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Characterization and Inactivation of Infectious Bursal Disease Virus for Use as a Vaccine and Immunodiagnostic Reagent

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A R T I C L E I N F O

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A B S T R A C T

Introduction: Poultry vaccines are used to immunize chickens against different diseases. Inactivated vaccines have been widely used to protect poultry against diseases such as infectious bursal disease (IBD). IBD is one of the most important viral immunosuppressive diseases in the poultry industry. This viral disease targets the immune organs. This study was aimed to evaluate the effect of an inactivated IBDV antigen on inducing the humoral immune response in Specific-Pathogen-Free (SPF) chickens. **Methods:** An infectious strain of bursal disease virus (IBDV) was isolated from an affected chicken bursa of Fabricius. Serological diagnostic tests and molecular experiments were carried out to identify the isolate. Different concentrations of formalin, beta propiolacton (BPL) and binary ethylenimine (BEI) were used for inactivation of IBDV. The samples of IBDV antigen were adjuvanted separately with ISA-70. Three-week-old SPF chickens were divided into four groups. Groups 1, 2, and 3 received 0.5 ml of the adjuvanted antigens subcutaneously and group 4 received PBS as negative control. Blood samples from each group were collected 4 weeks post-inoculation and the targets were measured by ELISA and serum neutralization test (SNT). **Results:** The lowest concentrations that could fully inactivate the infectivity of IBD virus were 2.5 mM for BEI, 0.15% for BPL and 0.1% for formalin. Examination of the inactivated samples with 0.1% formalin showed a decrease in antigenicity after 12 months. Treatment with 2.5 mM BEI and 0.15% BPL showed no apparent adverse effect on IBDV infectivity and showed a reliable inactivation. In the SPF chickens of all experimental groups, the antibody titers raised against IBDV were detected by ELISA. **Conclusion:** In the group which the virus was inactivated using BEI, the antigenicity stability was much better than others. Hence, BEI-inactivated IBDV is suggested for preparing more immunogenic, efficient and stable vaccines against IBD.

Citation:

INTRODUCTION

Infectious bursal disease (IBD) is one of the acute and highly contagious viral diseases in young birds. It leads to immunosuppression, poor immune responses to other infections and negative immunization interventions [1-4]. The IBD viral agent (IBDV), belongs to the Avibirinavirus of Birnaviridae family which is characterized by a double-stranded RNA genome including small and large segments and a non-enveloped icosahedral capsid [5, 6]. IBDV strains target proliferating B lymphocytes of the bursa of Fabricius and cause up to 20% mortality in chickens of 3 weeks of age or more [7].

Exposure of birds to the IBDV-contaminated farm causes the rapid spread of the disease [8-10]. Due to the significant economic impact of IBD on the poultry industry, traditional and next-generation vaccines including viral vectors, subunit and genetically-engineered vaccines, have been developed to prevent the disease [11-14].

Typically, primary protection of chickens against IBD is achieved through maternal antibody, transmitted to the newly-hatched chicks. These antibodies protect the offspring until the adaptive immune response becomes activated. The active immunity against IBDV involves a critical vaccination program with live-attenuated and inactivated vaccines. Live IBDV vaccines may exhibit inadequate efficacy in the presence of maternal antibodies [15-17]. To gain full protection against IBDV, chickens should be revaccinated with an inactivated vaccine [13, 18]. It is expected that high levels of virus-

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neutralizing antibodies maintained through laying, protect the young chickens against clinical or sub-clinical IBD [15].

Inactivated vaccines provide a high and uniform level of protection, especially when administered after priming with a live vaccine. This should be considered in the implementation of vaccination programs for breeder and layer flocks due to the fact that they require high and long-term immunity for the protection during the laying period [19]. The aim of this study was to prepare inactivated IBDV antigen and evaluate its effects on the humoral immune response in Specific-Pathogen-Free (SPF) chickens. In this regard, i) a local IBDV was isolated and identified; ii) the effects of different concentrations of three chemicals were examined in different treatment times on the inactivation procedure; iii) An adjuvanted (ISA-70) IBDV antigen was injected to the SPF chicken groups subcutaneously and the induction of immunity was assayed by ELISA and serum neutralization test (SNT).

MATERIALS AND METHODS

Ethical Statement

International and institutional guidelines for the care and use of animals were performed in the study.

