

## Adjuvant Effects of Platycodin D on Immune Responses to Infectious Bronchitis Vaccine in Chickens

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Adjuvants are common vaccine components. Novel adjuvants may improve the protective immunity conferred by vaccines against poultry diseases. Here, a less-hemolytic saponin, platycodin D (PD), isolated from the root of *Platycodon grandiflorum* was investigated as a potential alternative adjuvant. PD was tested as an adjuvant in the infectious bronchitis (IB) vaccine, because the existing IB vaccine has often failed to induce effective immune responses. The adjuvant activity of PD in conjunction with IB vaccine was evaluated in this study. Compared to control treatment, PD treatment significantly increased the proliferation of chicken peripheral blood mononuclear cells, concentration of interferon- $\gamma$  in culture supernatants, and anti-IB antibody titer. In chickens pre-challenged with the Mass 41 infectious bronchitis virus (IBV), PD administration resulted in fewer and less severe clinical signs, lower mortality rate, and higher protection compared to control treatment. Histopathological examination showed that the lungs and kidneys of PD-treated chickens displayed fewer pathological lesions than those of control chickens. Our results also demonstrated that this new vaccine adjuvant improved chicken humoral and cellular immune responses without any side effects. Hence, our findings suggest that PD might serve as an effective adjuvant in IBV vaccines.

**Key words:** adjuvant, immunogenicity, infectious bronchitis, platycodin D, *Platycodon grandiflorum*

*J. Poult. Sci.*, 57: 160–167, 2020

### Introduction

Infectious bronchitis (IB), caused by the infectious bronchitis virus (IBV), is an acute and highly contagious disease of chickens; IBV leads to severe economic losses in the poultry industry (Bermudez and Stewart-Brown, 2003; Amarasinghe *et al.*, 2017). Since IB pathogenesis is characterized by respiratory symptoms that often lead to complex infections and secondary bacterial disease, IB is an important concern for the poultry industry (Abd El Rahman *et al.*, 2010; De Wit *et al.*, 2017). Though both live-attenuated and inactivated vaccines are widely used to control IBV, outbreaks occasionally occur in endemic areas (Mase *et al.*, 2010; Dhama *et al.*, 2014). Several strategies, including use of powerful adjuvants to enhance immunogenicity, optimizing delivery methods, selecting appropriate immunization routes, and targeting effective antigen presentation have been

employed to improve the efficacy of the IBV vaccine (Wang *et al.*, 2009; Chen *et al.*, 2010; Zeshan *et al.*, 2011).

Adjuvants elicit immune responses through different signaling pathways, and can improve vaccine formulations for better protection (Aghasadeghi *et al.*, 2011; Czajka *et al.*, 2012; Antúnez *et al.*, 2016). However, as alum or oil adjuvants may cause local post-vaccination reactions in animals, these widely used chemical adjuvants are often associated with several disadvantages (Liu *et al.*, 2013; Martinez-Lopez *et al.*, 2014). Therefore, it is crucial to develop novel adjuvants for poultry vaccines.

Traditional Chinese medicine (TCM) uses natural products that have been employed by humans for centuries. TCMS contain numerous bioactive ingredients with beneficial effects, and have thus attracted much attention (Han *et al.*, 2011). Saponins, which are commonly found in plants, are a highly heterogeneous group of glycosides that have been known to exhibit adjuvant properties since the 1920s (Sjölander and Cox, 1998). The addition of saponins to drinking water was shown to increase vaccination efficacy against Newcastle disease, avian influenza, and infectious bursal disease in chickens (Yu *et al.*, 2015; Chi *et al.*, 2017).

The root of *Platycodon grandiflorum* A. DC (Campanulaceae) is a well-known TCM that has been employed as an expectorant for pulmonary diseases and as a remedy for respiratory disorders (Nyakudya *et al.*, 2014). Platycodin D

Received: August 15 2018, Accepted: August 16, 2019

Released Online Advance Publication: October 25, 2019

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(PD) is a less hemolytic and more stable saponin isolated from the root of *P. grandiflorum* (Choi *et al.*, 2018). Although PD has been previously shown to act as a potent adjuvant by enhancing specific immune responses to ovalbumin (Xie *et al.*, 2008) and a recombinant hepatitis B surface antigen (Xie *et al.*, 2009), it is unclear whether PD is an effective adjuvant for poultry vaccines.

