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## Effects of Polysaccharide from *Chuanminshen violaceum* on Immune Response of Newcastle Disease Vaccine in Chicken

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### ABSTRACT

**Background:** Newcastle disease (ND), caused by avian paramyxovirus serotype 1 (APMV-1), also known as Newcastle disease virus (NDV), is regarded as one of the two most devastating diseases of poultry with the characteristics of serious disease and high flock mortality. It causes severe economic losses in domestic poultry, especially in chickens. At present, there were no effective drugs to treat this disease and the main method to control ND was vaccination. Moreover, new strains of virus resistant to chemotherapy continue to emerge, so does the need for a safe and effective vaccine. The immune adjuvant can make vaccine generate a strong immune response providing long-term protection against infection. With commonly usage of some immune adjuvants (e.g. mineral oil and aluminium hydroxide), many problems were occurred, such as side effects, strong local stimulation and carcinogenesis, together with complicated preparation, or failure to increase immunogenicity of weak antigen and so on. Botanical polysaccharides, as a new type of adjuvant or immunopotentiator, had become the hot research area because of their less side effects and no toxicity. The purpose of this research was to observe whether *Chuanminshen violaceum* polysaccharide (CVPS) possessed synergistical immunoenhancement, and offer the theoretical evidence for developing potential new-type adjuvant.

**Materials, Methods & Results:** 200 three-yellow chickens at one day of age were randomly divided into four groups and reared in separated pens. On 7 days old, the average maternal serum hemagglutination inhibition (HI) antibody titer was less than 3 log<sub>2</sub>, all chickens of each group (the average body weight (BW) was 120 g) were vaccinated with ND live vaccine through nasal drip and eye-drop. At the same time, the chickens in three CVPS groups (high, medium and low doses of CVPS) were orally administered with 0.5 mL of CVPS at concentrations of 100 mg/kg BW, 50 mg/kg BW, 25 mg/kg BW respectively, once a day for five successive day, in negative control group (NC), with 0.5 mL of physiological saline, once a day for five successive day. On days 14, 21 and 28, the serum antibody titer, erythrocyte-C3b receptor rosette rate (E-C3bRR), erythrocyte-C3b immune complex rosette rate (E-ICRR), peripheral lymphocyte proliferation and peripheral CD4<sup>+</sup>/CD8<sup>+</sup> ratio were measured. The results showed that the antibody titer, E-C3bRR, elimination rate of immune complex and peripheral lymphocyte proliferation in three CVPS groups and peripheral CD4<sup>+</sup>/CD8<sup>+</sup> ratio in medium dosage of CVPS group were significantly higher ( $P < 0.05$ ) than those in control group throughout the process of whole experiment almost.

**Discussion:** The CVPS not only improved the E-C3bRR and accelerated the elimination rate of CIC, but also induced higher antibody titer, peripheral lymphocyte proliferation and peripheral CD4<sup>+</sup>/CD8<sup>+</sup> ratio in chickens vaccinated against ND live vaccine. This indicated that CVPS possessed immune-enhancement efficacy of ND live vaccine and might be expected as a candidate of new-type adjuvant.

**Keywords:** *Chuanminshen violaceum*, antibody titer, peripheral lymphocyte proliferation, CD4<sup>+</sup>/CD8<sup>+</sup> ratio, erythrocyte immunity, ND vaccine.

## INTRODUCTION

Newcastle disease (ND), caused by avian paramyxovirus serotype 1 (APMV-1), also known as Newcastle disease virus (NDV), is regarded as one of the two most devastating diseases of poultry with the characteristics of serious disease and high flock mortality [1]. It causes severe economic losses in domestic poultry, especially in chickens [2]. At present, there were no effective drugs to treat this disease and the main method to control ND was vaccination [3].

Nowadays, the immune adjuvant was widely used to make vaccine generate a strong immune response and long-lasting protection against infection [4]. Botanical polysaccharides, as a new type of adjuvant or immunopotentiator, had become the hot research area because of their less side effects and no toxicity. Previous research has shown that polysaccharides, derived from *Epimedium*, *Astragalus*, *Isatis* root and so on, possessed effect of improving immune response to ND vaccine in chickens [4-6].

CVPS was composed of D-carubiose and D-glucose in the ratio of 1: 16.2. The weight average molecular weight and the number-average molecular weight of CVPS were  $9.7632 \times 105$  Da and  $5.2270 \times 104$  Da respectively. The purpose of this research was to observe whether *Chuanminshen violaceum* polysaccharide possessed synergistical immunoenhancement, and offer the theoretical evidence for developing potential new-type adjuvant.

