獸醫學博士 學位論文

# Evolutionary genetics and immunological characterization of *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum

*Salmonella* Gallinarum 진화의 유전적 분석 및

변이주의 백신 가능성

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(수의미생물학)

김 남 형

# Evolutionary genetics and immunological characterization of *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum

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# Evolutionary genetics and immunological characterization of *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum

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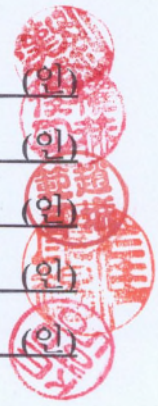

## Abstract

# Evolutionary genetics and immunological characterization of *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum

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*Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum (SG) is the causative agent of fowl typhoid, which causes substantial economic loss to the poultry industry due to high mortality. SG is known to be difficult to eradicate as it spreads not only through horizontal transmission but also egg transmission and possible to survive within cells as a latent infection. Currently, several vaccines are adopted, but fowl typhoid is still not eradicated in the field. The introduction of SG9R had a great effect, but recently despite the regular vaccination, fowl typhoid has still been reported in a large number of farms. Accordingly, there is a suspicion of the emergence of a pathogenic revertant of vaccine strain or field strain variants, resulting in increasing demand to improve existing vaccine strains and vaccine programs.

The evolutionary process of SG adapting to chicken as a specific host and its lifestyle within the cell drive SG to have lost various functions and accumulate pseudogenes. These pseudogene profiles could be used to clarify the evolutionary relationship between field strains. Also, the length of the polyproline linker (PPL) of the *spvB* gene, which plays an important role in *Salmonella* pathogenic mechanisms, varied from 11 to 21 prolines in domestic isolates, which were longer than those of the foreign strains. According to the growth competition in chickens, SG with 17 prolines dominated an SG with a PPL length of 15 prolines. Furthermore, it was confirmed that pseudogene profiles of the domestic field strains constituted their own cluster different from the foreign strains. Considering the pseudogene profiles and PPL length, the molecular epidemiology of SG in Korea was elucidated to be subdivided into 7 pseudogene subgroups, and two closely related SG isolates were speculated to be transformed into several subgroups. The

origin of transmission of the first SG in Korea was unclear, but considering the identical mutation in the *fliC* gene, it was surmised that the SG isolated in the Middle East appeared to share the origin.

A study was conducted to compare differences in humoral immune responses to various types of SG antigens. The outer membrane proteins with high immunogenicity were analyzed by 2D-gel electrophoresis and western blotting with rough strain vaccine SR2-N6 antisera. OmpA and OmpX were identified as a result of LC-MS/MS analysis. The epitopes of two proteins were analyzed by comparing the B-cell epitope prediction program with the protein tertiary structure, and peptide-ELISAs were established. The humoral immune responses of the groups inoculated with SG002 (smooth strain) and SR2-N6 (rough strain) killed oil vaccines were compared, showing significantly higher antibody titers in SR2-N6-inoculated group. The humoral immunity was compared in the same manner by inoculating various forms of the vaccine as live, killed, live + killed, and oil-adjuvanted killed. The mixed vaccine maintained high antibody titers until after 3 weeks of inoculation, but the killed form did not stimulate the humoral immunity. Infection with the field strain detained the immune response by about 2 weeks. The mucosal immunity was tested with bile juice, and immunity was not induced in all compositions, except when challenged after the live vaccine.

The rough strain vaccine SR2-N6, produced based on SG002, is currently commercialized, but its mechanism of attenuation and characteristics have not been studied. SR2-N6 was compared with SG002 through comparative genomics and proteomics studies. It was confirmed that SR2-N6 became a rough mutant due to the deletion of the 13-

nucleotide of the *rfaL* gene. Mutations in the quinolone resistance-determining region of *gyrA* gene increased the quinolone antibiotic resistance by about 8 times compared to SG002. In addition, *spvB* and *spvC*, the core virulence genes present in the large virulence plasmid, were also not detected. SR2-N6 was more susceptible to cold shock than SG002, probably due to the decrease in the expression of DNA starvation/stationary phase protection genes (*dps*).