The adjuvant performance of PD with the inactivated IBV vaccine was investigated in the present study. We aimed to test the efficacy and safety of PD, and to determine its optimum dosage. Our results may provide a theoretical basis for the development of novel adjuvants for poultry vaccines.

## Materials and Methods

### Animals

All experimental protocols were approved by the Institutional Animal Care and Ethics Committee of Nanjing Xiaozhuang College (permit number IACECNAU20140221), and were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals.

### Virus and Adjuvant

Two IBV strains, H120 and Mass 41 (M41), obtained from Jiangsu Provincial Academy of Agricultural Science, were propagated in the allantoic cavities of 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs, and the allantoic fluid from each egg was harvested 36 h post inoculation. The 50% embryo infection dose (EID<sub>50</sub>) was calculated by inoculating serial 10-fold dilutions of each virus strain into 10-day-old SPF embryonated chicken eggs following the protocol of Reed and Muench (1938). PD (C<sub>57</sub>H<sub>92</sub>O<sub>28</sub>; molecular weight: 1224.5854) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA; purity: HPLC ≥ 98%; product number: SMB00424).

### Preparation of Vaccines

Allantoic fluid aliquots from 10-day-old embryos inoculated with the IBV H120 vaccine strain (minimum titer: 10<sup>6</sup> EID<sub>50</sub>/mL) were harvested and used without modification. The live purified virus was inactivated by incubation in 0.4% formaldehyde for 2 h with constant stirring at 20°C and was stored for 24 h at 37°C. PD and the inactivated IBV H120 antigen were combined at a ratio of 3:1 (vol:vol). Three sets of inocula were prepared, with final PD concentrations of 0.25, 0.5, and 1 mg/mL. Three additional sets of inocula were also prepared: inactivated IBV H120 antigen alone (vaccine group), Al(OH)<sub>3</sub> (0.5 mg/mL) plus H120 antigen (positive control), and phosphate buffered saline (PBS; negative control).

### Chicken Immunization

We randomly divided 180 SPF chickens (age: 7 d; average body weight: 95 g) into six treatment groups; each treatment included three replicate cages (ten birds per cage). Chickens in each treatment group were individually inoculated via intramuscular injection with 100× the mean embryo infectious dose of one of the vaccine preparations in 0.2 mL. The initial vaccination was boosted with an equivalent dose of the same vaccine preparation at day 14 post initial in-

oculation.

### Isolation and Culture of Chicken Peripheral Blood Mononuclear Cells (PBMCs) for Proliferation Assay

Peripheral blood samples were obtained from chicken wing veins at 0, 14, and 28 days post immunization (dpi), and transferred immediately into aseptic capped tubes containing sodium heparin. Each sample was diluted with an equal volume of Hank's solution, and carefully layered over a lymphocyte separation medium (Ficoll-Hypaque;  $\rho$ : 1.077 ± 0.002; Rong Sheng Biostix Inc, Nanjing, Jiangsu, China). After centrifugation for 20 min at 400×g, the white cloud-like lymphocyte band was collected and washed twice with RPMI 1640 medium (Gibco, Waltham, Massachusetts, USA) without fetal bovine serum. The resulting mononuclear cell preparations were washed twice with RPMI 1640 medium, followed by centrifugation at 400×g. Cell viability was determined using trypan blue vital staining. The resulting pellet was resuspended in RPMI 1640 medium containing benzyl penicillin (100 U/mL), streptomycin (100 mg/mL), and 10% fetal bovine serum to achieve a final concentration of 5×10<sup>6</sup> cells/mL. Cells were seeded on 12-well plates (Nunc<sup>TM</sup> Delta) at a concentration of 10<sup>5</sup> cells/well. After a 12 h incubation at 37°C, the PBMCs were restimulated with various substances, including inactivated IBV H120 antigen (10<sup>2</sup> HA units/mL) (vaccine group), inactivated IBV H120 antigen (10<sup>2</sup> HA units/mL) plus 0.5 μg PD at one of three different doses (PD group), 0.5 μg Al(OH)<sub>3</sub> [Al(OH)<sub>3</sub> group]; or 100 μL PBS (PBS group). Cell proliferation was quantified based on restimulated PBMCs and assessed through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The entire analysis was performed in triplicate.