Sample Preparation

The samples were collected from 5 week-old Bovans pullet farm which had suffered from hemorrhagic and edema of bursa of Fabricius. The bursa was grinded in PBS, containing penicillin (1000 units/ml) and streptomycin (1000 µg/ml), homogenized with tissue blender and centrifuged for 20 min at 4000 rpm at 4ºC. The supernatant was harvested and used for the virus isolation.

Virus Isolation and Identification

The prepared sample (0.2 ml) was inoculated onto chorio-allantoic membrane (CAM) of six 10-day-old SPF embryonated eggs (Razi Vaccine and Serum Research Institute (RVSRI), Iran). The eggs were incubated at 37°C and controlled daily up to 7 days post-inoculation (PI) for IBD pathological lesions. Serological and molecular tests were conducted for the characterization of the isolated virus.

Agar Gel Precipitation (AGP) Test

The 1.2% noble agar plate containing 8% sodium chloride and 0.5% phenol was prepared. The wells of 2.5 mm diameter and 2.5 mm interspace were cut using a template and cutter with wells in a circle around a center well. The peripheral wells were spilled with the samples of prepared CAM and centralized with positive serum. IBD positive and negative samples were also added as controls. The plate was incubated in humid chamber for 48 h at 37°C.

Immunofluorescence (IF) Assay

Cover-slips containing the collected allantoic fluids at 5 days PI were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. They were then incubated with chicken polyclonal anti-serum to IBDV at 4°C in a humidified chamber overnight. The slides were washed three times with Tris-buffered saline (TBS) and incubated with Fluorescein isothiocyanate (FITC)-labeled secondary antibody for 30 minutes at 37°C. For the negative control, a sample with no primary antibody was used under the same condition. The slides were washed with TBS, mounted and examined with a B-353FL confocal laser scanning microscope (Optika, Italy).

Molecular Identification

High Pure RNA Extraction Kit (RNeasy Mini Kit, Qiagen, Germany) was used to extract viral RNA. RT-PCR was performed to amplify IBDV VP2 gene during ONE-STEP RT-PCR PreMix Kit (iNtRON Biotechnology, South Korea) and the following primers:

Forward: 5′-GAA TTC CCT GGA GAA GCA CAC TCT CAG-3′
Reverse: 5′-GGT ACC GTC TTT GAA GCC GAA TGC TCC-3′

The reaction conditions were as follows: 1 cycle (50°C for 30 min), 35 cycles (94°C for 30 s, annealing at 61°C for 30 s, and elongation at 72°C for 60 s), and final extension at 72°C for 10 min for one cycle. The PCR product was electrophoresed on 1% Safe-Red stained agarose gel (AMPLIQON, Denmark) and sequenced in both directions by Metabion Company (Germany).

Virus Titration

The isolated IBDV was injected into the allantoic cavity of 10-day-old SPF chicken embryonated eggs. The infective embryos were harvested, pooled, and clarified. Serial dilutions of IBDV suspension were performed from 10^{-1} to 10^{-7}. Five SPF eggs were inoculated on the CAMs with each dilution (0.1 ml/egg) and the eggs were incubated at 37°C up to 7 days. After incubation, the pathological lesions were tested to confirm the presence of IBDV. Infection titer was calculated with the standard Reed & Muench formula [20].

Antigen Inactivation

The IBDVs were inactivated with three chemicals as described below:

I) Formalin treatment: Formaldehyde solution (Merck, Germany) was diluted 10 times (v:v) in double-distilled water (DDW). The concentrations of 0.05%, 0.1%, 0.15, 0.2% and 0.25% were attained in the virus. The samples were incubated for 16 h at 37°C on shaker.

II) Beta ropiolacton treatment: Beta ropiolacton (BPL; Sigma Aldrich, Germany) was diluted 1:10 in cold (4°C) DDW immediately before use. The final concentration was attained by making a 1:10 dilution into the virus solution. Concentrations of 0.05%, 0.1%, 0.15%, 0.2% and 0.25% (v:v) of BPL were evaluated. The samples were mixed by shaker, and incubated for 120 min at 37°C.

III) Binary ethylenimine treatment: Binary ethylenimine (BEI) was prepared by cyclization of 0.1 M 2-bromoethylamine hydrobromide (Fluka, Germany) as a 0.1 M solution in 0.2N NaOH at 37°C for 30 min. The 1, 1.5, 2, 2.5 and 3 mM concentrations of BEI were made in the test materials. The treated material was incubated at 37°C up to 16 h.