## MATERIALS AND METHODS

### *Extraction and purification of polysaccharides*

The dried powder of *Chuanminshen violaceum* was defatted with 95% EtOH at 75°C under reflux for 6 h. The residue was decocted 3 times with distilled water; the combined decoction was concentrated and precipitated by alcohol. The polysaccharides was deproteinized with sevag's reagent, filtrated by chromatography on macroporous adsorption resins D101 and dialyzed against distilled water for 3 days in turn to remove pigment and other impurity. The content of polysaccharides was 93.85% measured by the phenol-sulfuric acid method [7].

### *Vaccine and Virus*

ND live vaccine (La Sota strain, No.100608) was offered by Liyuan Bio co., LTD, Guangxi, China. ND virus (La Sota strain) was supplied by Key labora-

tory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Ya'an, China.

### *Chickens and Experimental Design*

Two hundred three-yellow chickens at one day of age, purchased from Wens Co., Ltd Guangzhou, China, were randomly divided into four groups and reared in separated pens at 37°C with 24h light during the first week, then gradually decreased to 25°C and illumination time was reduced to 12 h per day. The chickens were fed with commercial chick starter and sufficient water.

On 7 days old, the average maternal serum hemagglutination inhibition (HI) antibody titer was less than 3 log<sub>2</sub>, all chickens of each group (the average body weight (BW) was 120 g) were vaccinated with ND live vaccine through nasal drip and eye-drop. At the same time, the chickens in three CVPS groups (high, medium and low doses of CVPS, CVPSH, CVPSM and CVPSL) were orally administered with 0.5 mL of CVPS at concentrations of 100 mg/kg BW, 50 mg/kg BW, 25 mg/kg BW respectively, once a day for five successive day, in negative control group (NC), with 0.5 mL of physiological saline, once a day for five successive day. On days 14, 21 and 28, six chickens were randomly selected from each group for determination of serum antibody titer by HI test, four chickens were randomly selected from each group for examination of E-C3bRR, E-ICRR by red cell yeast rosette formation test and peripheral lymphocyte proliferation by MTT method, three chickens were randomly selected from CVPSM and NC group for determination of peripheral CD4<sup>+</sup> /CD8<sup>+</sup> ratio by flow cytometric assay.

### *Serum HI antibody titer assay*

The serum HI antibody titer was determined as previously described [8]. Briefly, blood sample (1 mL per chick) from jugular vein was collected and the serum was separated by centrifugation. After inactivated at 56°C for 30 min, twofold serial dilution of serum, ranged from 1:2 to 1:2048, was added in a 96-well, V-shaped bottom microtiter plate containing 50 µL of CMF-PBS in each well and then 50 µL of NDV antigen (4 HA units) was added into all wells except for the last row served as the controls. The antigen-serum mixture was incubated at 37°C for 10 min. Then, 50 µL of 1% chicken erythrocytes suspension was added into each well and re-incubated for 30 min. The condition of agglutination was monitored and recorded. The

highest dilution of serum causing complete inhibition was considered the endpoint. The geometric mean titer was expressed as reciprocal log<sub>2</sub> values of the highest dilution that displayed HI.

#### *E-C3bRR and E-ICRR assay*

Heparinized blood sample (1 mL per chick) from jugular vein was centrifuged at 2400 g for 5 min and erythrocytes were collected. After washed 3 times with Hanks' buffer (PH7.2), the erythrocytes were re-suspended with Hanks' buffer at a concentration of  $1.25 \times 10^7$  cell/mL. E-C3bRR and E-ICRR were determined as previously described [9]. Briefly, 1% yeast suspension was prepared and boiled in thermostat water bath for 20 min. After filtration, part of the yeast suspension was adjusted to  $1 \times 10^8$  cell/mL and to be served as unsensitized yeast. The rest of yeast suspension was opsonized with an equal volume of fresh rat serum at 37°C with water bath for 20 min and then washed with Hank's buffer. The yeast was re-suspended in Hank's buffer at a concentration of  $1 \times 10^8$  cell/mL and to be served as sensitized yeast. Then, 50  $\mu$ L erythrocytes suspension were incubated with 50  $\mu$ L yeast suspension (sensitized yeast was used for measuring E-C3bRR, unsensitized yeast for measuring E-ICRR) at 37°C for 30 min by shaking periodically, then 100  $\mu$ L Hank's buffer and 25  $\mu$ L 0.25% glutaraldehyde were added and shaken gently and continually. Five min later, three smears were prepared. After fixed with methanol and stained with Wright's stain, two hundred erythrocytes were counted from each smear under a microscope. E-C3bRR and E-ICRR = the number of erythrocytes binding to more than two yeast cells/200  $\times$  100%. The average percentage of three smears was calculated to obtain the results for each sample.