After a 44 h incubation at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> (Revco), 20 μL filter-sterilized thiazolyl blue tetrazolium bromide (5 mg/mL in calcium- and magnesium-free PBS) was added to each well, and the plates were reincubated for 4 h. The plates were centrifuged for 10 min at 1000×g at room temperature, and the supernatant was carefully discarded. Then, 10 μL dimethyl sulfoxide was added to each well, and the plates were shaken for 5 min to dissolve the formazan crystals. The optical density at 570 nm (OD<sub>570</sub>) of the cells in each well was measured using a microtiter enzyme-linked immunosorbent assay (ELISA) reader (Model DG-3022; East China Vacuum Tube Manufacturer), and the stimulation index (SI) was calculated as the ratio of the OD<sub>570</sub> values of the stimulated cells to those of the negative control, as described previously (Jiang *et al.*, 2006; Zhou *et al.*, 2010).

### Cytokine Assay

Approximately 1×10<sup>6</sup> PBMCs were stimulated with the corresponding reagent for 24 h as described above, and the culture supernatants were collected for the measurement of interferon (IFN)- $\gamma$  concentration. Supernatant cytokine concentration was determined using a sandwich ELISA kit (IFN- $\gamma$  Chicken Antibody Pair; Cat. No. CAC1233; Invitrogen, Waltham, Massachusetts, USA), following the manufacturer's instructions. Briefly, the coating antibody was prepared

(Anti-Chicken IFN- $\gamma$ ; 0.25 mg/0.5 mL) and added to the plate. Then, 100  $\mu$ L of the diluted cytokine standard, 100  $\mu$ L of the sample, and 50  $\mu$ L of the working detection antibody (Anti-Chicken IFN- $\gamma$  Biotin; 0.031 mg/0.2 mL) was added to each well. Plates were incubated for 2 h at 37°C. After washing five times, 100  $\mu$ L of the working streptavidin-HRP (Horseradish Peroxidase) solution was added to each well, and plates were incubated for 30 min at 37°C. The plates were washed five times, and 100  $\mu$ L of Tetramethylbenzidine (TMB) was added to each well. After incubation in the dark for 20 min, 100  $\mu$ L of stop solution (2 mol/L H<sub>2</sub>SO<sub>4</sub>) was added to each well, and the absorbance was measured at 450 nm using an automatic ELISA reader (Bio-Tek, Winooski, VT, USA). Standard curves were drawn, and the concentration of cytokines in each well was calculated with respect to the standards. Each serum sample was tested in duplicate.

#### **Serum Hemagglutinin Inhibition (HI) Antibody Assay in Chickens**

We randomly selected eight chickens from each group, and determined the serum HI antibody titer of each selected chicken. In brief, 0.5 mL of blood was drawn from the main brachial vein of each selected chicken into Eppendorf tubes at 0, 14, 21, and 28 dpi. The blood was allowed to clot at 37°C for 2 h. The serum was separated by centrifugation at 3860  $\times$  g for 15 min at 4°C and inactivated at 56°C for 30 min before use. The HI test was performed as stipulated by the Office of International des Epizooties (OIE, 2008) to determine the highest dilution of serum at which complete inhibition was still observed. The geometric mean titer was expressed as the reciprocal log<sub>2</sub> value of the highest dilution that displayed HI (Zeshan *et al.*, 2011; Habibi *et al.*, 2017).

#### **Challenge**

The M41 strain of IBV was grown in 10-day-old embryonated chicken eggs until the allantoic fluid titer reached  $5 \times 10^6$  EID<sub>50</sub>/mL. After vaccination, as described above, 35-day-old chickens were challenged with  $10^4$  EID<sub>50</sub> of M41 in 0.3 mL via the ocular-nasal route. Birds were monitored for 5 d post-challenge for any clinical signs or mortality. Dead chickens were necropsied to confirm that death was due to IBV infection. All remaining live chickens were euthanized at 5 d post-challenge. The kidney and lung tissues were collected from all chickens, and their viral RNA levels were quantified using RT-PCR. Cumulative mortality over the 5 d challenge was calculated as the number of birds that survived the challenge divided by the total number of birds challenged (Meir *et al.*, 2010; Khan *et al.*, 2017).