Infectivity Assay

To confirm the inactivation of IBDV antigen, 0.2 ml of each treatment was inoculated to 10-day-old SPF embryonated eggs. For virus infectivity assay, 3 consequent inoculations were applied. Each of the inactivated IBDV antigens was stored at 4°C for up to 12 months.

Chicken Immunization

Each of the formalin-, BPL-, and BEI-inactivated IBDV antigens was mixed with ISA-70 (SEPPIC Co., France) adjuvant at the ratio of 30/70 (w/w). Three-week-old SPF chickens were randomly divided into 4 equal groups (n = 20).

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Group A received 0.5 ml of the adjuvanted formalin-inactivated antigen subcutaneously. Groups B and C received the adjuvanted BPL-inactivated antigen and BEI-inactivated antigen, respectively and group D was considered as a negative control. Chickens were placed in the separate cages under the controlled conditions. Blood samples were collected 4 weeks PI and induction of specific antibody titers against IBDV was evaluated by SNT and ELISA (IDEXX, US). This procedure was repeated five times at 3-month intervals using another SPF chicken groups and the stored IBDV inactivated antigens up to 12 months.

ELISA

The negative and positive controls were dispensed (100 µl/each) undiluted in duplicate wells of a microtiter plate. The diluted sera samples were dispensed into each well (100 µl of 1:500). The plate was incubated at room temperature for 30 min. The wells were washed 3 times with deionized water. This was followed by the addition of 100 µl of conjugate into each well. The plate was allowed for incubation at room temperature for 30 min. After washing steps, TMB substrate was added into each well and the plate was left at room temperature for 15 min. The stop solution was added to each well. The absorbance value was measured using ELISA reader (BioTek, US) at 650 nm. The results were validated based on the manufacturer's recommendation that mean OD value of NCX must be ≤0.150, and when it is subtracted from mean OD of PCX, the result must be > 0.075. The endpoint titer of the samples was calculated using Log10 Titer = 1.09 (Log10 S/P)+C formula where, S/P (sample to positive ratio) = (Sample mean−NCX) / (PCX−NCX) and C is 3.36 (relates S/P at a 1:500 dilution to an endpoint titer). The presence of IBD antibody was reported as positive when S/P ratio was > 0.2 and negative when S/P ratio was ≤ 0.2. Furthermore, the antilogarithm of Log10 titer was calculated (IDEXX® software) and recorded as the quantity of IBD antibody in each sample.

Statistical Analysis

Statistical analysis of the data was done by t-test and Pearson's Chi-square using SPSS software ver. 22 (IBM, US). The statements of statistical significance were based upon $P < 0.05$.

RESULTS

The Effects of IBDV Infection on the Birds and the Embryos

The affected birds showed dehydration, and hemorrhages in the thigh and pectoral muscles. The cloacal bursa had an edema and hyperemia and gelatinous yellowish transudate covering the cream color serosal surface. Longitudinal striations on the surface became prominent. The infected bursa often had ecchymotic hemorrhages on the mucosal surface and extensive hemorrhage throughout the entire bursa (Fig. 1).

The lesions including congestion, intracranial hemorrhages, subcutaneous edema and abnormalities of internal organs were observed in the embryos infected by IBDV (Fig. 2).

Detection of IBDV Antigen in the Allantoic Fluid

In AGP test, the IBDV positive samples showed a line of precipitation between the antigen and the serum wells while negative samples had no precipitation line. The results of immunofluorescent staining of the virus isolation showed the presence of cells displaying bright apple-green fluorescence (Fig. 3) while no positive signal was detected in the negative control.

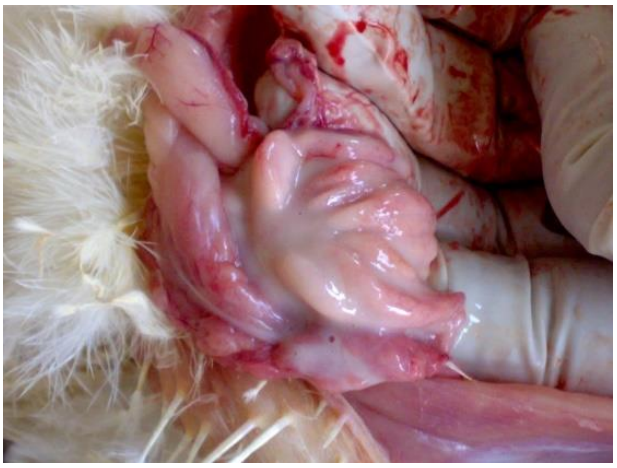

Fig. 1. Infected bursa of Fabricius from the affected chicken. The hemorrhages, creamish exudate and thickened longitudinal laminae were seen.