#### *Peripheral lymphocyte proliferation assay*

The peripheral lymphocyte proliferation was determined as previously described [10]. Briefly, the peripheral lymphocyte, separated by lymphocyte separation medium from heart blood, was suspended with RPMI 1640 media (containing 10% fetal bovine serum)<sup>1</sup> at a concentration of  $5 \times 10^6$  cell/mL; 80  $\mu$ L of cell suspension and 20  $\mu$ L of Con A<sup>2</sup> was added into each well of a 96-well microtiter plates, each sample seeded 4 wells. The plates were incubated at 39.5 °C with a humidified 5% CO<sub>2</sub>. After incubation for 44 h, 20  $\mu$ L of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)<sup>3</sup> was added into each

well, and the plates were re-incubated for another 4 h. Then the plates were centrifuged at 1200 g for 10 min to remove the supernatant carefully and 100  $\mu$ L of Dimethyl Sulfoxide were added into each well to dissolve the formazan crystal. The OD<sub>570nm</sub> was measured by an ELISA reader (Model 680, Bio-Rad Instruments) as the index of peripheral lymphocytes proliferation.

#### *Peripheral CD4+ /CD8+ ratio assay*

The peripheral CD4+ /CD8+ ratio was determined as previously described [11]. Briefly, the peripheral lymphocyte, separated by lymphocyte separation medium<sup>4</sup> from venous blood, was suspended and transferred to another centrifuge tube, then 2 mL PBS was added and centrifuged at 2400 g for 5 min. The supernatant was discarded. The cell concentration was determined using the normal counting method of blood cells and then diluted to  $1.0 \times 10^6$  cells/mL with PBS. The aforementioned 1 mL cell suspension was transferred to another centrifuge tube and centrifuged at 2400 g for 5 min. The supernatant was discarded. The cells were respectively stained with 10  $\mu$ L mouse anti-chicken CD3-SPRD<sup>5</sup>, 10  $\mu$ L mouse anti-chicken CD4-FITC<sup>5</sup>, 10  $\mu$ L mouse anti-chicken CD8 $\alpha$ -RPE<sup>5</sup> for 15-20 min at RT, and then 2 mL PBS added and centrifugal elutriation performed once. The supernatant was discarded. The cells were resuspended in 0.5 mL PBS and determined by fluorescence-activated cell sorter<sup>5</sup>. Flow cytometry analyses were performed on a BD FACSCalibur<sup>TM</sup>.

#### *Statistical analyses*

Data are shown as means  $\pm$  SD and provided to Statistical analyse using SPSS 17.0. Duncan's multiple range tests was used to evaluate the difference. Differences between means were considered significant at  $P < 0.05$ .

## RESULTS

#### *The dynamic changes of antibody titer*

The changes of serum HI antibody titer were summarized in Table 1. On day 14, the antibody titers in the three CVPS groups were significantly higher ( $P < 0.05$ ) than that in negative control group, however there were no significant differences in the three CVPS groups. On day 21, the antibody titer in CVPS<sub>L</sub> group was significantly higher than those in negative control group and another two CVPS groups

( $P < 0.05$ ); no significant differences were exhibited in the CVPS<sub>H</sub>, CVPS<sub>M</sub> group and negative control group. On day 28, the antibody titers in the three CVPS groups were significantly higher ( $P < 0.05$ ) than those in negative control group; compared with CVPS<sub>H</sub> group, the CVPS<sub>L</sub> group had higher effect on antibody titer ( $P < 0.05$ ).

*The dynamic changes of peripheral lymphocyte proliferation*

The changes of peripheral lymphocyte proliferation of each group were displayed in Table 2.

On day 14, peripheral lymphocyte proliferation in the three CVPS groups was significantly higher ( $P < 0.05$ ) than that in negative control group; significant differences ( $P < 0.05$ ) were exhibited between CVPSH and CVPSL group. On day 21, the peripheral lymphocyte proliferation in the three CVPS groups was significantly higher ( $P < 0.05$ ) than that in the negative control group; the CVPSH group had higher effect on peripheral lymphocyte proliferation ( $P < .05$ ) in comparison with CVPSM and CVPSL groups. On day 28, no significant differences were detected in the four experimental groups.

