Salmonella Serovars and Vaccination Effect on the Immune Responses of Male and Female Layers

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Salmonella Enteritidis, S. Gallinarum and S. Pullorum are common serovars to infect poultry and cause diseases differently. The antibody production and cellular immune responses of male and female layers were evaluated before and after inoculation. Before inoculation, S. Gallinarum and S. Pullorum could survive and grow in 10% sera from 6-week-old layers, and S. Enteritidis and E. coli were completely eliminated. The weights of the male and female layers were increased the lowest by inoculation with S. Gallinarum, followed by S. Pullorum, and S. Enteritidis. Inoculation with S. Enteritidis, S. Gallinarum and S. Pullorum increased the antibody titer in the males depending on the serovars and maintained same higher antibody level in females. Furthermore, an increased anti-Salmonella IgG titer was associated with bactericidal ability and the level was reduced by serovars and complemente. Despite the vaccination and serovars, the male layers expressed more IgG2a than IgG1, indicating preferential activation of the Th1 pathway. The inoculation number affected the expression level of IFN-γ and IL-12 in the blood not in the secretion of the peripheral blood mononucleated cells (PBMCs) and more inoculations increased the expression of both cytokines. Inoculation increased more reactive oxygen species (ROS) production in polymorphonuclear (PMN) cells, not the PBMCs. ROS production was greater in cells from the males than from the females and greater in the cells treated with S. Enteritidis than S. Gallinarum and S. Pullorum. These three serovars and their vaccinations differed in sera killing and immune responses.

Key words: cytokine, IgG antibody, PBMC, PMN, reactive oxygen species, Salmonella Infection

J. Poult. Sci., 53: 318-328, 2016

Introduction

Zoonotic non-typhoid Salmonella causes foodborne salmonellosis in humans through the consumption of contaminated poultry meat, eggs and their products (Harrison et al., 1992; Hogue et al., 1997). From 2004 to 2012, S. Enteritidis and S. Typhimurium were two of the most prevalent serovars that infected humans in Taiwan (Kuo et al., 2014). In poultry, S. Enteritidis, S. Pullorum, and S. Gallinarum of serogroup D Salmonella are common serovars to cause salmonellosis with different symptoms. Broad-host-range S. Enteritidis with conserved genome and 60-kb virulence plasmid causes mostly asymptomatic in poultry and may transfer from layers to humans (Chu et al., 2009). However, poultry-specific and flagella-less S. Gallinarum causes typhoid fever in chickens of all ages, while S. Pullorum mainly infects young chicks and is associated with high mortality.

The antibody production and cellular immunity of the avian immune system against bacterial infections have been extensively reviewed (Wigley et al., 2013). The major heterogeneous immune groups involved in cellular immunity include lymphocytes, monocytes and macrophages of peripheral blood mononucleated cells (PBMCs), and neutrophils (heterophils), eosinophils, basophils, and mast cells of polymorphonuclear leukocytes (PMN). In poultry, heterogeneous PMN cells consist of heterophil, which can express Toll-like receptor (TLR)-induced My88-dependent pathway to activate avian innate immunity against S. Enteritidis infection (Kogut et al., 2012), utilize Toll-like receptors to detect pathogens and mediate oxidative burst (Farnell et al., 2003), stimulate expression of proinflammation genes (He et al., 2005). Although monocytes and macrophages of the innate immune system function to remove pathogens directly, the intestinal microbiota can minimize Salmonella infections by preventing pathogen growth and adhesion to epithelial
cells (Lawley et al., 2008; Qureshi et al., 2000).

Although chickens infected with S. Enteritidis can increase their expression levels of proinflammatory genes, such as IFNγ, IL-1β, and IL-6, and antibodies IgA and IgG (Cranova et al., 2011; Matulova et al., 2013), S. Enteritidis is still observed at the highest level in the cecum, followed by the spleen and liver (Matulova et al., 2013). Additionally, an oral vaccination with S. Gallinarum increases plasma IgG, intestinal secretory IgA and cell-mediated responses (Jeon et al., 2013). In study of immune responses, S. Enteritidis, S. Gallinarum and S. Pullorum stimulated expressions differently of cytokine and chemokine gene of cecal tonsil in newly hatched chicks and older chickens (Setta et al., 2012).