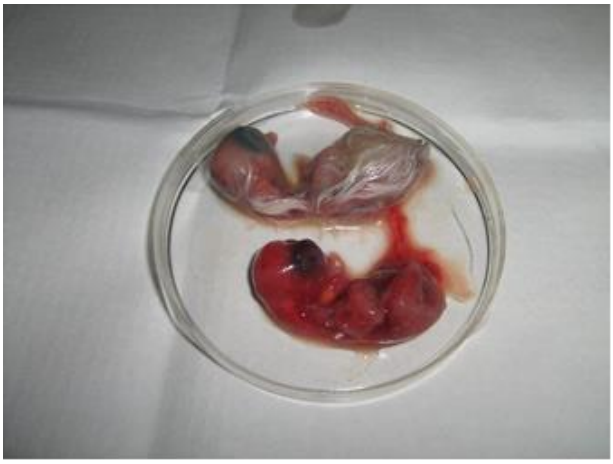

Fig. 2. Chicken embryo at 14 days old. Up: normal, down: infected with IBDV.

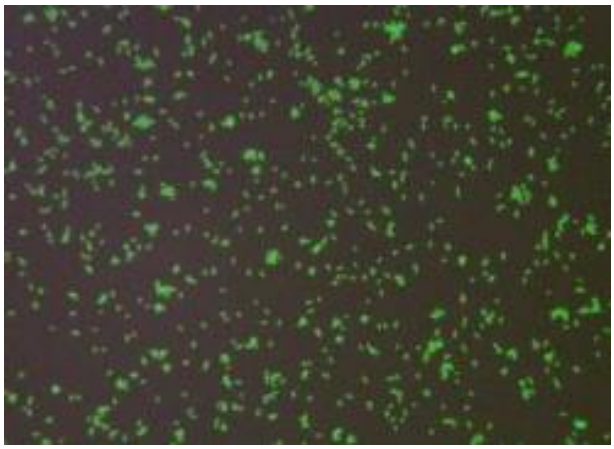

Fig. 3. The immunofluorescence detection of IBDV antigen in the infected allantoic fluid (IFT, × 20). The positive signals of the IBDV antigen with a wide distribution appeared in green color.

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The Phylogenetic Relationship of IBDV VP2 Gene Sequences

The VP2 gene of IBDV amplified at the corresponding band (1244 bp) was sequenced at both directions and deposited in GenBank under KT633995 accession number. The phylogenetic relationship between the isolate and other IBDVs isolated from Iran and vaccine strains was determined using the minimum evolution analysis with 1000 bootstrapping using MEGA6 software (US) (Fig. 4). Taken together, the serological and molecular results indicated that the isolated virus was a classic intermediate IBDV and the virus titer in the propagated sample was calculated to be $10^{7.83}$ EID_{50}/ml.