**Table 1.** The changes of serum HI antibody titer in each group (Log<sub>2</sub>).

group	D <sub>14</sub>	D <sub>21</sub>	D <sub>28</sub>
CVPS <sub>H</sub>	4.50 ± 0.55 <sup>b</sup>	4.17 ± 0.41 <sup>a</sup>	4.00 ± 0.63 <sup>b</sup>
CVPS <sub>M</sub>	4.33 ± 0.52 <sup>b</sup>	4.33 ± 0.52 <sup>a</sup>	4.17 ± 0.41 <sup>bc</sup>
CVPS <sub>L</sub>	4.17 ± 0.41 <sup>b</sup>	5.33 ± 0.52 <sup>b</sup>	4.67 ± 0.52 <sup>c</sup>
NC	3.17 ± 0.41 <sup>a</sup>	4.33 ± 0.52 <sup>a</sup>	3.33 ± 0.52 <sup>a</sup>

The superscripts within a column with different letters (a-c) differ significantly ( $P < 0.05$ ).

**Table 2.** The changes of peripheral lymphocyte proliferation in each group (OD<sub>570</sub> value).

group	D <sub>14</sub>	D <sub>21</sub>	D <sub>28</sub>
CVPS <sub>H</sub>	0.197 ± 0.025 <sup>c</sup>	0.287 ± 0.031 <sup>c</sup>	0.243 ± 0.026 <sup>a</sup>
CVPS <sub>M</sub>	0.171 ± 0.012 <sup>bc</sup>	0.230 ± 0.020 <sup>b</sup>	0.229 ± 0.024 <sup>a</sup>
CVPS <sub>L</sub>	0.154 ± 0.018 <sup>b</sup>	0.224 ± 0.021 <sup>b</sup>	0.218 ± 0.020 <sup>a</sup>
NC	0.106 ± 0.019 <sup>a</sup>	0.162 ± 0.018 <sup>a</sup>	0.223 ± 0.021 <sup>a</sup>

The superscripts within a column with different letters (a-c) differ significantly ( $P < 0.05$ ).

*The dynamic changes of E-C<sub>3b</sub>RR*

The E-C<sub>3b</sub>RR value in each group was shown in Table 3. On day 14, the E-C<sub>3b</sub>RR values in three CVP<sub>S</sub> groups were significantly higher ( $P < 0.05$ ) than that in the negative control group, and in CVPS<sub>H</sub> group, the E-C<sub>3b</sub>RR value was significant higher ( $P < 0.05$ ) than those of CVPS<sub>M</sub> and CVPS<sub>L</sub> group. On day 21, the E-C<sub>3b</sub>RR values in three CVPS group were significantly higher ( $P < 0.05$ ) than those in negative control group; the CVPS<sub>L</sub> group had higher effect on E-C<sub>3b</sub>RR in comparison with CVPS<sub>M</sub> and CVPS<sub>H</sub> groups ( $P < 0.05$ ). On day 28, no significant differences were observed in all four experimental groups.

*The dynamic changes of E-ICRR*

The E-ICRR value in each group was exhibited in Table 4. On day 14, the E-ICRR value in CVPS<sub>H</sub> group was significantly lower ( $P < 0.05$ ) than those in CVPSM, CVPS<sub>L</sub> and negative control group, however there were no significant differences in these three groups. On day 21, the E-ICRR values in three CVP<sub>S</sub> groups were significantly lower ( $P < 0.05$ ) than that in negative control group; the E-ICRR value in CVPSH group was significantly lower ( $P < 0.05$ ) than those in CVPS<sub>M</sub> and CVPS<sub>L</sub> groups. On day 28, significant differences were exhibited between the three CVPS groups and the negative control group, but no significant differences were observed in the three CVPS groups.

*The dynamic changes of peripheral T-lymphocyte CD4<sup>+</sup>/CD8<sup>+</sup> ratio*

The changes of peripheral T-lymphocyte CD4<sup>+</sup>/CD8<sup>+</sup> ratio were shown in Figure 1. On day 14, no

changes about the CD4<sup>+</sup>/CD8<sup>+</sup> ratio was observed in CVPSM and negative control group. On days 21 and 28, the CD4<sup>+</sup>/CD8<sup>+</sup> ratio in CVPSM group was significantly higher ( $P < 0.05$ ) than that in the negative control group