#### **Pathological Examination**

The collected kidney and lung tissues were fixed in 10% buffered neutral formalin, processed using standard histological procedures (Jain *et al.*, 2015), and embedded in paraffin. The paraffin-embedded tissues were then cut into 5  $\mu$ m-thick sections and stained using a standard hematoxylin and eosin (H&E) histological staining protocol.

#### **Statistical Analysis**

Statistical analyses were performed by Student's *t*-test and a one-way repeated measures analysis of variance (ANOVA) using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

Data are shown as mean  $\pm$  standard error (SE). The means of treatments were compared using Tukey's multiple range tests. Differences were considered significant at  $P < 0.05$ .

## **Results**

### **Effect of PD on Chicken PBMC Proliferation**

PBMC proliferation at 28 dpi was significantly higher in chickens treated with 0.5 and 1 mg/mL PD compared to that in control group chickens ( $P < 0.05$ ) (Fig. 1). Chickens treated with 0.5 mg/mL PD showed the highest SI across all immunized groups.

### **Cytokine Responses in Chickens**

The concentrations of IFN- $\gamma$  at 14 and 28 dpi were significantly higher in chickens vaccinated with inactivated IBV H120 plus PD or Al(OH)<sub>3</sub> than those in control chickens mock vaccinated with PBS or H120 only (Fig. 2). Of the three PD concentrations tested, chickens treated with 0.5 mg/mL PD showed the highest IFN- $\gamma$  concentration.

### **Effects on Serum Antibody Titer**

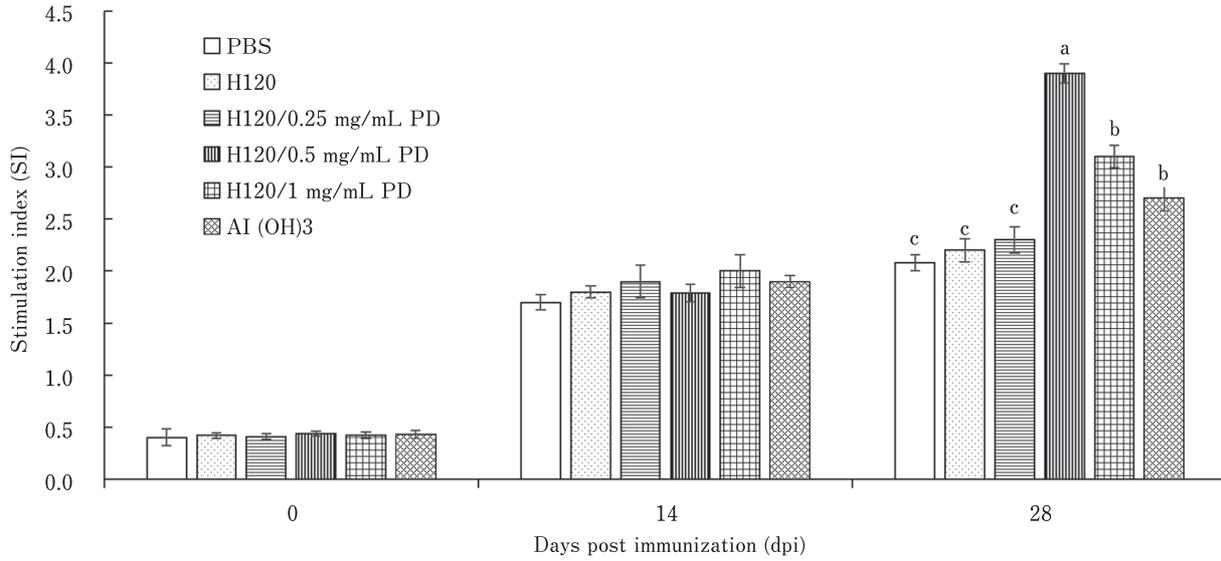
No significant differences ( $P > 0.05$ ) in HI antibody titer were identified among the PD treatment groups at 0 dpi, and no specific IBV antibodies were detected in the PBS group (Table 1). Antibody levels increased slowly in the group vaccinated with 0.25 mg/mL PD, but these levels were not significantly different from those of the control groups. The groups vaccinated with 0.25 and 1 mg/mL PD produced significantly more antibodies to IBV at 14 dpi, as compared to all other groups ( $P < 0.05$ ). At 21 and 28 dpi, the antibody titers in the 0.5 and 1 mg/mL PD groups were significantly higher than those in the H120 group ( $P < 0.05$ ).