Most recent study mainly reported the effects of oral infection of vaccine strains on cecal and intestinal immune response. Therefore, we investigated the effects of inoculation number and vaccination with S. Enteritidis, S. Gallinarum, and S. Pullorum on the IgG, production and serum killing, activation of Th1 or Th2 pathway associated IgG1 and IgG2a antibodies and cytokines and ROS production of PBMC and PMN cells from male and female layers against these three serovars.

Materials and Methods

Bacterial Isolates

Serovars S. Enteritidis OU7586, an invasive strain of S. Enteritidis OU7439 to chicks (Chen et al., 2008), S. Pullorum OU7115 and OU7116, S. Gallinarum OU7113 and OU7114 of serogroup D Salmonella, and Escherichia coli OU4092 were used. The use of all bacteria and the animal experiments were approved by the Biological Security Committee, the Animal Use Protocol (IACUC No.99022), and the Institutional Animal Care and Use Committee of National Chiayi University in accordance with the laws of Taiwan (ROC).

Vaccination with Pathogenic Serogroup D Serovars

Salmonella Gallinarum OU7113, S. Pullorum OU7115, and S. Enteritidis OU7586 were used to inoculate Hendrix male and female layers, which were housed in the Animal House at National Chiayi University. Based on sex and age, the three chicken groups were the M1 (male), M2 (male) and F2 (female) groups. M2 and F2 chicks were purchased at same age. Chicks were randomly separated into the Control group (n=3/3/2 for M1/M2/F2, respectively), S. Gallinarum (SG) group (n=4/4/3 for M1/M2/F2, respectively), S. Pullorum (SP) group (n=3/4/3 for M1/M2/F2, respectively) and S. Enteritidis (SE) group (n=3/3 for M1/M2, respectively) at 16 weeks of age.

Three inoculations were performed by injecting 5×10^5 colony-forming units (CFU) of OU7113, OU7115, and OU7586 with complete Freund’s adjuvant (F5881, Sigma-Aldrich, Shanghai, China) into muscle at the first inoculation. Next, two consecutive injections of 5×10^5 CFU were performed three weeks apart with incomplete Freund’s adjuvant (F5506, Sigma-Aldrich). Ten milliliters of blood were collected into a heparin tube (REF 366480, Beckman Dickson, Franklin Lakes, NJ, USA) one day before the first inoculation and 3 weeks after the three inoculations. After centrifugation at 3,000 rpm (RA-6-62 rotor, Eppendorf 5810, Eppendorf, Hamburg, Germany) for 10 min, plasma was collected and stored at −80°C. Some of the plasma was treated at 56°C for 30 min to inactivate the complement activity.

Anti-Salmonella IgG, IgG1 and IgG2a Measurement

Antibody titers were measured for IgG in all groups and for IgG1 and IgG2a in M1 and M2 groups. To remove non-specific antibodies, 1×10^6 CFU of E. coli was reacted with 500 μl plasma by shaking at 100 rpm and 37°C for 1 h. The supernatant was then collected after centrifugation at 10,000 rpm (rotor F-5-45-30-1, Eppendorf 5417C) for 5 min. These steps were repeated three times. An overnight culture was inactivated at 56°C for 30 min, and then, 1×10^8 cfu of a bacterial culture were coated on the wells of a 96-well plate at 4°C overnight. After blocking with PBS buffer plus 5% non-fat milk, each well was washed three times with 200-μl wash buffer (PBS plus 0.05% Tween 20, PBST). After addition of 1,000-fold diluted sera, 45,000-fold diluted horse-radish peroxidase (HRP)-labeled anti-chicken IgG, IgG1 and IgG2a antibody (Sigma-Aldrich) and 3', 3', 5', 5'-tetramethyl benzidine (TMB) (T0440, Sigma-Aldrich) were added to the wells. Finally, the reaction was inactivated with 0.5 N H2SO4. The OD450 was measured using an Anthos 2010 ELISA reader (Anthos Labtech Instrument, Australia). The anti-Salmonella antibody titer was calculated.