**Table 3.** The changes of E-C<sub>3b</sub>RR in each group (%).

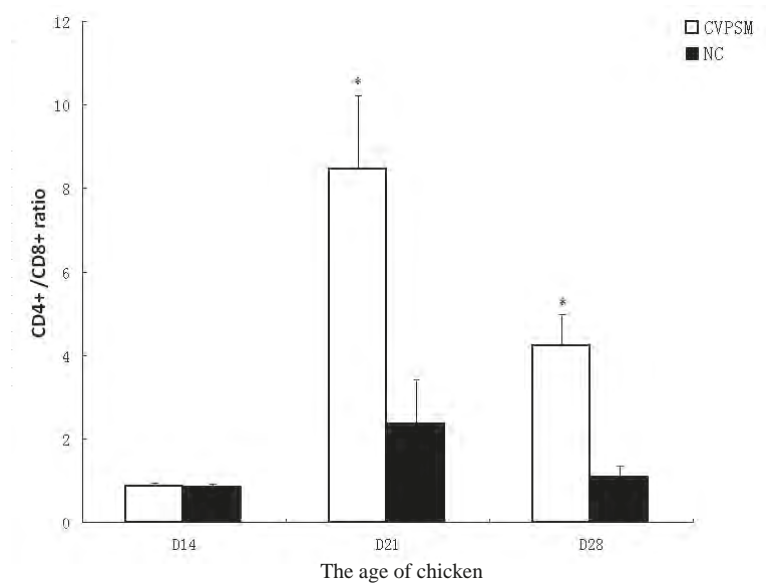
group	D <sub>14</sub>	D <sub>21</sub>	D <sub>28</sub>
CVPS <sub>H</sub>	7.50 ± 0.71 <sup>c</sup>	6.38 ± 0.48 <sup>b</sup>	3.75 ± 0.64 <sup>a</sup>
CVPS <sub>M</sub>	4.62 ± 0.49 <sup>b</sup>	5.12 ± 0.85 <sup>b</sup>	3.50 ± 0.58 <sup>a</sup>
CVPS <sub>L</sub>	4.24 ± 0.65 <sup>b</sup>	7.38 ± 0.75 <sup>c</sup>	3.12 ± 0.63 <sup>a</sup>
NC	2.75 ± 0.29 <sup>a</sup>	3.12 ± 0.48 <sup>a</sup>	3.00 ± 0.41 <sup>a</sup>

The superscripts within a column with different letters (a-c) differ significantly ( $P < 0.05$ ).

**Table 4.** The changes of E-ICRR in each group (%).

group	D <sub>14</sub>	D <sub>21</sub>	D <sub>28</sub>
CVPS <sub>H</sub>	12.83 ± 0.71 <sup>b</sup>	3.71 ± 0.34 <sup>c</sup>	3.92 ± 0.57 <sup>b</sup>
CVPS <sub>M</sub>	14.33 ± 1.03 <sup>a</sup>	6.21 ± 0.60 <sup>b</sup>	4.38 ± 0.28 <sup>b</sup>
CVPS <sub>L</sub>	14.79 ± 0.92 <sup>a</sup>	7.08 ± 0.63 <sup>b</sup>	4.66 ± 0.45 <sup>b</sup>
NC	15.12 ± 1.07 <sup>a</sup>	10.12 ± 0.96 <sup>a</sup>	6.74 ± 0.50 <sup>a</sup>

The superscripts within a column with different letters (a-c) differ significantly ( $P < 0.05$ ).



**Figure 1.** The changes of peripheral T-lymphocyte CD4<sup>+</sup>/CD8<sup>+</sup> ratio in medium dose of CVPS group and negative control group. Significant differences between medium dose of CVPS group and negative control group was reported as \* $P < 0.05$ .

## DISCUSSION

The changes of serum antibody titer in poultry reflected the state of humoral immunity [5]. Antibody usually appears within 6-10 days after infection in blood and local tissues [12]. The high level of antibody titer has always been associated with better protection against ND [13]. In the present study, the serum antibody titer of three CVPS groups was almost higher at the three experimental time points in comparison with negative control group, and the low dosage of CVPS group possessed better activity in three CVPS groups. Treated with low dosage of CVPS could induce the antibody titer increasing from day 14 to day 21 and still maintain a higher level on day 28, which indicated the CVPS could generate the stronger and longer lasting humoral immunity.

