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Differences in immune responses of pigs vaccinated with *Salmonella* Typhimurium and *S.* Choleraesuis strains and challenged with *S.* Choleraesuis

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ABSTRACT

S. Choleraesuis (Choleraesuis) and *S.* Typhimurium (Typhimurium) cause salmonellosis in pigs and humans. The effects of vaccine strains pSV-less Typhimurium OU5048 and Choleraesuis OU7266 and SPI-2-mutant Choleraesuis SC2284 on the immune responses of pigs against Typhimurium, Choleraesuis, and *S.* Enteritidis (Enteritidis) with or without the virulence plasmid (pSV) were determined. After oral vaccination of three vaccine groups and challenge with Choleraesuis CN36, the level of *Salmonella*-specific IgG in sera and the bactericidal effects and superoxide generation of peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs) against the above strains were determined using ELISA and NBT assay, respectively. Among three vaccine strains tested, OU7266 stimulated the highest *Salmonella*-specific IgG levels. Complement inactivation increased IgG concentration, while *E. coli* absorption reduced IgG levels. The pSV-containing strains were less resistant to serum killing than the pSV-less strains, and Enteritidis exhibited the lowest resistance to serum killing. Serovars tested, vaccine strains, and timeline periods postvaccination and challenge were important factors affecting superoxide production. The two Choleraesuis vaccine strains stimulated greater levels of superoxide from PMNs and PBMCs than the Typhimurium strains. The PMNs and PBMCs in challenged and vaccinated pigs reduced more superoxide than those in challenged hosts. In vaccinated hosts, pSV-less *Salmonella* strains triggered lower levels of PMN/PBMC-generated superoxide upon challenge than strains with pSV against Enteritidis and Choleraesuis. Overall, Choleraesuis OU7266 may be better than the other vaccine strains in generating the greatest IgG levels, serum bactericidal activity and superoxide levels. The pSV likely influences the immune responses.

1. Introduction

Salmonella can survive in different environments and cause zoonotic infection through the consumption of *Salmonella*-contaminated foods and water via the fecal/oral pathway. In the intestine, *Salmonella* responds to attack by macrophages and dendritic cells recruited from Peyer's patches and mesenteric lymphoid tissue [1] and triggers an inflammatory response resulting in diarrhea and inflammation [2]. Differences in the host ranges for *Salmonella* may be associated with gene acquisition through the transfer of plasmids, transposons, and phages; the loss of genes or gene inactivation (pseudogenes); and the interaction with the host's immune system and commensal organisms during early stages of infection in intestines [3].

Immune responses and symptoms of the hosts are associated with serovars [4,5]. Further, the seroincidence in humans differs from 0.06 infections per person-year in Sweden to 0.61 among 13 European countries and is correlated with the prevalence of *Salmonella* in slaughtered pigs (P = 0.03) [6]. Enteritidis, Typhimurium, and Choleraesuis can also cause gastroenteritis and even bacteremia or death in humans [7,8], while Choleraesuis, Typhimurium and Terby are the common serovars in pig infections [4,9–11]. For Typhimurium, infection alters protein expression in host cells, tissue morphology, and molecular transport at the early stages of infection [12]. Furthermore, pSVs play a role in enhancing the virulence for hosted serovars. For examples, Rck prevents complement C9 binding to the bacterial surface to form the membrane attack complex (MAC) [20,21], the *pef* operon,

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containing the genes for plasmid-encoded (PE) fimbriae to assist bacterial attachment to intestinal cells, and Mig-5, a carbonic anhydrase, aids in bacterial survival within macrophages [22].

After phagocytosis of the bacteria, neutrophils and macrophages synthesize reactive oxygen species (ROS) to kill intracellular bacteria; however, *Salmonella* can inhibit ROS production, enabling survival in these phagocytized cells [13,14] by expressing superoxide dismutases or catalase to convert ROS into water; additional genes on the *Salmonella* pathogenicity island 2 (SPI-2) can suppress ROS synthesis [15,16]. Furthermore, protein-isoaspartyl methyltransferase (PIMT) inhibits H_2O_2 and hypochlorite (HOCl) attack to assist the intracellular survival of the Typhimurium inside phagocytic cells. In a mouse model, the Δ*pimt* mutant strain was less abundant in the spleen and liver and had a 10-fold greater LD_{50} than the wild-type strain [17]. Complement and antibodies in serum can assist the lysis of pathogens by forming membrane attack complexes (MAC) and enhancing opsonization. Furthermore, host-specific antibodies can prevent *Salmonella* infection [18], and antibody IgG binding on the bacterial surface proteins can increase phagocytosis by macrophages [19].