The popular rough strain vaccine SG9R became rough mutant by a single nonsense mutation of the *rfaJ* gene, making it possible to recover virulence on the field. In the present study, the *rfaJ* gene removed mutant (Safe-9R) was generated for the vaccine stability. Live Safe-9R showed similar protective efficacies to SG9R, but although inactivated oil adjuvanted Safe-9R (OE Safe-9R) stimulated the humoral immune response, it caused severe body weight loss in 1-week-old chicks. As the endotoxin was suspected to be the cause of the weight loss, lipid A synthesis and modification genes were removed to detoxify the endotoxin. *phoP/phoQ*, *lpxL*, *lpxM*, and *pagP* were eliminated and tested for the expression of pro-inflammatory cytokines in chicken macrophage cells (HD11). Among the knock-out mutants, *ΔlpxL* (Dtx-9RL) and *ΔlpxM* (Dtx-9RM) showed a significant decrease in cytokines expression levels, and especially, Dtx-9RL did not show weight loss in 1-week-old chicks. The live Dtx-9RL also showed no liver lesion in immunocompromised chickens. However, when challenged after the live detoxified strains inoculation, the protective efficacy of Dtx-9RL was immaculate, showing a similar mortality to the negative control. Dtx-9RM, on the other hand, provided full protection and demonstrated lesser liver lesions than the conventional vaccine strains without recovery of

inoculated strains.

In conclusion, as the Korean field SG is speculated to have evolved into more pathogenic variants by accumulating mutations in both chromosomes and the *spvB* gene, it has to be prepared. The improved SG9R mutants, Safe-9R, Dtx-9RL, and Dtx-9RM, are expected to be useful in establishing more effective vaccine strategies.

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***Keywords****: fowl typhoid; Salmonella Gallinarum; comparative genomics; humoral immunity; vaccine; lipid A detoxification*

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# Abbreviations

| | |
|---|---|
| **CFU** | Colony forming unit |
| **CONT** | Negative control |
| **d-o** | Day-old |
| **dpi** | Day post inoculation |
| **Dps** | DNA starvation/stationary phase protection protein |
| **ELISA** | Enzyme-Linked Immunosorbent Assay |
| **FT** | Fowl typhoid |
| **IM** | Intramuscular |
| **KCDC** | Korea Centers for Disease Control and Prevention |
| **LC-MS/MS** | Liquid Chromatograph-Tandem Mass Spectrometer |
| **LPS** | Lipopolysaccharide |
| **MALDI-TOF** | Matrix-Assisted Laser Desorption/Ionization time-of-flight |
| **MAPK** | Mitogen-activated protein kinase |
| **MIC** | Minimum inhibitory concentration |
| **MX** | Mixed vaccine of PBS suspension killed vaccine and live SG9R |
| **O-Ag** | O-antigen |
| **OD** | Optimal density |
| **OE** | Oil-emulsion |
| **OMP** | Outer membrane protein |
| **PCR** | Polymerase chain reaction |

| | |
|---|---|
| **PEM** | Protein malnutrition model |
| **PPL** | Polyproline linker |
| **PS** | PBS-suspension |
| **QIA** | Animal and Plant Quarantine Agency |
| **QRDR** | Quinoline resistance domain region |
| **SE** | *Salmonella enterica* subsp. *enterica* serovar Enteritidis |
| **SG** | *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum |
| **SLS** | Sodium lauroyl sarcosine |
| **SNU** | Seoul National University |
| **SP** | *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Pullorum |
| **SPF** | Specific pathogen free |
| **ST** | *Salmonella enterica* subsp. *enterica* serovar Typhimurium |
| **w-o** | Week-old |
| **Wpi** | Week post inoculation |

# General Introduction

*Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum (SG) causes fowl typhoid in poultry and is analogous to *Salmonella* serovar Typhi in humans (Thomson et al., 2008). Since the first outbreak of fowl typhoid in 1992, it has become enzootic in Korea (Lee et al., 2003). Although live attenuated vaccines have been extensively used in the field, virulent isolates continue to be isolated. For this reason, the appearance of novel and more virulent SG or the reversion mutation of a vaccine strain has been suspected (Kwon and Cho, 2011).