Both humoral immunity and cellular immunity play an important role in defense against NDV. In chickens, Cell-mediated immune responses play an important role to decrease the excretion and dissemination of the NDV [14]. Lymphocytes proliferation is the indicator reflecting the state of cellular immunity [15]. In the present study, mitogen-induced lymphocyte proliferation in responses to the T-lymphocyte mitogen conA was significantly augmented in three CVPS groups with a dose-dependent manner in comparison with the control group on days 14 and 21. These results suggested CVPS could promote the cellular immunity and the effect was strengthened gradually with the increase of dosage. The higher peripheral cellular immunity induced by CVPS might be involved in a greater proportion of specific T-lymphocytes [3]. Studies in *Astragalus* polysaccharide and *Codonopsis pilosula* polysaccharide had shown the similar results [4,6].

The immune function of erythrocytes play the major carriers of immune complexes(IC) in the circulation via their complement receptor(E-C3b, CR1), these erythrocytes bind C3b bearing immune complexes and transport them to the organs of the monocyte phagocytic system and reticuloendothelial system where the IC were eliminated, including the liver and spleen [17,18]. Defect of E-C3b could lead to tissue injury by IC which escaped from the red cell immune system [19]. In this study, the E-C3bRR of chickens treated with CVPS was always higher than that of negative control group on days 14 and 21, thus it suggested that CVPS could increase the activity of CR1, and enhance the expression of E-C3b [18]. Treat-

ed with high dosage of CVPS could rapidly cause the E-C3bRR rising to a high level, then began to decline, while treated with medium and low dosages of CVPS led to relatively low E-C3bRR in the beginning and then the E-C3bRR increased along with the extending of time. This indicated that different dosage of CVPS had different mode of action on E-C3bRR.

After vaccinated against ND vaccine, ND-specific antibodies were abundantly produced by chick immune organs and many circulating immune complexes (CIC) were formed in blood circulation. The CIC were adhered by erythrocytes via CR1 causing the E-ICRR rising, then quickly carried to be cleaned up [9,19]. In our research, the E-ICRR in all four experimental groups was reached a higher level after vaccination, and then it went down gradually. On day 14, the E-ICRR in high dosage of CVPS group was significantly lower than that in negative control group, and it was turned to normal level on day 21, while the medium and low dosage of CVPS groups were recovered on day 28. At the same time, the negative control group was still higher on days 21 and 28 than the three CVPS groups. These results demonstrated the CVPS could accelerate clearing the CIC in a dose dependent way. This was agreement with the studies in Selenium, Sodium Selenite and *Epimedium* polysaccharides [20-23].

The CD4<sup>+</sup> /CD8<sup>+</sup> ratio has been widely used to estimate the state of immune system [24]. In chickens, low CD4<sup>+</sup> /CD8<sup>+</sup> ratio has been associated with decreased humoral immunocompetence. In our study, treatment with medium dosage of CVPS caused an increase in the percentage of CD4<sup>+</sup> cell and the decreased percentage of CD8<sup>+</sup> cell, resulting in markedly increasing CD4<sup>+</sup> /CD8<sup>+</sup> ratio. These changes in distribution of CD4<sup>+</sup> and CD8<sup>+</sup> cells were observed at days 21 and 28, which indicated that chickens treatment with CVPS may induce a higher immune status. Studies in *Dioscorea opposita* polysaccharide and *Isatis* root polysaccharide had shown the similar changes of lymphocyte subsets [25,26].

The correlation of immunoenhancement efficacy with the dosage of CVPS was also investigated in this research. Our observations shown that different dosage of CVPS possessed a dose-dependent relationship in peripheral lymphocyte proliferation and E-ICRR, however this relationship did not exist in serum antibody titer and E-C3bRR. Similar effects of

some Chinese herbal medicinal ingredients on serum antibody titer and *Epimedium* polysaccharides on E-C3bRR in chickens immunized with ND vaccine was reported [21,27].

#### CONCLUSION

The CVPS not only improved the E-C3bRR and accelerated the elimination rate of CIC, but also induced higher antibody titer, peripheral lymphocyte proliferation and peripheral CD4+ /CD8+ ratio in chickens vaccinated against ND live vaccine. These results confirmed that CVPS could enhance the immune response of ND vaccine in chicken, and it would be expected as a candidate of new-type immunologic adjuvant.

#### SOURCES AND MANUFACTURERS

<sup>1</sup>Gibco, Shuanghai, China.

<sup>2</sup>Sigma-Aldrich Co., St. Louis, Missouri, USA

<sup>3</sup>Amersco, Ohio, USA.

<sup>4</sup>Haoyang Biological manufacture co., LTD, Tianjin, China.

<sup>5</sup>BD Pharmingen, New Jersey, USA.

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**Declaration of interest.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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