on microscope slides that were stained with hematoxylin and eosin then covered with cover slips.

Immunohistochemistry: The tissue mounts were deparaffinized in 2 changes of xylene and hydrated serially through different dilutions of ethanol and finally in water. After rinsing for 5 minutes in distilled water and 10 minutes in wash buffer, tissue endogenous peroxidase activity was blocked by blocking solution for 10 minutes. The sections were then washed with distilled water and transferred into wash buffer for 5 minutes. The tissue sections were covered first 1:100 dilution of rabbit IBD virus hyperimmune serum (determined after standardization) for 1 hour. Primary antibody was rinsed off and sections were covered with peroxidase conjugated anti-rabbit for 30 minutes at room temperature, rinsed in wash buffer and Immunoperoxidase staining Novusbio® HRP system were used for immunoperoxidase staining. Washing of sections was again done in wash buffer, treated with substrate3, 3' Diaminobenzidine DAB and hematoxylin was used as a counterstain for 3 minutes and rinsed with distilled water. Sections were rinsed in 2-propanol, air-dried and mounted with cover-slip in depex as followed by Cruz *et al*. [23].

Viral RNA Extraction: The collected bursal tissues were homogenized in phosphate buffered saline with penicillin and streptomycin (1000 µg/ml each) as a 30% homogenate and the suspension was vortexed prior to three rounds of freeze-thawing [16]. The total viral RNA in the samples was extracted from 250 µl of homogenized bursa tissue using 750µl of TRIZOL LS reagent (Invitrogen), according to the manufacturer's protocol [24].

Reverse Transcription PCR: RT-PCR was done using QIAGEN One-Step RT-PCR by amplification of the hypervariable region of VP2 with the following forward and reverse primers: the sense primer VP2F ´5CGC CAG GGT TTT CCC AGT CAC GAC AAC AGC CAA CAT CAA CG3´ and the antisense primer VP2R ´5TCA CAC AGG AAA CAG CTA TGA CGC TCG AAG TTR CTC ACC C3´.The expected fragment size was 650 bp according to Zierenberg *et al*. [24]. 10 µl of a reaction buffer, 2.0 µl of dNTP mix, 2 µl of enzyme mixture, 100 pmol of each oligonucleotide and 5 µl of RNA were mixed with RNase-free water to a final volume of 50 µl. The RT-PCR program is as follows: 30 min at 50C (RT reaction); 94C for 2 min (initial PCR activation); 39 three-step cycles of 94C for 30 sec, 58C for 1 min and 68C for 2 min; and 68C for 7 min (final extension).

RESULTS

Clinical and Postmortem Examinations: Necropsied broiler birds showed substantial gross lesions of bursa which appeared enlarged, grayish white, edematous and covered by slimy material. On incision, the bursal folds were edematous, covered by gelatinous exudates. In few cases, the bursae were atrophied. The liver appeared pale. Congestion and hemorrhages of thymus were observed in a number of birds. Hemorrhagic lesions were also seen at the Junction between proventriculus and gizzard in a few birds. Hemorrhages, particularly in the thigh and breast muscle, were detected in many cases. Congestion and hemorrhages in the intestine and cecal tonsils were also observed.

Histopathology: Histologically the bursa of Fabricius revealed a reduction of lymphoid cells in the follicles, medullary necrosis with an accumulation of homogenous eosinophilic masses. Cystic dilatation of follicle with mild to severe infiltration of heterophils and plasma cells (Fig. 1A). Marked hyperplasia of epithelium was characteristic. Hypercellularity of intrafollicular connective tissue, as well as interfollicular hemorrhages and a glandular transformation, were also noticed (Fig.1 B). Spleen showed depletion of lymphoid cells of splenic pulps and apoptotic picture were observed in the cortex of spleen pulps. (Fig.1 E, F) congestion and diffuse hemorrhages observed in the thymus. Degeneration and necrosis of lining tubular epithelium, focal and diffuse hemorrhages with mild heterophilic infiltration in the interstitium of the kidney were noticed.