Vaccination using dead Typhimurium stimulates IgG production to protect host animals from infection [18]. Administration of an avirulent live Choleraesuis reduced the *Salmonella* and seroprevalence in lymph nodes [23]. The aim of this study was to investigate the effects of three *Salmonella* vaccine strains and challenge IgG production and serum mediated bacterial killing in pigs. Additionally, we investigated superoxide-mediated killing of the three serovars with and without pSVs by PMN and PBMC cells before and after vaccination and challenge.

2. Materials and methods

2.1. Bacterial sources

All bacterial strains are listed in Table 1 and include three vaccine strains pSTV-less Typhimurium OU5048, pSCV-less Choleraesuis OU7266, and the *Salmonella* pathogenicity island-2 (SPI-2) mutant Choleraesuis SC2284 and the challenge strain Choleraesuis CN36 collected from ill pigs. Furthermore, the pSV-containing strains Enteritidis OU7130, Typhimurium OU5045, and Choleraesuis OU7085 and their pSV-less derivative strains Typhimurium OU5046, Enteritidis OU7067 and Choleraesuis OU7266 were included in this study. *E. coli* pir116 was utilized as a control.

2.2. Vaccination experimental design

Twenty 9-week-old female LYD x Landrace x Yorkshire x Duroc hybrid pigs from the Animal House of National Chiayi University were separated into five groups, including three vaccine groups, one challenge group and one control group, with four pigs in each group. The experimental design was described in Supplementary Fig. 1. All pig groups were raised separately in an individual unit of a pig farm with identical feeding and water regimens. Pigs were orally fed with 2×10^9 bacteria of three vaccine strains at Weeks 0. At the end of week 2 after blood collection, pigs in the vaccine groups and challenge group were orally fed strain CN36 at a dosage of 1×10^{10} bacteria per pig. Blood samples were collected from all pigs at first week and second weeks after vaccination (weeks 1 and 2) and after challenge (weeks 3 and 4). After the end of the experiment, the pigs remained in the Animal House. This project was approved by the IACUC of our university (96,012).

2.3. Blood collection and cell separation

Blood (7–8 ml) was collected from the carotid artery and stored in a tube with anticoagulant lithium heparin and centrifuged at 3000 rpm (Eppendorf 5810R, A-4-62 rotor) for 10 min. Serum of the upper layer was transferred into a new 15 ml tube and stored at -80℃ for future use. The remaining blood cell layer was mixed with 0.9% NaCl thoroughly; buffy coats and red blood cell layers were collected after centrifugation at 3000 rpm for 10 min to purify PBMCs and PMNs. For PBMCs, buffy coat cells were mixed with 5 ml of RPMI 1640 solution (RPMI 1640 media with 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin) (Sigma) in a 15 ml tube. Next, 2.5 ml of Ficoll solution (1.077 g/ml, Histopaque-1077, Sigma) was added slowly. After centrifugation at 400 × *g* for 30 min, the middle remaining plasma layer was transferred into a 1.5 ml Eppendorf tube and mixed with 1 ml RPMI 1640 solution. The mixture was centrifuged at 4 °C and 800 × *g* for 10 min, and then the pellet was suspended in 1.5 ml RBC lysis buffer (0.83% NH_4Cl, 0.1% $KHCO_3$, 0.009% EDTA) and incubated on ice for 10 min. After centrifugation at 4 °C and 800 × *g* for 5 min, the pellet was mixed with 1.5 ml RPMI 1640 solution and centrifuged at 4 °C and 800 × *g* for 5 min. Finally, the cell pellet was mixed with 1 ml RPMI 1640 solution, and cell number counts were determined. For PMNs, the red blood layer was mixed completely with RBC lysis buffer and then incubated on ice for 15 min. After centrifugation at 800 × *g* and 4 °C for 5 min, the pellet was washed twice with 1 ml RPMI 1640 solution and then centrifuged at 800 × *g* and 4 °C for 10 min. Finally, the cells were mixed with 1 ml RPMI 1640 solution, and the cell number was counted.

