JPSA
Japan Poultry Science Association

http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.0150167

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 consistof 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 S*almonella* infections by preventing pathogen growth and adhesion to epithelial

Received: November 17, 2015, Accepted: March 29, 2016
Released Online Advance Publication: May 25, 2016
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cells (Lawley *et al*., 2008; Quresh *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 (Crhanova *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-Alderich). 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-4-62 rotor, Eppendorf 5810, Eppendorf, Hamburg, Germany) for 10 min, plasma was collected and stored at −80℃. Some of the plasma was treated at 56℃ 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, 1×10^9 CFU of *E. coli* was reacted with 500 μl plasma by shaking at 100 rpm and 37℃ 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℃ 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℃ 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 horseradish peroxidase (HRP)-labeled anti-chicken IgG, IgG1 and IgG2a antibody (Sigma-Alderich) and 3′, 3′, 5′, 5′-tetramethyl benzidine (TMB) (T0440, Sigma-Alderich) were added to the wells. Finally, the reaction was inactivated with 0.5 N H_2SO_4. The OD_{450} 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 1×10^4 CFU/m*l* 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℃ for 30 minutes to achieve serum concentrations of 20%, 10%, and 5%. After an culture for 16−18 h, the OD_{595} was measured using an ELISA reader (Anthos 2010, Biochrom, Cambridge, UK). The bactericidal capability was calculated using the following formula: Growth ratio (%)=[(OD_{595} of mixture of MHB and defined sera with bacteria − OD_{595} of mixture and defined serum without bacteria)/(OD_{595} of MHB with bacteria - OD_{595} 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/m*l*; SI10771, Sigma-Aldrich) after centrifugation at 25℃ and 600×g for 6 minutes. PBMCs were washed with 1 m*l* PBS three times and mixed with 1 m*l* RPMI of a solution (R6504, Sigma-Alderich). To purify the PMN cells, the

layer under the buffy coat was mixed with 10 m*l* PBS and then centrifuged at 350×g for 10 minutes. The pellet was lysed with 30 m*l* of RBC Lysis buffer (0.19 M NH_4Cl, 4 mM K_2HCO_3, 0.39 mM EDTA) for 10 minutes. After centrifuging at 350 g for 10 minutes, the pellet was washed three times with 1 m*l* PBS buffer. The pellet was mixed with 1 m*l* 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℃ and 5% CO_2 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 (Catalougue 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-

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

Challenge		Chicken groups (Mean±standard error)[1]		
		M1	M2	F2
Group	SG	$0.93\pm0.01^{b,y}$	$0.64\pm0.01^{c,x}$	0.65 ± 0.01^{x}
	SP	$0.71\pm0.01^{a,z}$	$0.37\pm0.01^{a,x}$	0.63 ± 0.01^{y}
	SE	$0.72\pm0.01^{a,y}$	$0.50\pm0.001^{b,x}$	
Vaccination[2]	0	$0.74\pm0.01^{a,z}$	$0.53\pm0.01^{a,y}$	$0.48\pm0.01^{a,x}$
	1	$0.86\pm0.01^{c,z}$	$0.53\pm0.01^{a,x}$	$0.60\pm0.01^{b,y}$
	2	$0.79\pm0.01^{b,z}$	$0.48\pm0.01^{b,x}$	$0.62\pm0.01^{b,y}$
	3	$0.78\pm0.01^{b,y}$	$0.48\pm0.01^{b,x}$	$0.87\pm0.01^{c,z}$

[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.

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^{b}	116.9±7.55^{a}	106.2±7.71^{a}
	1	ND	131.9±11.56	138.2±19.57	140.9±16.62^{b}	105.1±12.29^{a}	112.1±7.30^{a}
	2	114.3±15.20	126.6±20.98	134.1±21.40	140.4±19.37^{b}	116.8±5.00^{a}	104.7±6.90^{a}
	3	119.9±15.38	136.8±24.72	138.4±22.90	148.1±20.78^{b}	114.1±3.69^{a}	110.3±4.86^{a}
IgG2a	0	160.4±4.39^{a}	168.2±13.38^{a}	175.4±5.40^{b}	176.8±7.57^{ax}	210.1±6.64^{c}	193.7±3.43^{b}
	1	ND	156.8±16.45	181.4±10.76	199.8±1.07^{y}	204.4±5.64	211.9±15.80
	2	158.2±10.41^{a}	165.2±14.09^{ab}	176.5±7.70^{b}	186.0±4.35^{ax}	203.3±6.45^{b}	192.5±6.20^{ab}
	3	157.9±5.24^{a}	176.9±12.29^{ab}	177.4±5.01^{b}	197.3±6.56^{y}	216.6±10.59	197.1±12.57