### **Protection of Chickens Against IBV Challenge**

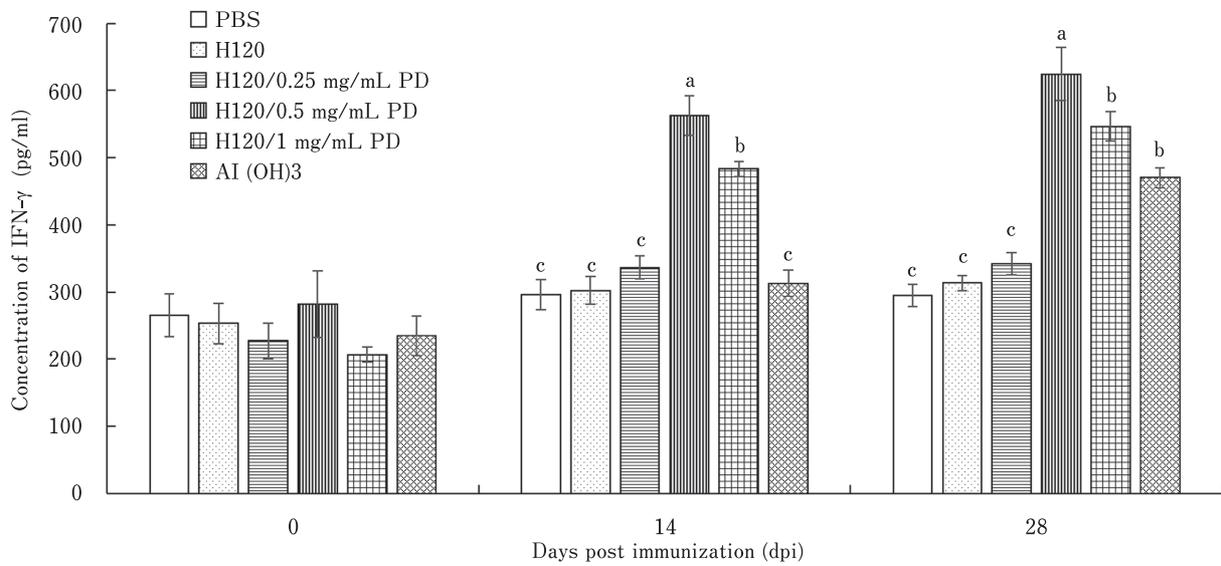
To evaluate the protective efficacy of the IBV vaccine with a PD adjuvant, all chickens were challenged with the IBV strain M41. Vaccination with IBV plus PD or Al(OH)<sub>3</sub> conferred greater protection than vaccination with IBV alone, as indicated by reduced morbidity, mortality, and clinical signs of IB in the chickens (Table 2). Chickens began to die or to show clinical signs of infection on day 4 after the challenge. The chickens injected with PBS alone were not protected against IBV infection, and developed cough, nasal discharge, and dyspnea. The death rate in the group injected with PBS was 43.3% at day 14 post-challenge, and the mortality rate in the 0.5 mg/mL group (0%) was significantly lower than that in all other groups ( $P < 0.05$ ). Moreover, the clinical signs and pathological changes observed in the affected chickens in the H120 and PBS groups were more conspicuous and severe than those observed in the affected chickens in the immunized group.