2.4. Bactericidal evaluation of the serum

One milliliter of overnight *E. coli* culture was centrifuged at 4000 x g for 5 min. The pellet was mixed with 1 ml of pig's serum and vortexed at 100 rpm. The mixture was incubated at 37 °C for 1 h to remove the common antibodies between *E. coli* and *Salmonella*. After centrifugation at 4000 × *g* for 5 min, the supernatant was stored at −80 ℃ for further use. For complement inactivation, serum was heated at 56 ℃ for 30 min and then stored at −80 ℃. Sera with active and inactive complements

Table 1
The *Salmonella* strains used in this study.

Classification	Serotype	Serogroup	Strains	Virulence plasmid (kb)	Description	Source*
Vaccinated strains	Typhimurium	B	OU5048	Deleted	derived from OU5045	a
	Choleraesuis	C1	OU7266	Deleted	derived from OU7085	a
			SC2284	50	destroy in SPI-2	b
Challenged Strain	Choleraesuis	C1	CN36	50	Wild type	a
Ex vivo Treatment strains	Enteritides	D1	OU7130	60	Wild type	a
			OU7067	Deleted	derived from OU7130	
	Typhimurium	B	OU5045	90	Wild type	
			OU5046	Deleted	derived from OU5045	
	Choleraesuis	C1	OU7085	50	Wild type	
			OU7266	Deleted	derived from OU7085	
	E. coli		pir116			

* Bacterial strain sources are as follows: a: laboratory stock; b: from Chang Yung-Fu College of Veterinary Medicine, Cornell University, Ithaca, New York.

were diluted with Mueller Hinton broth (MHB) to concentrations of 20%, 10%, 5%, and 2.5%, respectively, and then each diluted sera was placed in each well of a 96-well plate (Corning Cat No. 3599). Each well contained 5×10^4 bacteria per 100 µl. The mixture was incubated at 37 °C overnight, and then the OD at 550 nm was recorded by an ELISA reader, and the MIC levels were analyzed.

2.5. Measurement of anti-Salmonella-specific IgG levels

Then, 500 µl of overnight bacterial culture was added to 5 ml LB broth and incubated at 37°C for 3 h with shaking at 150 rpm. After centrifugation at 4000 × *g* for 5 min, the pellet was diluted to 10^8 bacteria per ml, and the solution was incubated at 65°C for 30 min to kill bacteria. Then, 1×10^7 bacteria were added to each well of a 96-well plate, and the mixture was incubated overnight. After removing supernatant, each well was washed with PBST (PBS with 0.05% Tween 20) thrice, and 100 µl of 5% skim milk was added for blocking at room temperature for 2 h. After removing the PBST, 100 µl of 100-fold-diluted serum was added as the first antibody, and the solution was incubated at room temperature for 3 h. Then, 100 µl of 5,000-fold-diluted goat polyclonal anti-pig IgG (AB6915, Abcam) was added, and the solution was incubated at room temperature for 1 h. After washing with PBST thrice, 100 µl of 3,3′,5,5′-tetramethyl benzidine (TMB, Sigma) was added to each well, and the plates were incubated in the dark for 5 min. 100 µl of 4 NH_2SO_4 was added to stop the reaction, and the $OD_{450\ nm}$ was measured. The serum IgG level was determined by the $OD_{450\ nm}$ results of each treatment minus those of the normal group.

2.6. Analysis of superoxide production using nitro blue tetrazolium

The 5×10^5 PMNs and PBMCs in RPMI 1640 solution with antibiotics were incubated in each well of a 96-well plate at 37 °C and 5% CO_2. After washing with PBS solution, 5×10^7 bacteria in new RPMI 1640 solution without antibiotics were added at an MOI = 100 at 37 °C for 1, 2 and 4 h, separately. At the end of each incubation, the mixture was centrifuged at 800 × *g* for 10 min, and the cell pellets were washed with 200 µl of PBS. After centrifugation at 800 × *g* rpm for 10 min and supernatant removal, 200 µl of 0.08 mg/ml NBT solution was added and reacted in the dark at 37 °C for 45 min. After centrifugation at 800 x g for 10 min, the cell pellets were washed with 200 µl PBS twice. Finally, 60 µl of DMSO and 60 µl of 2 M KOH were added and mixed for 5 min. The $OD_{620\ nm}$ of each reaction was measured and analyzed.

2.7. Statistical analysis

Student's *t*-test, analysis of variance (ANOVA), and Tukey's honestly significant difference (HSD) test were used to analyze the differences among treatments with SPSS 12.0. A significant difference was indicated as $p < 0.05$.