Mean±SD.
[a, b, c] Different letters indicate significant difference between different *Salmonella* serotype.
[x, y] 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[1]				Plasma concentration		
		SG	SP	SE	*E. coli*	20%	10%	5%
M1	SG5	44%^{a}	41%^{a}	105%^{c}	78%^{b}	51%^{a}	64%^{b}	86%^{c}
	SP11	41%^{a}	46%^{b}	67%^{d}	64%^{c}	45%^{a}	56%^{b}	62%^{c}
	SE15	50%^{a}	65%^{c}	61%^{b}	62%^{b}	52%^{a}	62%^{b}	64%^{c}
M2	SG8	46%^{a}	75%^{ab}	71%^{b}	77%^{c}	59%^{a}	71%^{b}	73%^{b}
	SP14	65%^{b}	60%^{a}	88%^{c}	92%^{d}	63%^{a}	80%^{b}	86%^{c}
	SE20	68%^{a}	78%^{c}	73%^{b}	94%^{d}	64%^{a}	82%^{b}	88%^{c}
F2	SG4	41%^{a}	48%^{b}	124%^{d}	102%^{c}	67%^{a}	81%^{b}	88%^{c}
	SP9	37%^{a}	48%^{b}	109%^{c}	120%^{c}	65%^{a}	80%^{b}	91%^{c}

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

lected from each group. In the presence of complement activity in the M1 group, sera from SG5 could effectively reduce the amount of *S.* Gallinarum and *S.* Pullorum, but not *S.* Enteritidis and *E. coli*. The sera from SP11 and SE could reduce all the serovars and *E. coli* (Table 3). In the M2 group, the sera from SG8 and SE20 could effectively reduce the growth of *S*. Gallinarum and *S*. Enteritidis, respectively, but not the other two species. The sera from SP14 could reduce the growth of *S*. Gallinarum and *S*. Pullorum, 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 and PBMCs determined 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 PBMCs, the highest ROS production was

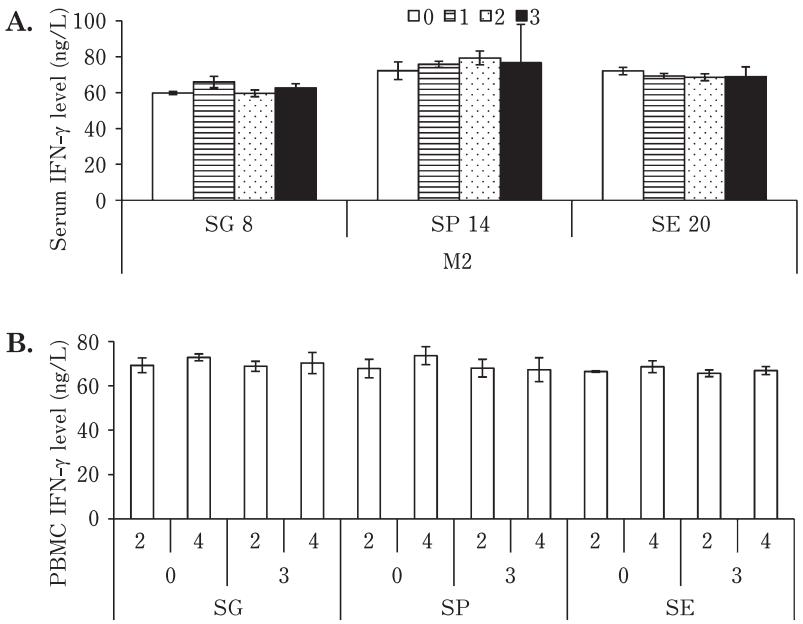

Fig. 1. **The IFN-γ expression levels in serum of M2 chickens inoculated with *S*. Gallinarum, *S*. Pullorum, and *S*. Eneteritidis before inoculation (0), and after first (1), second (2), and third (3) inoculation (A) and in the secretion by PBMCs from M2 chicken that were interacted with *S*. Gallinarum,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