The large virulence plasmid of *Salmonella* varies in size for each serotype but has a well-conserved virulence sequence known as *spv*. The *spv* locus is crucial for the pathogenicity of *Salmonella*, and particularly the *Salmonella* plasmid virulence genes, *spvB* and *spvC*, are the core (Matsui et al., 2001). The *spvB* gene encodes an actin-ADP-ribosylating toxin, which depolymerizes actin filaments resulting in the instability of cytoskeleton of cells and enhancing the proliferation of *Salmonella* (Lesnick et al., 2001; Otto et al., 2000). The C terminal of *spvB* mainly triggers this toxic effect, and the N terminal was identified to promote extracellular secretion (Gotoh et al., 2003). There is a polyproline linker (PPL) connecting the N- and C-terminal domains of SpvB, and the previous research found that the length of PPL determines the pathogenic activity of SpvB, and is variable among *Salmonella* serovars (Kwon and Cho, 2011; Pust et al., 2007). PPL was longer in SG than other *Salmonella* serotypes, and SG isolated in Korea showed elongated aspects than reference strains deposited in GenBank (Kwon and Cho, 2011). Considering that the length of PPL, which is important for pathogenicity, has various

lengths, it could be used as an indicator of the evolution study against SG in Korea.

*Salmonella* Gallinarum (SG) is predicted to share recent common ancestries with serovars Choleraesuis, Paratyphi C, Enteritidis and Typhimurium. Among serovars, SG is assumed to have evolved from *Salmonella enterica* serovar Enteritidis based on shared genes and pseudogenes (Langridge et al., 2015; Thomson et al., 2008). *Salmonella* Pullorum (SP) is also very genetically close to SG, and they were speculated to have gained much more pseudogenes than SE in the process of being restricted to host (Feng et al., 2013). Pseudogenes occur randomly but might be generated for efficiency in particular circumstances, such as host adaptation or intracellular lifestyle. (Kuo and Ochman, 2010). With pseudogene profiling, it could be understood the evolutionary status of SG and the molecular epidemiology of FT.

Although *Salmonella* is an intracellular antigen, the role of humoral immunity against *Salmonella* should not be undervalued. The protective efficacy of outer membrane protein vaccines (OMP) has already been established, and protective OMPs of *S.enterica* serovars have been identified for vaccine development (Gil-Cruz et al., 2009; Yang et al., 2018). LPS is known to assist a strong humoral immune response to concomitantly inoculated antigens, but LPS may also conceal or compete with outer membrane proteins (OMPs), resulting in decreased immunogenicity of OMP (Johnson et al., 1956; Seppala and Makela, 1984). Therefore, the rough strain vaccine, might have the potential to demonstrate increased immunogenicity for OMP compared to the smooth strain.

The lipopolysaccharide (LPS) rough vaccine strain SG9R, which lacks outer core and O-side chain repeats of LPS due to a single nonsense mutation in *rfaJ*, has been used