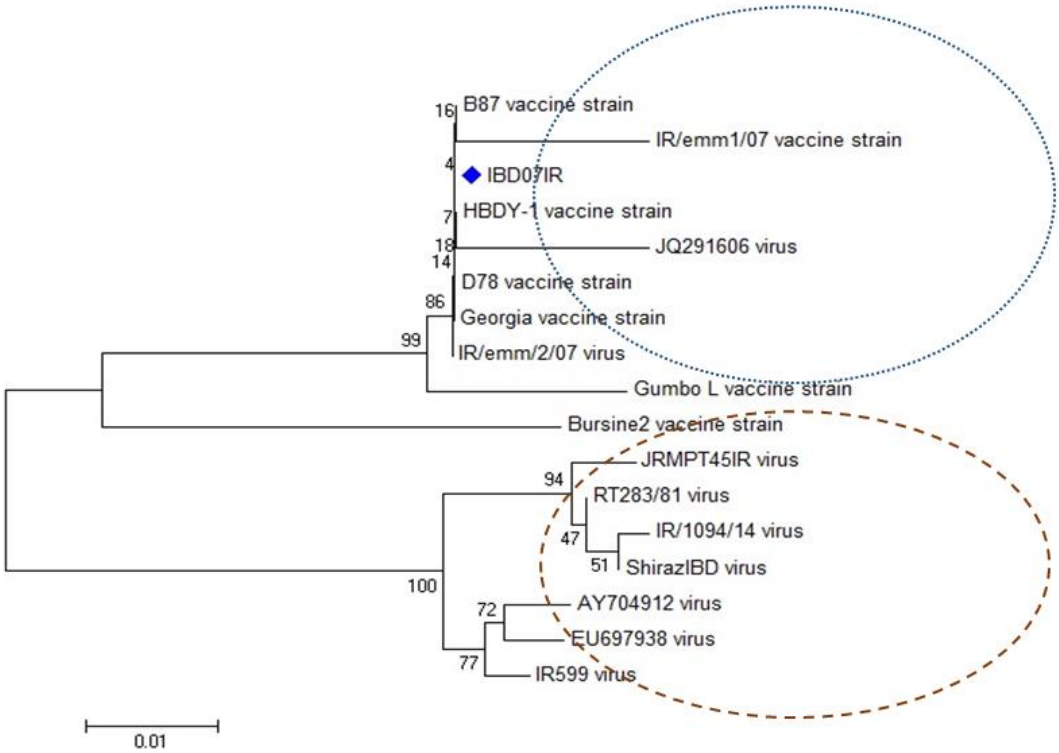

Fig. 4. The phylogenetic relationship of IBDV VP2 gene sequences of vaccine strains and virus isolates deposited in GenBank. The neighbor joining tree was constructed with 1000 replicates. The upper branch shows the low pathogenic or intermediate IBDVs and the lower branch shows the very virulent IBDVs. ◆: depicts the isolated virus in this study.

The Effects of Chemical Treatments on the Infectivity of IBDV

The effects of chemical treatments with formalin, BPL and BEI on infectivity of characterized virus are summarized in Table 1.

The concentration 0.1% or more of formalin inactivated the infected fluid. Examination of the stored inactivated samples with ≤0.1% formalin showed a decrease in antigenicity after 12 months. Treatment with 2.5 mM BEI and 0.15% BPL showed no apparent adverse effect on IBDV infectivity and showed a reliable inactivation. To ensure inactivation, no signs and traces of the virus were observed after 3 passages.

Table 1. Effects of formalin, beta propiolactone (BPL) and binary ethylenimine (BEI) on infectivity of IBDV.

Formalin		BPL		BEI	
Con.(%)	**Infectivity**	**Con.(%)**	**Infectivity**	**Con.(mM)**	**Infectivity**
0	+	0	+	0	+
0.05	+	0.05	+	1	+
0.1	-	0.1	+	1.5	+
0.15	-	0.15	-	2	+
0.2	-	0.2	-	2.5	-
0.2.5	-	0.25	-	3	-

Con: concentration

Antibody Titers Against IBDV

In each trial, antibody titers against IBDV were raised in all treated groups compared to the control group. The mean serum antibody titers of chickens vaccinated with the inactivated candidate vaccine increased 4 weeks after the vaccination. The antibody titer in chickens that received BEI-inactivated antigen was slightly higher than in chickens that received BPL-inactivated antigen. Although the titer for formalin group was significantly ($P < 0.05$) lower than the other two treated groups (Fig. 5).

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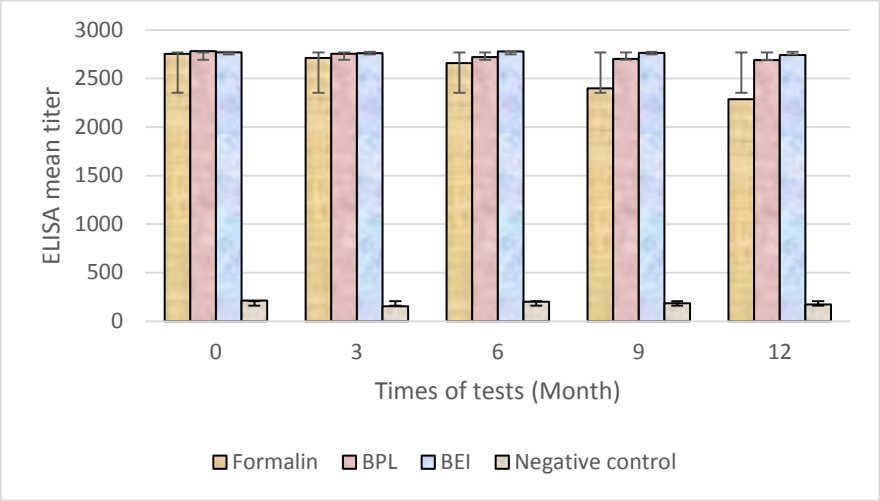

Fig. 5. Chicken sera titers detected by ELISA. They were injected with inactivated IBDV up to 12 months after treatments with different inactivating reagents as indicated.