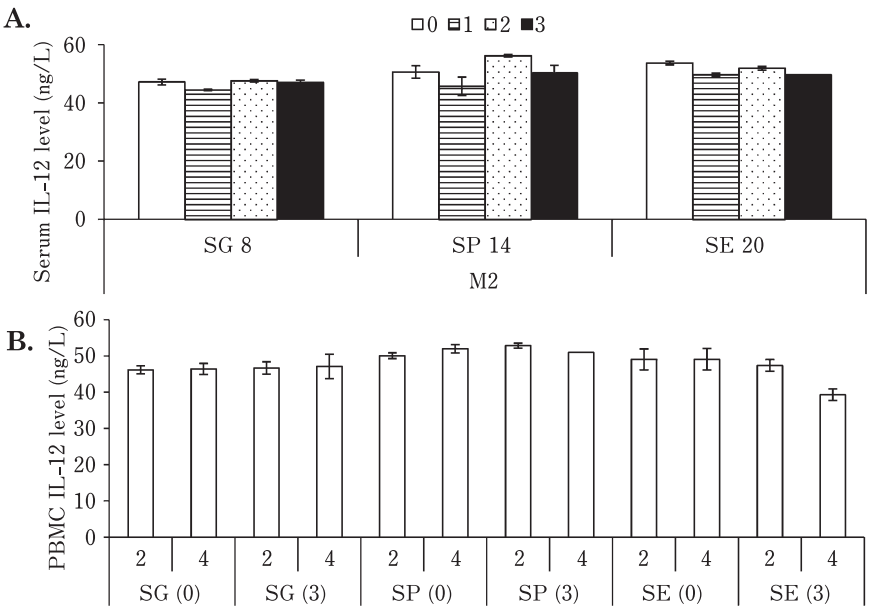

Fig. 2. **The IL-12 expression level in in serum of M2 chickens inoculated with *S*. Gallinarum, *S*. Pullorum, and *S*. Eneteritidis before inoculation (0), first (1), second (2), and third inoculation (3) (A) and PBMC cells from M2 chicken that were interacted with *S*. Gallinarum, *S*. Pullorum, and *S*. Eneteritidis for 2 and 4 hours before (0) and after third (3) inoculation (B).**