To evaluate the level of protection after IBV challenge, the collected lung and kidney tissue samples were examined for IBV infection using RT-PCR. Results showed that the protective rates in the 0.5 and 1 mg/mL groups (96.7%) were significantly higher than those in the other three groups ( $P < 0.05$ ).

On day 5 post-challenge, pathological lesions in the lungs and kidneys of chickens inoculated with PBS were characterized by epithelial degeneration, mild, moderate, or pro-



**Fig. 1. The influence of PD on the proliferation of chicken PBMCs, as measured by MTT assay.** The culture medium served as the negative control. Error bars represent  $\pm$ SE ( $n=8$ ). Means within a treatment group labeled with different letters (a, b, c) are significantly different ( $P < 0.05$ ).



**Fig. 2. The concentration of IFN- $\gamma$  in the sera of vaccinated chickens.** Error bars represent  $\pm$ SE ( $n=8$ ). Means within a treatment group labeled with different letters (a, b, c) are significantly different ( $P < 0.05$ ).

nounced lymphoid accumulation in the epithelial tissue, focal necrosis, and increased amounts of exudates. In the PD-immunized group, lesions were more moderate than those in the control groups (Fig. 3). Thus, our results suggested that PD confers resistance against virulent IBV.

### Discussion

Inoculation with commercially available inactivated IBV vaccines is not sufficient to prevent IBV infection due to the short duration of protection and limited cellular immune responses (Smith *et al.*, 2015). It has been shown that ad-

Table 1. Dynamic variations in antibody titers in chickens (log<sub>2</sub>)

Treatment group	Days post immunization (dpi)			
	0	14	21	28
PBS	0	0	0	0
IBV H120	0.5±0.2	3.2±0.4 <sup>b</sup>	4.1±0.3 <sup>c</sup>	4.3±0.1 <sup>c</sup>
IBV H120 + 0.25 mg/mL PD	0.5±0.3	4.0±0.4 <sup>a</sup>	4.5±0.6 <sup>c</sup>	4.3±0.6 <sup>c</sup>
IBV H120 + 0.5 mg/mL PD	0.3±0.1	3.7±0.4 <sup>b</sup>	5.9±0.4 <sup>b</sup>	5.9±0.4 <sup>b</sup>
IBV H120 + 1 mg/mL PD	0.4±0.2	4.7±0.7 <sup>a</sup>	6.5±0.7 <sup>a</sup>	6.9±0.3 <sup>a</sup>
IBV H120 + Al(OH) <sub>3</sub>	0.6±0.4	3.1±0.5 <sup>b</sup>	5.7±0.3 <sup>b</sup>	5.7±0.7 <sup>b</sup>

PBS, phosphate buffered solution; IBV, infectious bronchitis virus.

Means within the same column appended by different letters (a, b, c) are significantly different ( $P < 0.05$ ).

Values are expressed as means±SE.

Table 2. Protective effects of platycodin D immunization against infectious bronchitis virus (IBV) strain M41 in 5-week-old SPF chickens

Treatment group	Number of dead chickens <sup>a</sup>	Number of affected chickens <sup>b</sup>	Mortality (%)	Protection (%) <sup>c</sup>
PBS	13/30	30/30	43.3	0
IBV H120	5/30	9/30	16.7	70.0
IBV H120 + 0.25 mg/mL PD	5/30	5/30	16.7	83.3
IBV H120 + 0.5 mg/mL PD	0/30	1/30	0	96.7
IBV H120 + 1 mg/mL PD	1/30	1/30	3.3	96.7
IBV H120 + Al(OH) <sub>3</sub>	2/30	5/30	6.6	83.3

(a) The number of deaths was recorded each day post-challenge, and is presented as the total number of dead chickens in each group.

(b) IBV infection was detected in all chickens by RT-PCR, as indicated by the expression of IBV N protein in the lung and/or kidney tissues.