DISCUSSION

Vaccination with live-attenuated and inactivated vaccines remains the most effective strategy to control IBD in poultry industry [21-23]. In order to induce the protective immunity in chickens, the inactivated IBDV vaccines should have either a high or an optimized antigenic content [15, 16, 13]. Thus, the method used to inactivate the virus is an important factor affecting the antigen quality. Formalin, a chemical that affects protein structures is the most commonly used compound for inactivation of viral vaccines; however, treatments with other components with less destructive effects on the protein structures are suggested [24-30]. Here, we determined the effects of three chemicals on inactivation of IBDV antigen using the same virus concentration to eliminate possible variations. Subsequently, the immunogenicity of the inactivated IBDV antigens was evaluated in chickens. At first, our results showed that the virus was inactivated with 0.1%, 0.15% and 2.5 mM of formalin, BPL and BEI, respectively. Applying the high concentrations of the chemicals caused a decrease in IBDV infectivity titer. Up to the end of the experiment, the BEI-inactivated virus retained its infectivity indicating that the chemical did not adversely affect the induced immune responses.

Classically, formalin has been used to inactivate viruses [29, 31]. The electrophilic agent affects both genome and proteins via nucleophilic addition, inter- and intra-molecularly crosslinking, and their combination, which prevent the virus genome transcription. The most problem in the application of formalin is the incomplete inactivation of virus particles, which can cause the outbreak of virus infection following vaccination [32]. It has been shown that outbreaks of foot-and-mouth disease in Western Europe [33] and Venezuelan equine encephalitis in Central America [34] are due to remaining of incomplete inactivated viruses in vaccines, possibly due to alkylation of amino and sulphydrilic groups of the proteins and purine bases and crosslinking with viral proteins following formalin inactivation [31]. The RNA-protein crosslink, viral proteins modification and change in the conformation of epitopes have also been reported for viruses inactivated by formalin and BPL. However, the role of pH, inactivation time and temperature should not be ignored [35].

The poor inductions of neutralizing antibody responses as well as the formation of immune complexes between viruses and antibodies have been reported in inactivation of viruses by formalin. Treatment of virus antigen with this chemical also results in weak and short immune responses in inactivated vaccines. For this reason, the use of alkylating inactivating agents such as BPL or BEI is recommended [36, 32]. These chemicals mainly act on viral DNA or RNA through a nucleophilic substitution mechanism. Although they display the same mode of action, BPL is much more toxic than BEI. For this reason, the use of alkylating inactivating agents such as BPL and BEI is favorable. BPL activates viruses properly which leads to the induction of neutralizing antibody and protection upon a challenge [32].

To test whether inactivation with formalin, BPL, and BEI interfered with the immunogenicity of IBDV, induction of humoral immune responses was evaluated in chickens. Compared to the control chickens, the specific IBDV antibodies increased in all of the vaccinated groups while decrease in antibody titer was detected in chickens that received the formalin-inactivated antigen [37]. The viral protein modification especially on Cysteine, Methionine, and Histidine as the most reactive amino acid residues may affect the virus infectivity. Such altering in the activity of proteins has been demonstrated in the enveloped viruses. It seems that BPL has a limited capacity to inactivate non-enveloped viruses which possibly lead to denaturation of viral immunogens and induction of short-time immunity [38]. Slightly reduction in the immunogenicity of BPL-inactivated IBDV compared to the BEI-inactivated virus may be due to their different inactivation capacities on viruses because IBDV is a non-enveloped virus. Despite formalin and BPL that alter the antigenic components of the inactivated viruses, no change in the antigenicity has been reported for both envelope and non-enveloped viruses inactivated by BEI [39]. The inactivation of different viruses such as Newcastle disease virus, avian influenza virus, foot-and-mouth disease virus, vesicular stomatitis virus, swine vesicular disease virus, sheep pox virus, hematopoietic necrosis virus and blue tongue virus with BEI did not affect their immunogenicity [24, 40-44]. Even in low concentration, BEI passes through the virus capsid and reacts with the N7-guanine of the genome then guanine becomes alkylated through the

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opening of the BEI ring. This reaction is faster in RNA nucleosides than that in DNA nucleosides, therefore, BEI does not interact with the proteins [32]. Considering the preservation of the viral structure and neutralizing epitopes in the BEI-treated viruses, the inactivated vaccines with BEI are suggested to be used in design of more immunogenic, efficient, and stable vaccines against IBD in future.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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