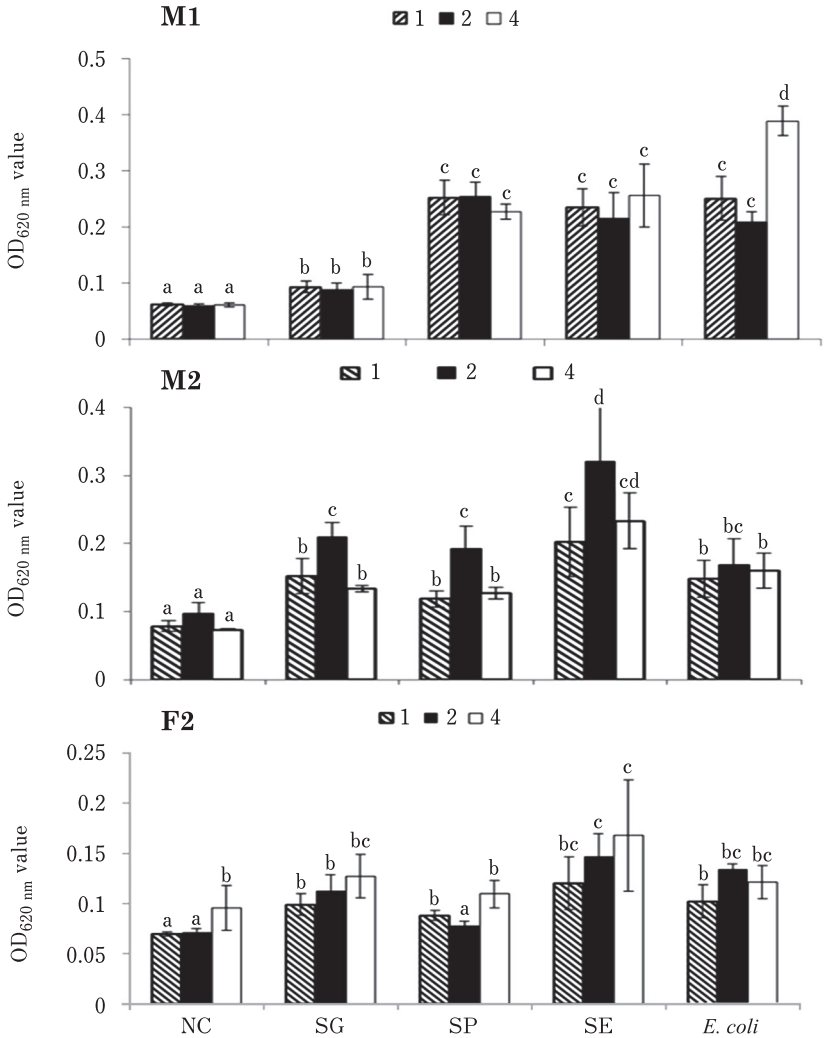

Fig. 3. **The superoxide production of the PBMC cells from non-chanllenged M1, M2 and F2 layers were interacted with *Salmonella* Gallinariun (SG), *S*. Pullorum (SP), *S*. Enteritidis (SE) and *E. coli* for 1, 2 and 4 hours.** NC: control. [a, b, c] Different letters indicate significant difference between different challenge time, $P<0.05$.

observed at 4 hours in the F2 group, at 2 hours in the M2 group for all serovars and at 2 hours for *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 *S*. Gallinarum could inhibit ROS production better than other serovars and *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.