In vitro Bactericidal Effect of Sera with or without Complement Activity

Before vaccination, a basic bactericidal capability was evaluated using the plasma from six-week-old female layers. The 10% serum was mixed with 1×10^8 CFU/ml of S. Gallinarum OU7113 and OU7114, S. Pullorum OU7115 and OU7116, S. Enteritidis OU7130 and OU7586, and E. coli was plated on XLD plates at 0, 40, 80 and 160 min. Viable bacteria were counted and analyzed. After inoculation, the effects of the complement and serum concentrations on the bactericidal capability of the sera against the above bacteria were evaluated. Mueller Hinton broth (MHB) was mixed with serum with or without complement activity that was inactivated at 50°C for 30 minutes to achieve serum concentrations of 20%, 10%, and 5%. After an culture for 16~18 h, the OD595 was measured using an ELISA reader (Anthos 2010, Biochrom, Cambridge, UK). The bactericidal capability was calculated using the following formula: Growth ratio (%)=[OD595 of mixture of MHB and defined sera with bacteria – OD595 of mixture and defined serum without bacteria)/OD595 of MHB with bacteria - OD595 of MHB without bacteria]×100%.

IFN-γ and IL-12 Levels and ROS Production of the PBMCs and PMN Cells

The lymphocytes were collected according the modification of method described previously (Barta et al., 1992). Briefly, buffy coats were collected in a Ficoll solution (1.077 g/ml; SI10771, Sigma-Aldrich) after centrifugation at 25°C and 600×g for 6 minutes. PBMCs were washed with 1 ml PBS three times and mixed with 1 ml RPMI of a solution (R6504, Sigma-Aldrich). To purify the PMN cells, the
layer under theuffy coat was mixed with 10 ml PBS and then centrifuged at 350 × g for 10 minutes. The pellet was lysed with 30 ml of RBC Lysis buffer (0.19 M NH4Cl, 4 mM K2HCO3, 0.39 mM EDTA) for 10 minutes. After centrifuging at 350 g for 10 minutes, the pellet was washed three times with 1 ml PBS buffer. The pellet was mixed with 1 ml RPMI, and the cell numbers were counted.

After the addition of 5 × 10^5 PBMCs into each well of a 96-well plate, bacteria were added at an MOI of 100. The mixture was incubated at 37°C and 5% CO2 for 1, 2, and 4 h. IL-12 and IFN-γ levels in the blood and PBMC supernatant were measured using a Chicken IFN-γ (Catalogue No. 201-16-0003) and Chicken Interleukin 12 (IL-12) (Catalogue No. 201-16-0009) ELISA kit (Shanghai Sunred Biological Technology Co., Shanghai, China). Next, we measured the effect of serovars on the superoxide production of PMN cells and PBMCs. A similar procedure was performed as above. However, the superoxide was measured at 1, 2, and 4 h using nitro blue tetrasodium (NBT; N5514, Sigma-Alderich) according to the method from the manufacturer.

**Statistical Analysis**

Statistical analyses were performed using SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA). A Tukey HSD test was used to rank the different treatments. P values < 0.05 were considered significantly.

**Results**

During the experimental period, the average weight per chicken increased from 1.7 kg to 2.0 kg in the M1 and M2 groups and from 1.3 kg to 1.5–1.6 kg in the F2 group. However, the average weight increased among the serovars without significant difference. The lowest weight increase was observed in the S. Gallinarum (SG) group, followed by the S. Pullorum (SP) and S. Enteritidis (SE) groups with a weight increase of 54 g/106 g/358 g for the SG group/SP group/SE group, respectively, in the M1 group vs. 74 g/96 g/138 g, respectively, in the M2 group. However, the weight increase for the SG group/SP group was 8 g vs. 46 g, respectively, in the F2 group.