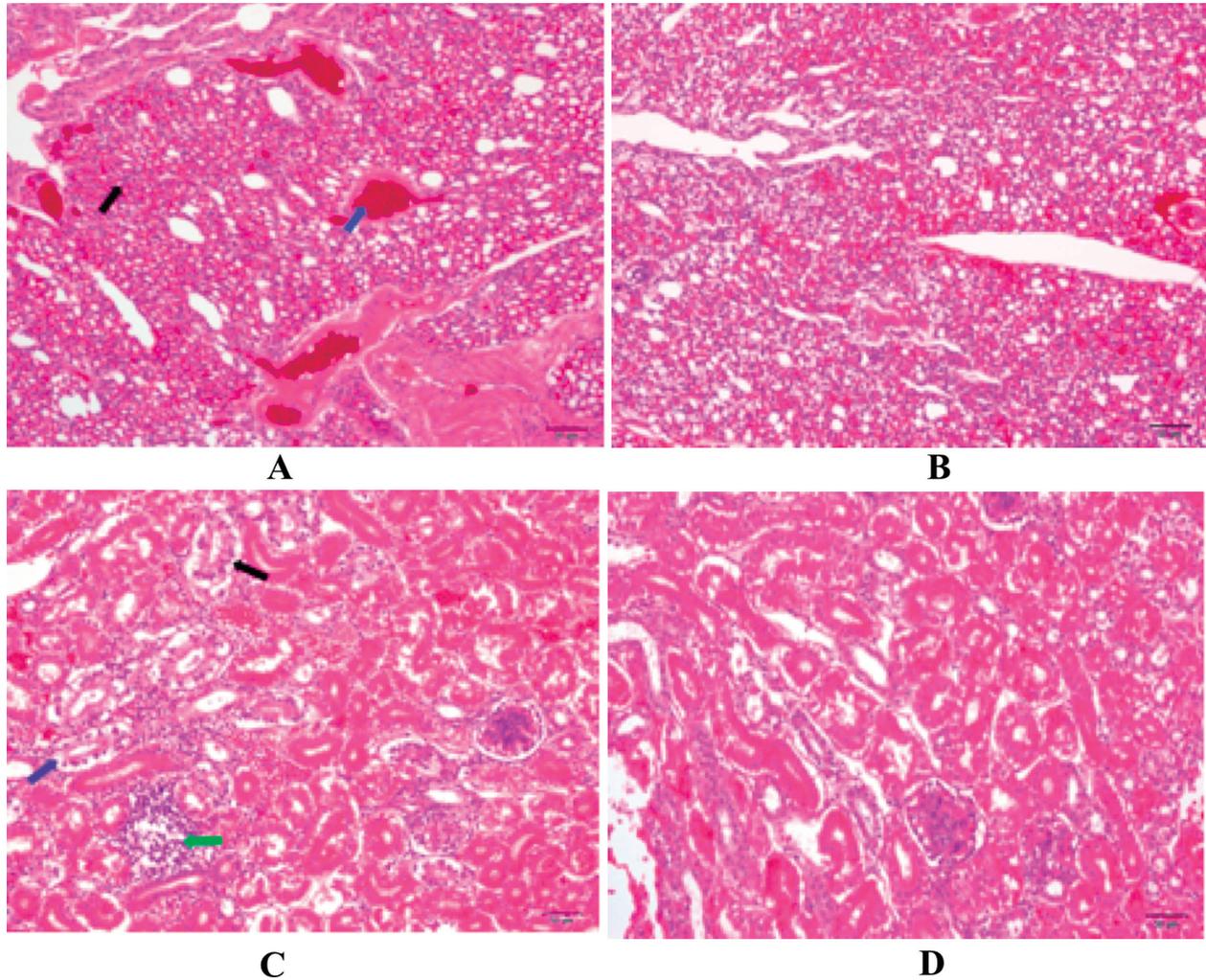
(c) Percent protection represents the number of unaffected chickens divided by the total number of chickens in each group.

juvants may enhance antigen-specific response and extend the duration of protection (Toro *et al.*, 2012). PD is a potential less-hemolytic saponin adjuvant isolated from the root of *P. grandiflorum*. PD treatment was shown to significantly enhance specific antibody and cellular responses in mice, and simultaneously elicit a Th1 and Th2 immune response (Sun *et al.*, 2011). Here, PD was used as an adjuvant. We evaluated how effectively PD induced immunity and protected chickens against IBV challenge. As expected, treatment with PD plus the inactivated IBV H120 antigen resulted in a higher IBV-specific antibody titer, greater T cell proliferation, and more robust protection against mortality. These results indicated that PD together with an IBV vaccine may confer preferably protection against IBV infection in chickens.