3. Results

3.1. Salmonella-specific IgG level

Vaccination with pSV-less OU7266 induced greater IgG levels than pSV-less OU5048 at weeks 1 and 2. However, the SPI-2 mutant SC2284 did not elicit IgG responses (Fig. 1A). After challenging with the strain CN36, a significant increase in anti-*Salmonella* IgG levels was observed in the CN36, OU5048, and OU7266 groups from weeks 3–4. Furthermore, the greatest IgG level was observed in the OU7266 group; by contrast, the lowest IgG level was elicited in the SC2284 group. We used *E. coli* to absorb the IgG common for *Salmonella* and *E. coli* to minimize possible *E. coli* infection through the intestine. After vaccination and challenge, changes of *Salmonella*-specific IgG levels were similar to the serum samples taken from hosts without *E. coli* absorption, with an increase of *Salmonella*-specific IgG in the pSV-less groups (Fig. 1B). However, we observed a decrease in the IgG levels in the OU7266 group at week 4 after challenge compared to serum from hosts without *E. coli* absorption. Furthermore, inactivation of the complement increased IgG levels in every group, especially in the OU7266 and SC2284 groups, and the greatest anti-*Salmonella* IgG level was observed for the OU7266 group (Fig. 1C and D). These results demonstrated that OU7266 stimulated the greatest IgG levels and the complement interfered with IgG binding to *Salmonella*.

3.2. Bactericidal effect of pig serum against three serovars

Sera from hosts at week 2 (vaccination) and week 4 (challenge) were used to evaluate bactericidal effects against three serovars with and without pSV. Treating with serum with active complement at week 2, we observed that in the normal group, the MIC was 5% against *E. coli* and 10% and 20% against wild-type Enteritidis OU7130 and its derivative pSV-less OU7067, respectively, and greater than 20% for Choleraesuis and Typhimurium with and without pSV (Table 2), indicating that the serum could inhibit the growth of *E. coli* and Enteritidis, not Choleraesuis and Typhimurium. In the Typhimurium OU5048 group, a deceased MIC level was found against wild-type Typhimurium OU5045 and Choleraesuis strains, while there was no difference in MIC levels between Cholerasuis groups. Compared to the MIC levels in week 2, those in week 4 were reduced mainly for Enteritidis OU7130. Vaccination, not challenge, increased the bactericidal effect on *Salmonella,* and the presence of pSV increased serum resistance levels. Furthermore, Complement inactivation in sera decreased the bactericidal activities of the sera (Table 2).

3.3. Factorial analysis of superoxide production from the PMNs and PBMCs

The effects of vaccination (weeks 1–2) and challenge (weeks 3–4) on superoxide levels of PMNs and PBMCs were analyzed using ANOVA analysis (Table 3). For PMNs, superoxide production was correlated with *ex vivo* treatment of the cells using serovars and *E. coli* in the vaccination groups. Additionally, challenge induced significantly more superoxide production than vaccination ($p < 0.001$). For PBMCs, similar results of vaccination effect and challenge effect were observed for PMNs.

3.4. Superoxide production of the PMNs and PBMCs among vaccination groups

The effects of vaccine strains pSV-less Typhimurium OU5048 and Choleraesuis OU7266 as well as SPI-2-deficient Choleraesuis SC2284 on the superoxide production of the PMNs and PBMCs differed. During vaccination, both Choleraesuis OU7266 and SC2284 stimulated significantly more superoxide production from both PMNs and PBMCs than Typhimurium OU5048 ($p < 0.001$). However, the superoxide amounts were not different between the two Choleraesuis strains (Table 4). Nevertheless, challenge stimulated more superoxide production from the PMNs and PBMCs than vaccination. The statistical patterns of superoxide production were almost identical to those of vaccination. In contrast to the vaccination effect, the challenge significantly increased superoxide production between the two *S*. Choleraesuis strains with greater superoxide levels for OU7266.