**Anti-Salmonella IgG, IgG1 and IgG2a Production**

The highest anti-Salmonella IgG antibody titer was observed in the SG group for both the M1 and M2 groups. There was no difference in the antibody titers between the SP and SE groups in the M1 group, but they were significantly different in the M2 group (Table 1). In the F2 group, there were no antibody differences between the SG and SP groups. An increase in the antibody titer associated with the inoculation number was only observed in the F2 group, while such an increase was not observed in the M1 and M2 groups. The statistical analysis of the antibody IgG titer revealed significant differences between the sera with and without complement activity (F=5643.02 for the M1 group, F=8709.56 for the M2 group; p < 0.01). There was no change in the F2 group (F=0.07; p=0.79). In addition, the antibody titer differed between the M1 and M2 groups (age difference) (F=3577.4; p<0.01) and between genders (M2 and F2 groups) (F=98.87; p<0.01).

Further, we analyzed the anti-Salmonella IgG1 and IgG2a levels in the sera of the M1 and M2 layers. Both antibody titers differed among serovars in the M1 and M2 groups (Table 2). In the M1 group, the highest IgG1 and IgG2a levels were observed for all serovars after the third inoculation. In the M2 group, the highest IgG1 titer was observed in SG8 and SP14 after the third inoculation and in SE20 after first inoculation, while the highest IgG2a level was observed in SG8 and SE20 after the first inoculation and in SP 14 after the third inoculation.

**Bactericidal Ability of Serum with or without Complement Activity**

After the interaction with 10% serum from 6-week-old female layers for 0, 40, 80, and 180 minutes, S. Enteritidis and E. coli pir116 were killed at 40 min, while S. Gallinarum and S. Pullorum could survive and propagate. All the bacterial species survived in 10% serum without complement activity.

In the vaccination experiment of layers older than 40 weeks, we examined the bactericidal activity of the serum from layers inoculated with S. Enteritidis, S. Gallinarum and S. Pullorum. The serum with the highest IgG titer was se-

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**Table 1. IgG antibody titer among different factors within three chicken groups**

| Challenge | Chicken groups (Mean ± standard error) 1 |
|-----------|------------------------------------------|
|           | M1                     | M2                     | F2                     |
| Group     |                          |                        |                        |
| SG        | 0.93 ± 0.01 b,y         | 0.64 ± 0.01 c,x        | 0.65 ± 0.01 a          |
| SP        | 0.71 ± 0.01 a,x         | 0.37 ± 0.01 b,x        | 0.63 ± 0.01 y          |
| SE        | 0.72 ± 0.01 b,y         | 0.50 ± 0.001 b,x       |                        |
| Vaccination 2 |                          |                        |                        |
| 0         | 0.74 ± 0.01 a,x         | 0.53 ± 0.01 b,x        | 0.48 ± 0.01 b,x        |
| 1         | 0.86 ± 0.01 c,x         | 0.53 ± 0.01 b,x        | 0.60 ± 0.01 b,y        |
| 2         | 0.79 ± 0.01 b,xy        | 0.48 ± 0.01 b,x        | 0.62 ± 0.01 b,y        |
| 3         | 0.78 ± 0.01 b,y         | 0.48 ± 0.01 b,x        | 0.87 ± 0.01 c,z        |

1 Mean ± standard deviation, Letters a, b in column or x, y, z in row indicates significant difference (p < 0.05) between means.

2: 0: before vaccination, 1, 2, and 3: first, second, and third vaccination, respectively.
reduced the amount of activity in the M1 group, sera from SG5 could effectively reduce the growth of both \( S. \) Pullorum and \( S. \) Gallinarum and \( S. \) Enteritidis, respectively. The sera from SP14 could significantly reduce the growth of \( S. \) Pullorum, but not the other two species. The sera from SP11 and SE could effectively reduce the growth of \( S. \) Gallinarum and \( S. \) Enteritidis, respectively, but not the other two species. The sera from SP15 could reduce the growth of both \( S. \) Gallinarum and \( S. \) Enteritidis, respectively, but not the other two species. In the F2 group, the sera from SG4 and SP9 could significantly reduce the growth of both \( S. \) Gallinarum and \( S. \) Pullorum, but not the other two species. The removal of complement activity reduced the bactericidal activity. Furthermore, the bactericidal ability was positively associated with the serum concentration.