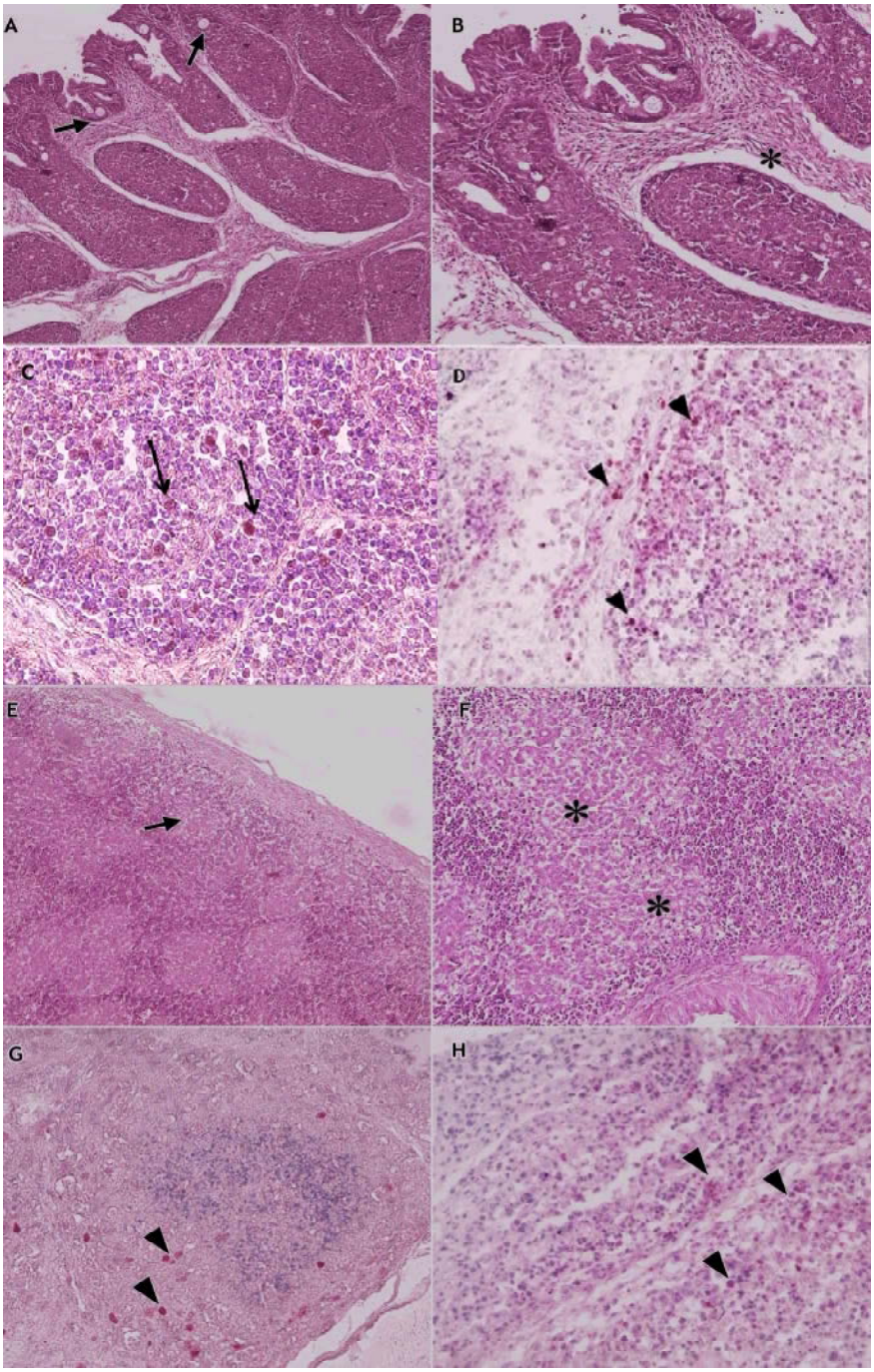

Fig. 1: Histopathology and immunoperoxidase staining of IBD viral antigen.
(A) Bursa showed depletion of lymphoid follicles and proliferation of cortico-medullary epithelium with cystic cavities (arrows) H&Ex100 (B) Bursal follicles showed lymphoid depletion and proliferation of interfollicular connective tissue (star) H&E x200.(C) Lymphocytic cell population showing reddish brown stained IBDV antigen (Ag) (arrows) x400. (D) Lymphocytic cells in lymphoid follicles showed positive IBDV antigen staining x200. (E) Spleen showing depletion of the lymphoid population of splenic pulp H&E x100. (F) Splenic pulps showing lymphoid depletion and apoptotic changes (stars) H&E x200. (G) Spleen showed IBD virus Ag staining to lymphoid cells population (heads of arrows) x100. (H) Lymphoid cells in splenic pulp showed positive stained of IBDV Ag.