3.5. The role of the pSVs in the superoxide production of the PMNs and PBMCs during infection

The effects of the pSVs on superoxide production of the PMNs and the PBMCs after vaccination and challenge were determined by infecting these cells with or without pSV of Choleraesuis, Enteritidis, and Typhimurium. In vaccinated animals, PMNs infected by pSV-containing Enteritidis and Choleraesuis, but not Typhimurium, produced significantly more superoxide than those infected by the pSV-less strains

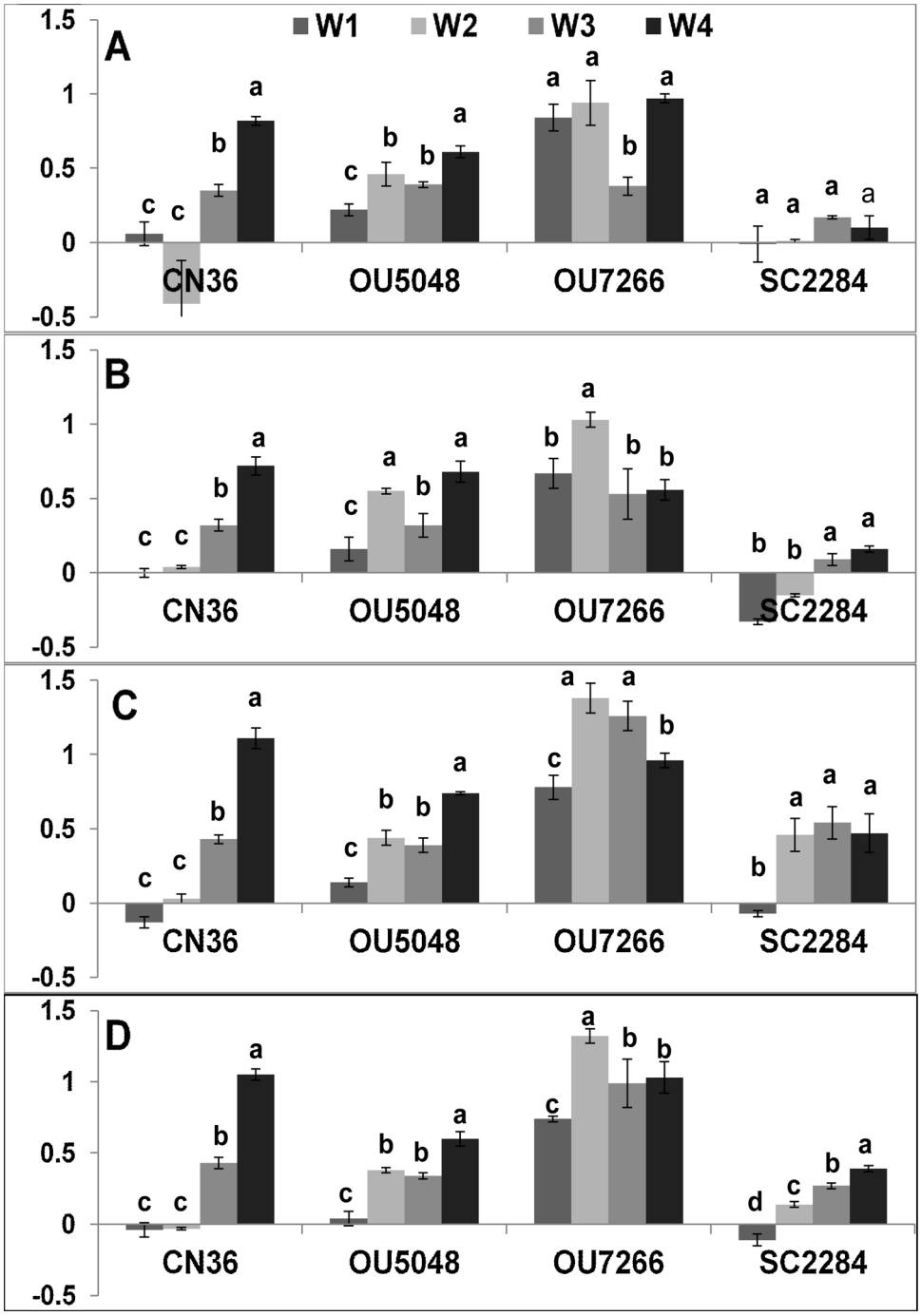

Fig. 1. The antibody titers to CN36, SC2284, OU7266 and OU5048 for the sera collected from weeks 1 - 4. The serum was separated into normal (A), *E. coli* absorption (B), heat inactivation (C) and absorption and inactivation (D) categories. Each value was compared to the control group. Each bar value was calculated by the original data - mean value of the control group. a–d Different letters indicate significant differences between different groups $p < 0.05$.