**IFN-γ and IL-12 Expression in the Blood and by PBMCs**

In this experiment, we only measured the IFN-γ and IL-12 levels in the blood and in the secretion of PBMCs in the M2 group that was vaccinated with different serovars. The increase of IFN-γ levels in the blood was associated with inoculation number: an increase in the first and third inoculation for SG 8 and a consecutive increase in the first and second inoculation and then a decrease for SP 14. No increase was observed in SE 20 (Fig. 1A). In the PMBCs, an increase of IFN-γ levels from the 2-h treatment to 4-h treatment was observed for all the serovars, while no increase was found after the third inoculation (Fig. 1B). An interesting phenomenon of a decrease in IL-12 level in the blood after the first and third inoculation was observed for all serovars (Fig. 2A). SP and SE induced higher IL-12 level by PBMCs than the SG group did. The IL-12 level was decreased from the 2-h treatment to 4-h treatment only in the SE group (Fig. 2B).

**ROS Production by the PBMCs and PMN Cells**

Before the inoculation, an *ex vivo* test of the PMN cells revealed more ROS was produced by the males than the females after a bacterial infection (Figs. 3 and 4). However, \( S. \) Enteritidis interaction induced the highest ROS production of all three serovars in the M2 and F2 groups. In the PMBCs, the highest ROS production was

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### Table 2. The IgG1 and IgG2a concentration of layer serum isolated from every challenge group

| Antibody type | Vaccination number | M1          | M2          |
|--------------|--------------------|-------------|-------------|
|              |                    | SG 7        | SP 8        | SE 12       | SG 8        | SP 14       | SE 20       |
| IgG1         | 0                  | 116.3 ± 16.93 | 124.1 ± 18.85 | 137.2 ± 20.51 | 143.1 ± 20.04<sup>b</sup> | 116.9 ± 7.55<sup>bc</sup> | 106.2 ± 7.71<sup>b</sup> |
|              | 1                  | ND          | 131.9 ± 11.56 | 138.2 ± 19.57 | 140.9 ± 16.62<sup>b</sup> | 105.1 ± 12.29<sup>a</sup> | 112.1 ± 7.30<sup>b</sup> |
|              | 2                  | 114.3 ± 15.20 | 126.6 ± 20.98 | 134.1 ± 21.40 | 140.4 ± 19.37<sup>b</sup> | 116.8 ± 5.00<sup>a</sup> | 104.7 ± 6.90<sup>b</sup> |
|              | 3                  | 119.9 ± 15.38 | 136.8 ± 24.72 | 138.4 ± 22.90 | 148.1 ± 20.78<sup>b</sup> | 114.1 ± 3.69<sup>c</sup> | 110.3 ± 4.86<sup>b</sup> |
| IgG2a        | 0                  | 160.4 ± 4.39<sup>a</sup> | 168.2 ± 13.38<sup>a</sup> | 175.4 ± 5.40<sup>b</sup> | 176.8 ± 7.57<sup>c</sup> | 210.1 ± 6.64<sup>c</sup> | 193.7 ± 3.43<sup>b</sup> |
|              | 1                  | ND          | 156.8 ± 16.45 | 181.4 ± 10.76 | 199.8 ± 1.07<sup>c</sup> | 204.4 ± 5.64<sup>b</sup> | 211.9 ± 15.80 |
|              | 2                  | 158.2 ± 10.41<sup>a</sup> | 165.2 ± 14.09<sup>b</sup> | 176.5 ± 7.70<sup>b</sup> | 186.0 ± 4.35<sup>c</sup> | 203.3 ± 6.45<sup>b</sup> | 192.5 ± 6.20<sup>b</sup> |
|              | 3                  | 157.9 ± 5.24<sup>a</sup> | 176.9 ± 12.29<sup>b</sup> | 177.4 ± 5.01<sup>b</sup> | 197.3 ± 6.56<sup>c</sup> | 216.6 ± 10.59 | 197.1 ± 12.57 |

Mean ± SD.