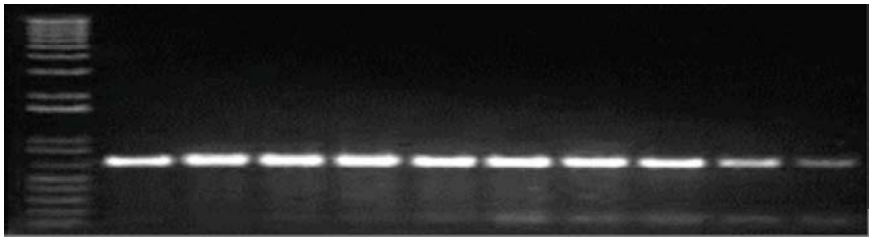

Fig. 2: Gel electrophoresis of RT-PCR showing 620 bp band of some positive samples

Table 1: Evaluation of produced HS against IBDV in Rabbit by AGIDT

	Protein measures mg/µl		AGID	
Animals	Group A	Group B	Group A	Group B
1	100	250	-	+
2	80	200	-	+
3	120	230	-	+
4	150	250	-	+
5	110	220	-	+

Group A five rabbits inoculated with live vaccine without (-ve) adjuvant
Group B five rabbits inoculated with live vaccine with (+ve) adjuvant

Immunohistochemistry: The presence of bound and stained IBD virus antigen was observed as red to brownish, fine or coarse granules or globules in the cytoplasm of infected cells while nuclei stained bluish. Positive reactions (stained cells) were absent in tissue sections from uninfected controls and also in control tissue sections from infected birds stained without rabbit anti IBDV serum i.e. primary antibody. Positive IBDV antigen staining was shown in tissue sections of bursa of Fabricius and spleen (Fig, 1c, d, g &h) negative staining for thymus and cecal tonsils. Negative control sections were showed negative staining.

Detection of IBDV by RT-PCR: Extracted Viral RNA was positive by RT-PCR using specific primers for the hyper variable region of VP2 gene. All RT-PCR positive samples showed specific bands at 650 bp on agarose gel as shown in Figure (2).

Confirmation of Anti-IBDV Hyperimmune Serum Raised in Rabbit by AGIDT: The prominent white line of precipitation was formed between bursal homogenates of the central well and known positive anti-IBDV hyper-immune serum of each of the five of the six peripheral wells due to antigen and antibody reaction (Table 1).

DISCUSSION

In this study, the obtained clinical and histopathological results indicate obvious pathotyping for vvIBDV infected broiler flocks. High mortality rates, severely depleted bursal follicles, hemorrhages, edema, heterophils infiltration together with proliferating interfollicular connective tissue in bursa are strongly evident in pathotyping vvIBD [25]. In contrast, mild to moderate lymphoid depletion, the absence of hemorrhages, edema or heterophils infiltration are indicants for the classic IBD [15, 26]

Earlier studies [27-29] have shown that IBD virus strains differ in tissue tropism which had been observed to be dependent on virulence. In the spleen and cloacal bursa of IBDV infected chickens, the antigen was detected in the cytoplasm of lymphocytes and inflammatory cells (mostly macrophages), as previously reported by Nunoya *et al*. [27]. However Jonsson and Engstrom [30] observed antigen only in bursal lymphocytes. Histopathological findings such as cellular infiltration, fibroplasia and formation of cystic follicles in the bursae of Fabricius of chickens are indications of bursal damage and the full susceptibility of chickens [31].

The production of antibodies is a complex biological phenomenon. It is not always followed any recommendations and guidelines outlined in literature, as procedures and protocols have to be modified depending on the antigen. For some purposes, a single injection may be sufficient but in general, higher antibody yields are obtained by administering a series of injections [32]. A number of vertebrate species, ranging from farm animal to rabbits, small laboratory rodent and chickens have been used over the years for choosing a species for raising polyclonal antibodies [33]. Rabbits are the single most used species because they are of a convenient size, easy to bleed and handle, have a relatively long lifespan and produce adequate volumes of antisera. Moreover, they are also free from antibodies against avian viruses.

Ideally, one follows the serum antibody titer in a hyperimmunized animal and gives a booster injection of antigen only after the antibody titer has begun to decline. However, when an animal has lesser responding, which is a usual situation for small doses of antigen, a booster

dose of antigen given at 2 to 3 weeks after the first antigen dose will usually increase the serum antibody titer [34].

Antibody formation is enhanced by the use of different adjuvants. They are supposed to prolong the exposure of the antigen to the immune system, protect it from degradation and stimulate the immune system [35]. Complete Freund's adjuvant is a water-in-oil emulsion of mineral oil has been strongly supported by Jurd and Hansen, [36]. As the infectivity titer also plays an important role in raising hyperimmune serum, therefore, possibilities may be that live adjuvanted virus inoculum of group B was stronger than the IBD vaccine of group A.

CONCLUSIONS

The overall results of this study suggested that Live IBDV vaccine with complete Freund's adjuvant provided best immune response in terms of highest antibody titers. This combination can be used for producing of hyperimmune serum in rabbits for the early diagnosis of IBD. The produced hyperimmune serum can be used as diagnostic tool in IHC. Lymphocyte populations, plasma cells and macrophages were the prime targets for IBDV antigen.

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