in fowl typhoid prevention. It has been found that not only rough LPS but also mutations of several virulence genes of SPI-1 and SPI-2, and pyruvate dehydrogenase subunit E1 (aceE) act on the attenuation of SG9R (Kang et al., 2012; Van Immerseel et al., 2013). However, still SG9R has pathogenic potency, showing mortality and severe lesions in immunocompromised chicken or chicks under 6 weeks of age (Kwon and Cho, 2011; Wigley et al., 2005). The development of an novel vaccines that can complement SG9R is required, but basic data on the differences in humoral immune responses to different forms of SG antigens (oil-emulsion, killed, smooth vs. rough SG; live vs. killed with or without oil adjuvant vs. a mixture of live and killed SG 9R; or field strain) should be elucidated first (Chaudhari et al., 2012; Kwon and Cho, 2011; Lee et al., 2007). In addition, humoral immunity against natural infection with field strains is unclear. It is known that *Salmonella* suppresses the stimulation of humoral immunity by inhibiting B lymphopoiesis (Slocombe et al., 2013). However, SG is a non-motile antigen without flagella, so it may show different aspects. In previous studies evaluating the humoral immunity of *Salmonella*, antibody titers were mainly evaluated using total OMP extracts, but it may provide better insight into the kinetics of humoral immunity when using single peptide epitopes.

To further attenuate virulence and develop the fowl typhoid vaccine, several pathogenic components can be modified. Recently, a new rough vaccine strain, SR2-N6, against fowl typhoid was verified to be as efficacious as SG9R. SR2-N6 did not cause any mortality or liver lesions and was not re-isolated in the immunocompromised model (Kwon and Cho, 2011). Despite the commercialization of SR2-N6 in Korea, the attenuation mechanism of SR2-N6 was unknown.

The another drawback of SG9R is the possibility of virulent revertant. As it became rough mutant via a single point mutation of *rfaJ* gene, SG9R has the potential to restore pathogenicity. In fact, several studies have reported that field smooth strains isolated from farms inoculated with SG9R had similar DNA finger prints (De Carli et al., 2017; Van Immerseel et al., 2013). In Korea, O-antigen ELISA based monitoring system is in operation to eradicate SE. The revertant of SG9R will be detected in ELISA due to the presence of O antigen, which can cause confusion in the monitoring system. Additionally, the inner core of LPS is known to be immunogenic, and SG9R vaccination may induce an anti-inner core antibody reaction to the O-Ag ELISA (Jakel et al., 2008; Kong et al., 2016). The elimination of *rfaJ* gene could be the alternative to the emergence of revertant. As the *rfaJ* is the pseudogene, the removal would not affect the phenotype of the mutant.

In addition to the pathogenic problem in inoculating SG9R to the young chicks, the bodyweight became significantly lower than that of the non-vaccinated group (Lee et al., 2005). Weight loss might occur due to the reduced feed intake by the virulence of live vaccines, but endotoxin-induced inflammatory reactions could be the reason (Blatteis, 1974; Xie et al., 2000). Endotoxin detoxification through modification of the lipid A synthesizing genes not only reduces the toxicity of endotoxin itself, but also reduces the virulence of pathogens (Fisseha et al., 2005; Lee et al., 2009). Proteins that attach acyl chain in the last biosynthesis of lipid A, *lpxL* and *lpxM*, have been studied for the vaccine candidates (Clementz et al., 1996; Clementz et al., 1997; Raetz and Whitfield, 2002). The *phoP/phoQ* regulatory gene that modifies lipid A and its sub-regulatory gene *pagP* also showed decreased virulence when eliminated (Dalebroux et al., 2014; Wang et al., 2013).

This study aimed to examine the cause of consistent outbreaks of fowl typhoid in Korea through genetic analysis of *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum. In addition, this study was to help overcome the fowl typhoid by developing ELISAs for evaluating the humoral immune response of the SG antigen, studying the characteristics of the conventional SG vaccine (SR2-N6), and presenting a new SG vaccine candidates. The molecular evolution of isolated SG in Korea was investigated by PPL lengths and pseudogene profiling, resulting that the Korean SG have evolved by accumulating pseudogenes and increasing their pathogenicity. As the emergence of more pathogenic variants have been suspected, a novel vaccine strategy should be established. However, the investigation of the humoral immune response to SG was insufficient. ELISA with immunogenic OMPs against SG was constructed to compare the humoral immune response of various SG antigens. Furthermore, characteristics of SR2-N6, which was not elucidated its attenuation mechanism, was analyzed by comparative genomics, and differential gene expression by a proteomic analysis. Studies have also been conducted to compensate for the shortcomings that SG9R is capable of recovering virulence and has pathogenic potency. The *rfaJ* removed mutant was developed, and its endotoxin synthesizing and modifying genes were removed to attenuate the virulence. Generated mutants were evaluated for cytokine expression in chicken macrophage cells, and promising mutants were tested for in vivo experiments, such as bodyweight comparison and vaccine efficacy.