<sup>a, b, c</sup> Different letters indicate significant difference between different *Salmonella* serotype.

<sup>a, b, c</sup> Different letters indicate significant difference between different challenge time.

### Table 3. Bactericidal capability of the sera from layers vaccinated with *S. Enteritidis*, *S. Gallinarum*, and *S. Pullorum*

| Chicken Group | Vaccination group | Bacterial species<sup>1</sup> | Plasma concentration<sup>2</sup> |
|--------------|------------------|-------------------------------|----------------------------------|
|              |                  | SG                            | SP                               | SE                               | E. coli                             | 20% | 10% | 5% |
|              |                  | SG5                           | 44%<sup>a</sup>                 | 41%<sup>a</sup>                  | 105%<sup>a</sup>                | 78%<sup>b</sup>                  | 51%<sup>a</sup> | 64%<sup>b</sup> | 86%<sup>c</sup> |
| M1           | SP11             | 41%<sup>a</sup>               | 46%<sup>b</sup>                 | 67%<sup>d</sup>                 | 64%<sup>c</sup>                | 45%<sup>a</sup>                  | 56%<sup>b</sup> | 62%<sup>c</sup> |
|              | SE15             | 50%<sup>a</sup>               | 65%<sup>c</sup>                 | 61%<sup>b</sup>                 | 62%<sup>b</sup>                | 52%<sup>a</sup>                  | 62%<sup>b</sup> | 64%<sup>c</sup> |
|              | SP8              | 46%<sup>a</sup>               | 75%<sup>b</sup>                 | 71%<sup>b</sup>                 | 77%<sup>c</sup>                | 59%<sup>a</sup>                  | 71%<sup>b</sup> | 73%<sup>c</sup> |
|              | SE12             | 68%<sup>a</sup>               | 78%<sup>a</sup>                 | 73%<sup>a</sup>                 | 94%<sup>d</sup>                | 64%<sup>a</sup>                  | 82%<sup>b</sup> | 88%<sup>c</sup> |
| M2           | SE8              | 46%<sup>a</sup>               | 124%<sup>a</sup>                | 102%<sup>c</sup>                | 67%<sup>a</sup>                | 81%<sup>b</sup>                  | 88%<sup>c</sup> |
|              | SP9              | 37%<sup>a</sup>               | 48%<sup>b</sup>                 | 109%<sup>a</sup>                | 120%<sup>c</sup>               | 65%<sup>a</sup>                  | 80%<sup>b</sup> | 91%<sup>c</sup> |

<sup>1</sup> SG: *S. Gallinarum*, SP: *S. Pullorum*, SE: *S. Enteritidis*. Bactericidal capability was calculated as the following formula: Growth ratio (%) = [(OD<sub>595</sub> of mixture of MHB and defined sera with bacterial growth - OD<sub>595</sub> of MHB without bacteria)]/OD<sub>595</sub> of MHB with bacterial growth × 100%. Letters a, b, c in row indicates significant difference (p < 0.05) between means.

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Fig. 1. The IFN-γ expression levels in serum of M2 chickens inoculated with *S. Gallinarum*, *S. Pullorum*, and *S. Enteritidis* before inoculation (0), and after first (1), second (2), and third (3) inoculation (A) and in the secretion by PBMCs from M2 chicken that were interacted with *S. Gallinarum*, *S. Pullorum*, and *S. Enteritidis* for 2 and 4 hours before (0) and after third (3) inoculation (B).

Fig. 2. The IL-12 expression level in in serum of M2 chickens inoculated with *S. Gallinarum*, *S. Pullorum*, and *S. Enteritidis* before inoculation (0), first (1), second (2), and third inoculation (3) (A) and PBMC cells from M2 chicken that were interacted with *S. Gallinarum*, *S. Pullorum*, and *S. Enteritidis* for 2 and 4 hours before (0) and after third (3) inoculation (B).
observed at 4 hours in the F2 group, at 2 hours in the M2 group for all serovars and at 2 hours for \textit{E. coli}. (Fig. 3). ROS production differed between the two male groups. The M2 group showed a similar ROS production pattern as the females, while the M1 group demonstrated that \textit{S. Gallinarum} could inhibit ROS production better than other serovars and \textit{E. coli}. In the PMN cells, the highest ROS production was observed at 2 hours for all the serovars in the F2 group, while the highest ROS production was observed at 4 hours for all the serovars in the M1 group (Fig. 4). However, no change was observed in the three periods for all the serovars in the M2 group.