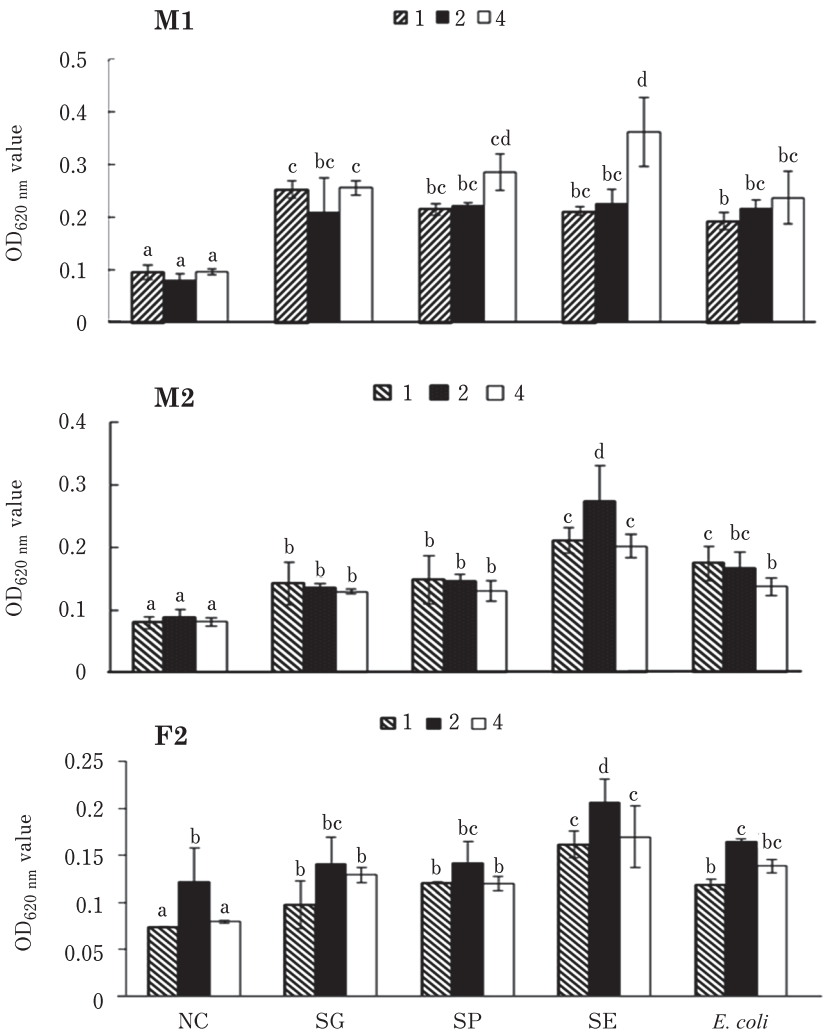

Fig. 4. **The superoxide production of the PMN cells from non-chanllenged M1, M2 and F2 layers were interacted with *Salmonella* Gallinariun (SG), *S*. Pullorum (SP), *S*. Enteritidis (SE) and *E. coli* for 1, 2 and 4 hours.** NC: control. [a, b, c] Different letters indicate significant difference between different challenge time, $P<0.05$.