(Table 5), suggesting that pSV may play an important role in an increase of superoxide production. This phenomenon was not observed in PBMCs after vaccination. Furthermore, vaccination did not change superoxide production between PMNs and PBMCs infected by wild type serovars. After challenge, we observed a significant increase in superoxide production from both PMNs and PBMCs compared to the levels stimulated by vaccination. All pSV-less strains stimulated lower superoxide levels than pSV-containing strains.

4. Discussion

Prevention of infection in pigs by the two most common pathogenic serovars Choleraesuis and Typhimurium was the primary goal in the development of an attenuated Typhimurium ΔznuABC vaccine strain applied in combination with an inactivated Choleraesuis vaccine [24]. However, these two serovars induced gene expression differently for innate immunity and inflammatory T helper 1 (Th1) responses in pigs with early transcriptional induction (8–24 h) for Typhimurium and significantly greater gene expression at later time points (48 h-21 d) [25]. Such differences in gene expression may be responsible for the variation in disease progression between these two serovars. Furthermore, Typhimurium may overcome early innate and adaptive immunity mechanisms and trigger apoptosis and pyroptosis in the mesenteric lymph node (MLN) [26]. *Salmonella* can survive in macrophages and

Table 2
The serum resistance of *Salmonella* serovars at different sera level.

Complement inactivation	Weeks	Groups	The inhibitory percentage (%) of sera to different *Salmonella* serovars							
			E. coli	*S.* Enteritides		*S.* Typhimurium		*S.* Choleraesuis		
				OU7130	OU7067	OU5045	OU5046	OU7085	OU7266	CN36
–	2	Normal	5	10	20	> 20	> 20	> 20	> 20	> 20
		OU5048	5	10	20	5	> 20	20	> 20	> 20
		OU7266	2.5	10	20	10	> 20	20	20	> 20
		SC2284	5	10	20	20	> 20	20	20	> 20
		CN36	ND	ND	ND	ND	ND	ND	ND	ND
	4	Normal	5	10	20	> 20	> 20	> 20	> 20	> 20
		OU5048	5	5	20	10	> 20	20	> 20	> 20
		OU7266	5	5	20	10	> 20	20	> 20	> 20
		SC2284	10	5	20	10	> 20	20	> 20	> 20
		CN36	5	10	20	10	> 20	20	20	> 20
+	2	Normal	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20
		OU5048	> 20	20	> 20	10	> 20	> 20	> 20	> 20
		OU7266	> 20	20	> 20	20	> 20	> 20	> 20	> 20
		SC2284	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20
		CN36	> 20	10	> 20	> 20	> 20	> 20	> 20	> 20
	4	Normal	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20
		OU5048	> 20	10	> 20	20	> 20	> 20	> 20	> 20
		OU7266	> 20	20	> 20	20	> 20	> 20	> 20	> 20
		SC2284	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20
		CN36	> 20	10	> 20	20	> 20	> 20	> 20	> 20

ND: No detection.

Table 3
The ANOVA analysis of the superoxide production from the PMN and PBMC cells against Salmonella infection from vaccination and challenge.

Cell types	Periods	Source	*p* value	
PMN	Week 1- 2	Serovars and species	< 0.0001	< 0.0001
	Vaccination	Interaction time	0.5543	
		Vaccine groups	< 0.0001	
		Week	< 0.0001	
	Week 3- 4	Serovars and species	< 0.0001	
	Challenge	Interaction time	0.9818	
		Vaccine groups	< 0.0001	
		Week	< 0.0001	
PBMC	Week 1-2	Serovars and species	< 0.0001	< 0.0001
	Vaccination	Interaction time	0.0515	
		Vaccine groups	< 0.0001	
		Week	< 0.0001	
	Week 3- 4	Serovars and species	< 0.0001	
	Challenge	Interaction time	0.9464	
		Vaccine groups	< 0.0001	
		Week	0.0159	

Table 4
Superoxide production of PMN and PBMC of vaccinated groups in week 1–2 and.3–4.