After inoculation, ROS production by the PBMCs was higher in the M2 group than the F2 group (Fig. 5). Furthermore, an increase of ROS production against SG and SP was observed for all interaction periods after second inoculation and ROS production patterns differed between SE group and SG/SP groups in the M2 group. Inoculation increased more ROS production by the PMN cells, not the PBMCs (Figs. 5 and 6). After first inoculation, SG vaccination decreased ROS production of PMN cells against SG infection in M2 and F2 groups, while SP inoculation exhibited different ROS production pattern between M2 and F2 groups. In the second inoculation, SG inoculation increased ROS production in M2 group and decreased ROS production in F2 group, while SP vaccination increased ROS production only in 1-h inoculation. Among three inoculations, third inoculation exhibited the lowest ROS production.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{The superoxide production of the PBMC cells from non-challenged M1, M2 and F2 layers were interacted with \textit{Salmonella Gallinarum} (SG), \textit{S. Pullorum} (SP), \textit{S. Enteritidis} (SE) and \textit{E. coli} for 1, 2 and 4 hours. NC: control. \textsuperscript{a,b,c} Different letters indicate significant difference between different challenge time, \textit{P}<0.05.}
\end{figure}
Discussion

Salmonella is the most common zoonotic pathogen that causes salmonellosis in humans, mainly through the consumption of Salmonella-contaminated eggs (Icgen et al., 2002; Phan et al., 2004; Cui et al., 2005). In poultry, Salmonella can be acquired from the feeding ground and feces (Marin et al., 2009) and can contaminate eggs via vertical transfer by the Salmonella-infected reproductive tract and ovary during systemic infection (Thiagarajan et al., 1994; Wigley et al., 2001) and via horizontal transfer by penetrating the egg shell after egg production. Salmonella spreads in the field because it can persist in the cecum of the chick by increasing IL-4, IL-5 and IL-13 of the Th2 response (Chaussé et al., 2014). In this study, first inoculation exhibited an increase of IFN-γ level and a decrease of the IL-12 level in the blood for SG and SP groups, compared to decrease of both levels for SE group, while a longer interaction with S. Gallinarum and S. Pullorum increased IL-12 and IFN-γ levels produced by the PBMCs and PMN cells (Figs. 1 and 2). Such an increase of both levels by the cells was not associated with inoculation number. These results imply that the serovars may regulate the IFN-γ and IL-12 expression in the blood and by the PBMCs and PMN cells.

Vaccination with S. Enteritidis, S. Gallinarum, and S. Typhimurium can increase concentrations of anti-Salmonella antibodies and T cell-related responses (Withanage et al., 2005; Berndt et al., 2006; Rana and Kulshreshtha, 2006). Additionally, an S. Enteritidis infection increases the expression of IgG, IFN-γ and IL-1β by 100-fold and reduces...
thenumberofviablebacteria12daysafteranoralinfection in chickens (Matulova et al., 2013). In older chickens, the elimination of Salmonella is not related to an increase in antibodies (Beal et al., 2006) but rather is regulated by T-cell responses (Beal et al., 2005), such as a reduction of CD4\(^+\) T cells and γδ-T lymphocytes after maturation in females that increases a Salmonella infection in the oviduct (Chappell et al., 2009; Johnston et al., 2012). Depending on the cytokines, Th1 and Th2 lymphocytes can induce the expression of immunoglobulin IgG2a and IgG1, respectively (Abbas et al., 1996), which are used as markers for Th1 and Th2 activation. In the present study, the inoculation number and serovars had different effects on the expression levels of IgG1 and IgG2a. Greater expression of IgG2a, not IgG1, was observed for all three serovars, demonstrating that all three serovars preferentially stimulate the Th1 pathway.