# Literature review

# Fowl typhoid

Fowl typhoid (FT) is septicemic disease affecting primarily chickens. It was first recognized in 1888, and the name fowl typhoid was applied in 1902 (Shivaprasad and Barrow, 2017). FT induces systemic infection in chickens, causing high mortality and substantial economic loss. Its major clinical signs may begin with a drop in feed consumption and show ruffled feathers. Death usually occurs after 5-10 days after exposure.

Several developed countries reported that the FT have been eradicated, but sporadic isolation of SG, which is the causative agent of FT, have been reported (Barrow and Neto, 2011). FT have constantly occurred in Central and South America, and many Asian countries. The fowl typhoid in Korea has frequently occurred since 1992 and is still emerging nationwide. Since the introduction of the fowl typhoid vaccine, SG9R, the prevalence in laying hens has rapidly decreased but is rather increasing in broilers. Most infections in broilers are 2 weeks old chickens, so vertical transmission is suspected. This change is speculated because of the full-scale introduction of vaccines (Kwon et al., 2010).

In some European countries, where SG was eradicated, SE emerged as a major egg-associated pathogen in the late 20th century, however, there are few reports of egg contamination by SE in Korea. The previous study affirmed that SG could competitively excludes SE, which might explain the low prevalence of SE in Korea. (Rabsch et al., 2000)

## 1. Pathology

The gross lesions of FT include enlarged liver, spleen, and kidney, and bronze liver by necrosis is the distinct lesion. Microscopic lesions of acute cases of FT show multifocal necrosis of hepatocytes with accumulation of fibrin and infiltration of heterophils in the hepatic parenchyma (Shivaprasad and Barrow, 2017). SG has strong host specificity and host specialization is often related to more acute pathogenicity (Barrow et al., 1994; Parkhill et al., 2003; Rohmer et al., 2007). Especially, SG is highly pathogenic among *Salmonella* serovars as it is facile to develop into a systemic infection without stimulating TLR5. SG was not able to enter Peyer's patch of mouse, which suggested to have a different phagocytic mechanism from *Salmonella* typhimurium (ST) (Pascopella et al., 1995). However, in chicken, SG did not demonstrate increased colonization in intestinal tissue or bursa than other *Salmonella* serotypes when inoculated orally. The multiplication and survival in chicken macrophages were also similar (Chadfield et al., 2003). Chicken pathogenic *Salmonella* have a different viability in macrophages according to the *SAL1* locus of chicken chromosome 5. The difference of SG according to the factor is more dramatic than SE or ST (Wigley et al., 2002). It is predicted to be the cause of the high susceptibility of brown egg-laying hens to FT. Since most of the laying hens are brown in Korea, it is more difficult to eradicate fowl typhoid (Kim et al., 2002; Smith, 1956).

## 1-1. Innate immunity

The innate immune response to *Salmonella* begins with releasing of several cytokines and chemokines. The nuclear factor-kappa B (NF-κB) regulates the epithelial