Cell types	Treatment	Mean	T value	*P* value
Week 1 -2 (vaccination)				
PMN	OU5048	0.16 ± 0.076	−4.164	< 0.001
	OU7266	0.22 ± 0.100		
	OU5048	0.16 ± 0.076	−5.28	< 0.001
	SC2284	0.22 ± 0.066		
	OU7266	0.22 ± 0.100	1.099	0.273
	SC2284	0.22 ± 0.066		
PBMC	OU5048	0.15 ± 0.087	−5,402	< 0.001
	OU7266	0.25 ± 0.065		
	OU5048	0.15 ± 0.087	−7.179	< 0.001
	SC2284	0.22 ± 0.067		
	OU7266	0.25 ± 0.065	−0.556	0.579
	SC2284	0.22 ± 0.067		
Week 3 -4 (challenge)				
PMN	OU5048	0.30 ± 0.185	−6.275	< 0.001
	OU7266	0.56 ± 0.456		
	OU5048	0.30 ± 0.185	−6.282	< 0.001
	SC2284	0.62 ± 0.573		
	OU7266	0.56 ± 0.456	−0.992	0.322
	SC2284	0.62 ± 0.573		
PBMC	OU5048	0.32 ± 0206	−6.368	< 0.001
	OU7266	0.57 ± 0.426		
	OU5048	0.32 ± 0.206	−2.470	0.014
	SC2284	0.41 ± 0.387		
	OU7266	0.57 ± 0.426	3.367	0.001
	SC2284	0.41 ± 0.387		

dendritic cells that present their antigens to B cells by inducing antibody production or activating cytotoxic T cells to attack macrophages with intracellular *Salmonella* [27,28]. The genes for intracellular *Salmonella* replication differ, including three genes *ydgT, recA*, and *corA* for cytosolic proliferation and to the gene *asmA* for a decrease in cytosolic replication [29].

Antibodies can prevent bacterial dissemination systemically, and *Salmonella*-specific antibodies can assist the host in reducing the severity of *Salmonella*-associated gastroenteritis and bacteremia. In this study on stimulating antibody production, Choleraesuis strains were proven to be more antigenic than Typhimurium, and the pSV-less strains elicited stronger antibody responses than the SPI-2 mutant strain (Fig. 1). Antibodies, particularly *Salmonella*-specific IgG, can inhibit *Salmonella* growth and assist in the activation of macrophages and $CD4^+$ T cells to kill *Salmonella* [18,20]. Choleraesuis and Typhimurium not Enteritidis, are more resistant to serum killing (Table 2). Further, the expression of genes on the pSV can prevent serum killing. Specific examples include outer membrane proteins Rck (a 19-kDa outer membrane protein encoded by the *rck* gene on the large virulence plasmid) and TraT (the transfer operon of the large plasmid harbors a gene called *traT* that encodes the TraT lipoprotein) inhibit C9 and C5b6 functions, thus preventing formation of the membrane attack complex (MAC), Rsk to enhance the function of Rck, and PagC to avoid complement attack [30]. These results imply that these two serovars may have evolved mechanisms other than those directed by products encoded by pSVs to survive in pigs, resulting in Choleraesuis and Typhimurium being the most prevalent porcine pathogens, rather than

Table 5
Superoxide production of PMNs and PBMC treated with different serovars.

Cell types	Serovars	Bacteria	Mean	T value	*P* value
Week 1 and 2 (vacination)					
PMN	Enteritides	OU7130	0.19 ± 0.069	3.638	< 0.001
		OU7067	0.14 ± 0.060		
	Choleraesuis	OU7085	0.18 ± 0.051	2.111	0.037
		OU7266	0.16 ± 0.044		
	Typhimurium	OU5045	0.18 ± 0.049	0.311	0.756
		OU5046	0.17 ± 0.045		
PBMC	Enteritides	OU7130	0.18 ± 0.053	1.587	0.115
		OU7067	0.16 ± 0.075		
	Choleraesuis	OU7085	0.18 ± 0.064	0.552	0.582
		OU7266	0.17 ± 0.059		
	Typhimurium	OU5045	0.16 ± 0.051	−1.045	0.298
		OU5046	0.17 ± 0.056		
Week 3 and 4 (Challenge)					
PMN	Enteritides	OU7130	0.34 ± 0.214	4.508	< 0.001
		OU7067	0.23 ± 0.100		
	Choleraesuis	OU7085	0.60 ± 0.623	2.761	0.007
		OU7266	0.39 ± 0.350		
	Typhimurium	OU5045	0.56 ± 0.553	3.205	0.002
		OU5046	0.36 ± 0.266		
PBMC	Enteritides	OU7130	0.33 ± 0.167	3.78	< 0.001
		OU7067	0.25 ± 0.133		
	Choleraesuis	OU7085	0.59 ± 0.506	4.515	< 0.001
		OU7266	0.33 ± 0.215		
	Typhimurium	OU5045	0.51 ± 0.408	4.411	< 0.001
		OU5046	0.30 ± 0.200		

Enteritidis. Furthermore, pSV may play an important role in serum killing as pSV-containing strains were less resistant than pSV-less strains.