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

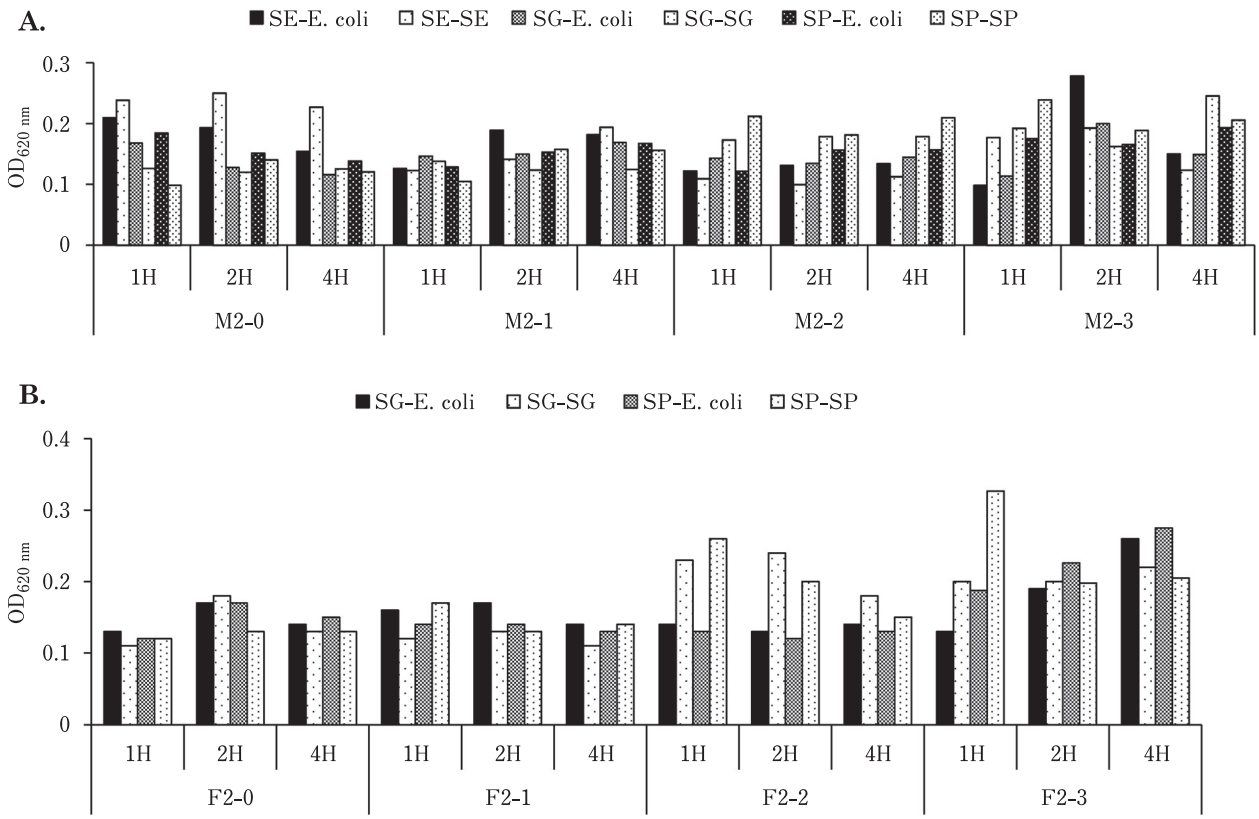

Fig. 5. **The average superoxide production of PBMC cells from M2 (A) and F2 (B) layers before (M2-0 and F2-0), and after first (M2-1 and F2-1), second (M2-2 and F2-2) and third (M2-3 and F2-3) inoculations with *S*. Enteritidis (SE), *S*. Gallinarum (SG), and *S*. Pullorum (SP), respectively.** The superoxide level was measured for the cells that were interacted with *E. coli* and the infected serovar for 1 H, 2 H, and 4 H. SE-*E. coli* and SE-SE meanthat the cells from thelayers inoculated with *S*. Enteritidis were interacted with *E. coli* and *S*. Enteritidis, respectively. SG-*E. coli* and SG-SG mean that the cells from the layers inoculated with *S*. Gallinarum were interacted with *E. coli* and *S*. Gallinarum, respectively. SP-*E. coli* and SP-SP mean that the cells from the layers inoculated with *S*. Pullorum were interacted with *E. coli* and *S*. Pullorum, respectively.

the number of viable bacteria 12 days after an oral infection 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 $\gamma\delta$-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; Pramoonjago *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

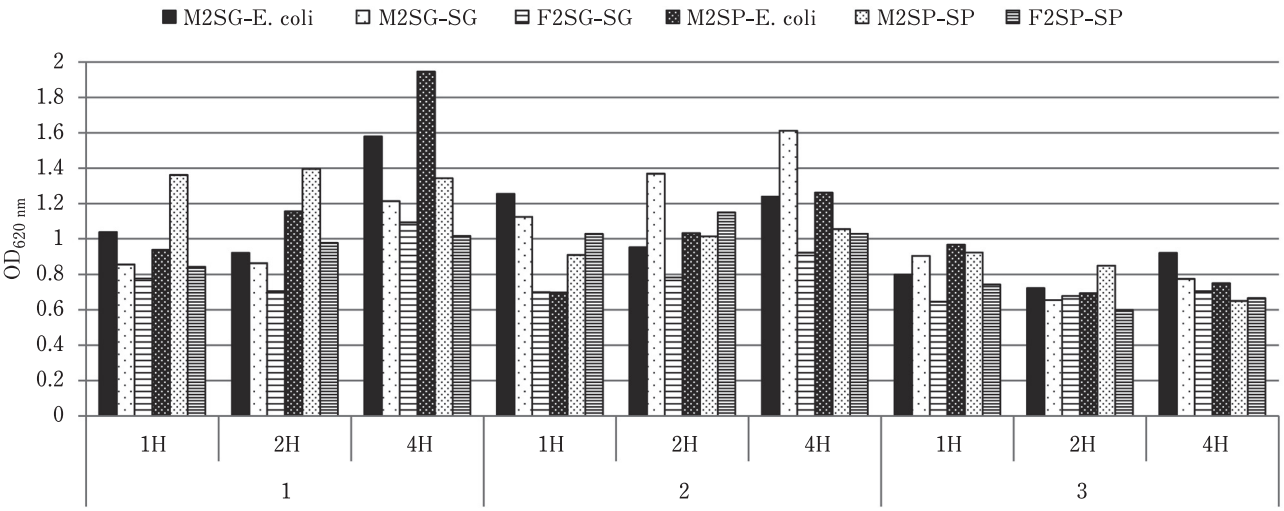

Fig. 6. **The average superoxide production of PMN cells from M2 and F2 layers after first (1), second (2) and third (3) inoculations with *S*. Gallinarum (SG), and *S*. Pullorum (SP), respectively.** The superoxide level was measured for the cells that were interacted with *E. coli* and the serovars for 1 H, 2 H, and 4 H. M2SG-*E. coli* and M2SG-SG mean that the cells from M2 layers inoculated with *S.* Gallinarum were interacted with *E. coli* and *S.* Gallinarum, respectively. M2SP-*E. coli* and M2SP-SP mean that the cells from M2 layers inolculated with *S.* Pullorum were interacted with *E. coli* and *S.* Pullorum respectively. F2SG-SG and F2SP-SP mean that the cells from F2 layers inoculated with *S.* Gallinarum and *S.* Pullorum were interacted with their infected serovars, respectively.

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 microenvironment 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 -004 -Executive Yuan, Taiwan (C. C).

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