pro-inflammatory response to *Salmonella* (Elewaut et al., 1999). Flagella is one of the major antigen which initiate the response. Epithelial cells secrete IL-8 to attract neutrophils, and flagellin-stimulated TLR5 also induces IL-8 expression (McCormick et al., 1995). However, without flagella, basolateral secretion of IL-8 does not occur, making it advantageous for non-motile *Salmonella* such as SG and SP to develop into a systemic infection. It is speculated that the IFN-γ and T cell response is correlated with the clearance. After inoculating the live attenuated vaccine SG9R, high IFN-γ level was followed (Wigley et al., 2005). The SG-infected macrophage and chicken kidney cells had lower expression of IL-6, CSCLi1 and iNOS (Setta et al., 2012). *Salmonella* mainly enters the oral route and invade through the M cell of Peyer's Patch (Galan, 1999). When the epithelial barrier is penetrated, infection begins throughout the body, such as the spleen or liver, through mesenteric lymph nodes. *Salmonella* has a well-developed mechanism to evade innate immunity, and in particular, the action of the PhoP/PhoQ regulator is essential to survive in macrophages (Miller et al., 1989). This system regulates over 40 genes. PhoP-activated genes act for survival in macrophages by modification of lipid A component, whereas PhoP repressed genes are suppressed in the phagosome (Guo et al., 1998). Furthermore, additional survival mechanisms exist, such as inactivation of reactive oxygen secreted by macrophages or increasing viability by TTSS, encoded by SPI-2 (De Groote et al., 1996). These mechanisms of *Salmonella* to evade innate immunity refers to the importance of innate immunity to the defense.

Autophagy is considered a pathway for the innate immune response to intracellular bacteria in the cytosol (Huang and Brumell, 2014). Autophagy may play a role

in protecting cells by upregulating innate immunity, but it is like a double-edged sword that may damage cells in certain diseases or environments (Shintani and Klionsky, 2004). Autophagy is a cellular pathway that transmits lysosomes that degrade them to proteins or structures in the cytoplasm and is upregulated by various stressors. It occurs in most tissues at the basal level and can also occur when environmental changes such as malnutrition occur (Jung et al., 2010). Every cell has an internal nutrient that can be used in starvation situations. Autophagy plays an important role when using these nutrients. Within 24 hours of starvation, autophagy increases, producing necessary nutrients through liver autophagy, cardiac autophagy, and muscle autophagy. Under normal circumstances, when an organism is infected with *Salmonella*, autophagy acts as a mechanism to protect the organism (Fig L.1). However, when the starvation period is prolonged, autophagy can destroy the host itself, so the TOR signal, which suppresses autophagy, is activated (Scott et al., 2004). Inhibited autophagy by the TOR signal enables the survival and replication of *Salmonella*. Therefore, in protein-malnutrition model, the attenuated SG can show extensive virulence showing mortality in chicks and severe liver lesions (Kwon and Cho, 2011).