Salmonella vaccination or infection increases CD4\(^+\) and CD8\(^+\) T cell numbers and antibody production to prevent the dissemination of Salmonella in mice (Mastroeni et al., 1993). Rck, Rsk and TraT on the virulence plasmid of S. Typhimurium inhibit the function of the MAC complex to evade host complement attack (Van den Bosch et al., 1989; Pramooyjago et al., 1992). Additionally, long chain lipopolysaccharides resulting in smooth colonies can resist complement activity (MacLennan et al., 2008). The interaction of outer membrane protein Rck with the functional recruitment of human complement inhibitor C4b-binding protein provide resistance to complement-mediated killing (Ho et al., 2011), which confers LPS-independent serum resistance. In the present study, S. Enteritidis could not
survive in the sera possibly due to a lack of TraT on the virulence plasmid of *S. Enteritidis* (Chu et al., 1999).

Vaccinated antibodies increase opsonization related to complement-mediated killing (MacLennan et al., 2008). In addition, anti-*Salmonella*-specific IgA, IgG and IgM are produced after vaccination (Zhang-Barber et al., 1999). Here, vaccination could increase the IgG titer, especially in the SG group (Table 1). We observed an increase in bactericidal abilities and IgG titers after the vaccination with *S. Gallinarum*, but not *S. Pullorum* and *S. Enteritidis* (Tables 1 and 3), indicating a serovar-specific immune response. Vaccination with *S. Pullorum* and *S. Gallinarum* increased the serum killing ability against *S. Gallinarum* and *S. Pullorum* for the female layers, but not the male layers, demonstrating that a *S. Gallinarum* vaccine can provide effective protection against infection by *S. Enteritidis*, *S. Gallinarum*, and *S. Pullorum*.

Antibodies (IgG and IgM) and complement are required for an optimal oxidative burst, phagocytosis, and killing of nontyphoidal *Salmonella* by peripheral blood cells (neutrophil and monocyte) (Gondwe et al., 2010). PMN cells can produce ROS in number- and time-dependent manners and through direct interaction with PBMCs (Ghrib et al., 2002). As an important member of innate immunity that eliminates pathogens and present antigens, macrophages are heterogeneous cells that react to multiple signals from the micro-environment to eliminate pathogens and present antigens (Van Ginderachter et al., 2006). *Salmonella* pathogen island-2 *Salmonella* SPI-2 can assist *Salmonella* in surviving in macrophages by inhibiting ROS production and decreasing acidity in the phagosome (Gallois et al., 2001). Earlier reports demonstrated that heterophil to lymphocyte ratios were higher in males than females and that age affected heterophils and lymphocyte numbers (He et al., 2005). Sex maturation not only changes cellular responses but also differentiates the expression of avian β-defensin gene-encoded antimicrobial peptides, important antimicrobial peptides in innate immunity in testis and vagina and against *S. Enteritidis* infection (Anastasiadou et al., 2013, 2014). In the present study, the ROS production by the PBMCs and PMN cells (Figs. 3 and 4) and antibody production may be regulated by sex. Additionally, such an increase of ROS production in PMN cells is strongly associated with inoculation number and serovars (Figs. 5 and 6). Whether such differences are regulated by physiological factors such as sex hormone or change of cell groups in PMN cells needs to be investigated.

In conclusion, different serovars affect the weight of the male and female layers. *S. Gallinarum*, *S. Pullorum* and *S. Enteritidis* survived differently in the sera and preferentially stimulated the Th1 pathway in male layer. Vaccination-mediated *Salmonella*-specific IgG production and the expression of IFN-γ and IL-12 in the blood was not associated
with the vaccination. However, such expression in the PBMCs was associated with longer interacation n with three serovars, not vaccination. ROS production differed depending on the serovars, and inoculation number and sex.

Acknowledgments

This work was funded by the Ministry of Science and Technology grant MOST 102-2320-B-415 -Executive Yuan, Taiwan (C. C.)

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