Superoxide production is involved in killing of invasive bacteria by PMNs and PBMCs. After phagocytosis, macrophages activate radical oxygen species (ROS) production, such as activation of NADPH oxidase to produce superoxide, facilitating bacterial killing [13,14,31]. Genes in SPI-2 can inhibit ROS production [12,32]. Furthermore, *Salmonella* can express catalase-related genes *katE*, *katG* and *katN*, alkylhydroperoxide reductase-related genes *ahpC* and *tsaA* to disrupt H_2O_2 generation [33,34], and superoxide dismutases-related genes *sodA*, *sodB*, *sodCI* and *tpx* whose products convert H_2O_2 to H_2O [35,36]. Furthermore, HOCl generated by myeloperoxidase from neutrophils is a key antimicrobial agent; MSRA increased the resistance to HOCl and neutrophil-mediated killing [37]. All of the above mechanisms can suppress superoxide production to allow *Salmonella* survival in macrophages. In this study, *Salmonella* can inhibit superoxide production from PMNs and PBMCs after vaccination and challenge (Tables 3–5). The PMNs and PBMCs from the Choleraesuis pSV-less OU7266 and SPI-2 mutant SC2284 groups responded differently regarding superoxide production against various serovars and after challenges. These differences may be the result of variations in the genetic background of OU7266 and SC2284 or differences in the expression of pSV and SPI-2. Three wild-type serovars stimulated more superoxide production in immune cell lines than pSV-less derivative strains, suggesting that pSV plays a negative regulatory role in host superoxide production defense mechanisms.

Vaccination and challenge can change immune cell types and populations. For example, granulocyte-macrophage colony-stimulating factor (CSF) stimulates the regeneration of granulocytes and monocytes and antigen presentation to helper T cells to differentiate between the Th1 and Th2 pathways. In pigs, vaccination with *Mycoplasma* vaccine increased the $CD4^+$ T population at day 3 and reduced the $CD8^+$ T population at days 3 and 7 [38]. *Ex vivo* bacterial treatment demonstrated different superoxide levels and timing among five groups; this may be associated with vaccination or challenge to induce the changes in the immune cell types and populations. Different serovars infecting same host may express different genes on SPIs [39,40]. A previous study demonstrated that IL8 and CCL20 expression was significantly greater in the cells infected by Typhimurium than in those infected by Choleraesuis after infection of pigs at 3 and 6 h [5]. After infection, Choleraesuis stimulates inflammatory reactions later, and these persist longer than in Typhimurium infections. In pigs, Choleraesuis infection stimulates the expression of INF-γ and IL-1β for 21 days, while Typhimurium only stimulated the expression of INF-γ and IL-1β for 48 h [39,41]. Therefore, challenge triggers greater superoxide levels from PMNs and PBMCs to kill bacteria with Choleraesuis than with Typhimurium vaccine application and triggers greater superoxide production with Choleraesuis and Typhimurium treatments than the Enteritidis treatment (Tables 4 and 5).

In the prime-boost strategy, an initial, or first, vaccine stimulates the immune system, and a second vaccine is applied to enhance a more specific immune response to challenge by a pathogen strain or serovar. Using the prime-boost method to apply the Typhimurium vaccine enhances antigen-specific $CD8^+$ T cells or antibody production [42,43]. In this study, challenge significantly increased superoxide production from PMN and PBMC cells from two Choleraesuis vaccine groups two weeks after challenge (Table 4), demonstrating that vaccination was effective in promoting superoxide production against *Salmonella* infection.

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Prime-boost vaccination with attenuated Salmonella Typhimurium ΔznuABC and inactivated Salmonella Choleraesuis is protective against Salmonella Choleraesuis challenge infection in piglets.

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5. Conclusions

Choleraesuis OU7266 may be a better vaccine strain in generating high IgG levels and stimulating serum bactericidal activity and superoxide production levels by PMN/PBMCs; the presence of pSV likely influences the magnitude of immune responses.

Conflict of interest

All authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2019.04.003.

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