It is well known that cell-mediated immunity plays a crucial role in anti-infection and anti-tumor mechanisms, and can promote lymphocyte-mediated antibody production through lymphocytes sensitized to the corresponding antigen (Zhang *et al.*, 2017). Vaccination induces cell-mediated responses by promoting lymphocyte proliferation and increasing cytokine production (Oliveira *et al.*, 2016). The cytokine profile induced by an immune adjuvant plays an important role in the polarization of immune responses (Gan *et al.*,

2019). Chicken IFN- $\gamma$  activates macrophages, increases the expression levels of major histocompatibility complex I and II antigens in various cell types, and neutralizes viral replication (Janardhana *et al.*, 2007; Kano *et al.*, 2009; Andersen *et al.*, 2017). Our proliferation assay indicated that PD treatment significantly increased lymphocyte proliferation in immunized chickens. In addition, PD treatment significantly increased IFN- $\gamma$  concentration in the culture supernatants. These results indicated that PD treatment enhanced T and B lymphocyte activation, and induced humoral and cell-mediated immune responses in chickens. In addition, co-immunization with PD led to the secretion of the Th1 cytokine IFN- $\gamma$ , suggesting that immunized chickens were able to elicit an adaptive immune response by synthesizing T cells and improving cell-mediated immunity.

Serological response is an important indicator of successful vaccination, as this response reflects the ability of the vaccine to attach, replicate, and induce immune responses, including humoral antibodies (Andoh *et al.*, 2015). Humoral responses are an important element of protective immune responses against IBV (Chhabra *et al.*, 2015), and it has been reported that high antibody levels are associated with protection against IBV infection (Okino *et al.*, 2017). In this study, HI was used for the detection of IBV humoral anti-



**Fig. 3. Histological lesions in the lungs and kidneys of chickens at day 5 post-challenge (200× magnification under a light microscope).** A and C, Representative (A) lung and (C) kidney micrographs of control chickens. B and D, Representative (B) lung and (D) kidney micrographs of chickens treated with PD. Control chickens showed more severe nephritis compared to chickens treated with PD. The arrows indicate areas of pathological lesions.

bodies, as HI antibody levels are commonly used as indicators of protection in inactive vaccine (Katz and Kohn 1976). Indeed, HI antibody levels are typically used to monitor the antibody response induced by an IBV vaccine (Awad *et al.*, 2015). Therefore, HI titer was an important factor for the assessment of vaccine adjuvant performance. Our results indicated that the mean HI antibody titer production was dependent on the PD concentration when injected intramuscularly (i.m.) Co-administration of the antigen and PD increased HI titers, and the titers of the PD groups were significantly higher than those of the control or Al(OH)<sub>3</sub> groups. This result strongly indicated that PD improved antibody formation.

The results of the viral challenge indicated that chickens treated with PD were better protected against IBV than

unvaccinated chickens or chickens treated with the inactive vaccine. The protection rate in the PD groups (0.5 and 1 mg/mL) was 96.7%, indicating protective immunity against viral infection. Immune response analysis indicated that viral challenge elicited a strong immune response and enhanced the protection rate in the PD-treated groups. These results suggest that use of PD as an adjuvant might increase protection against IBV. The enhancement of immune responses with the PD adjuvant was slightly higher than that with the Al(OH)<sub>3</sub> adjuvant, thereby suggesting that PD can be used as an adjuvant in IBV vaccines, and it might provide better protection against IBV by stimulating both the humoral and cellular immune responses. Hence, these findings may have important implications in the design of IBV vaccine adjuvants.

### Acknowledgments

This work was supported by a cooperation project in industry, education, and research of the Science and Technology Department of Jiangsu Province (No. BY2018026), and a cooperation project in industry, education and research of Nanjing Agricultural Committee (No. 2018RHJD10). Supported by the earmarked fund for Jiangsu Agricultural Industry Technology System (No. JATS [2019] 020)

### Conflicts of Interest

The authors declare no conflict of interest.

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