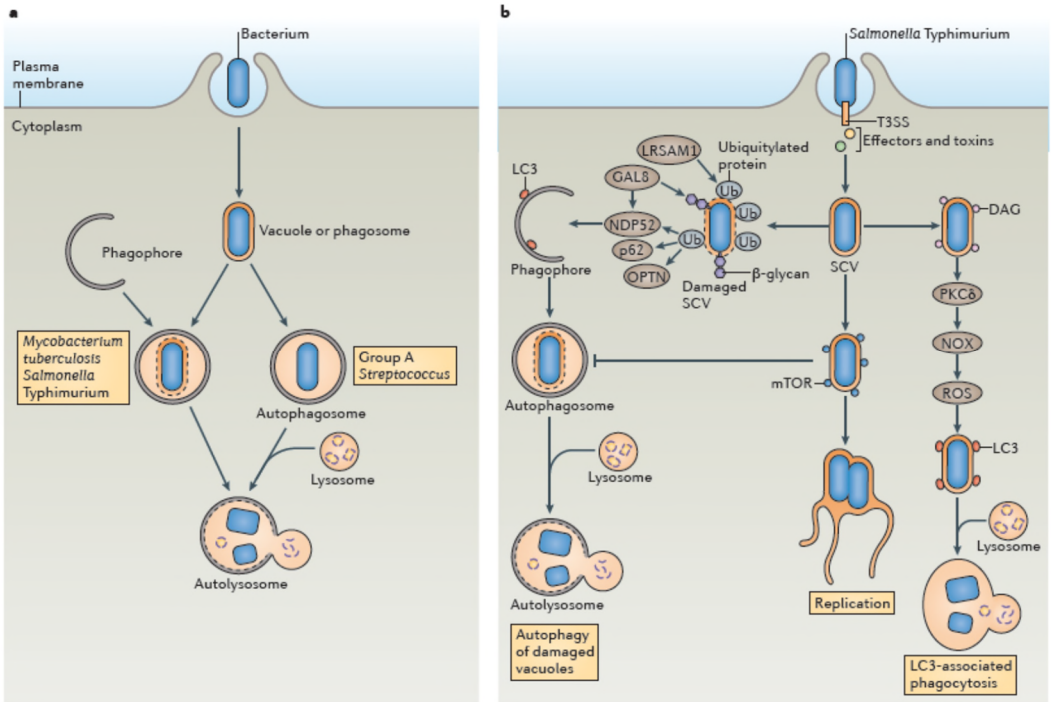


**Figure L.1.** Autophagy is an antibacterial mechanism. (a) Schematic diagram of the ways that autophagy targets intracellular bacteria. (b**)** Model of the antibacterial autophagy of *S.* Typhimurium. Adapted from Huang, Ju, and John H. Brumell (2014)

## 1-2. Humoral immunity

Since *Salmonella* is an intracellular antigen, the importance of humoral immunity to *Salmonella* had been undervalued. *Salmonella* inhibits B lymphopoiesis, which interferes with the rise of humoral immunity, so it is difficult to expect the role of humoral immunity during initial infection (Slocombe et al., 2013). However, many studies show that humoral immunity is important for the defense of *Salmonella* as well as cellular immunity, and studies for *Salmonella* protection through humoral immunity are actively being conducted (Kaufmann, 1993; Mastroeni et al., 1993). In a study examining the role of B cells in *Salmonella* defense by removing bursa of Fabricius from chickens, *Salmonella* reisolation increased in various immune organs (Mastroeni et al., 2001). While the killed whole-cell vaccine for enhancement of humoral immunity in *Salmonella* did not show sufficient protective efficacy, it is partially protected against intestinal colonization, faecal shedding, and systemic infection, and its efficacy is increased when used with an appropriate adjuvant (Gast et al., 1992; Mastroeni et al., 2001). In SG, antibody titer was hardly increased when inoculated with PBS, but high humoral immunity was shown when used with oil emulsion adjuvant (Kim et al., 2020b). However, the protective effect showed about 50% during initial vaccination and then decreased with time. Protecting *Salmonella* with humoral immunity has shown clear limitation, but given the potential risks of an attenuated live vaccine, it is worth persisting research.

## 1-3. Cellular immunity

*Salmonella* is an intracellular antigen that can survive and proliferate in macrophages, making it difficult to defend compared to other pathogens, and therefore, cellular immunity has a major role in defense. *Salmonella* enters non-phagocytic cells by *Salmonella* pathogenicity island1-type 3 secretion system, and uptake via M cells, dendritic cells and other phagocytic cells also bring *Salmonella* to the host (Figure L.2) (Jantsch et al., 2011). Bacteria, which was internalized by phagocytic cells but able to survive, spread throughout the host resulting in systemic infection. Cytotoxic T cells (CTL) respond to the *Salmonella* infection, but little is known about the bacterial antigens that are recognized by the CTL (Sztein et al., 1995). Clearance of the *Salmonella* from the reticular endothelial system needs the activation of T-cells, which is dependent on the CD28 (O'Brien et al., 1982). The late resistance and bacterial clearance play a more important role in $CD4^+$ T cells than $CD8^+$ T cells. B cells in salmonellosis not only produce antibodies, but also modulate the immune response, presenting *Salmonella* antigens to activate the *Salmonella*-specific T cells and also play a role in the establishment of long-lasting Th1 type T cell (Sztein et al., 1995). The Th1 type T cells play a role in inducing IL2 and IFN-γ production (Harrison et al., 1997).

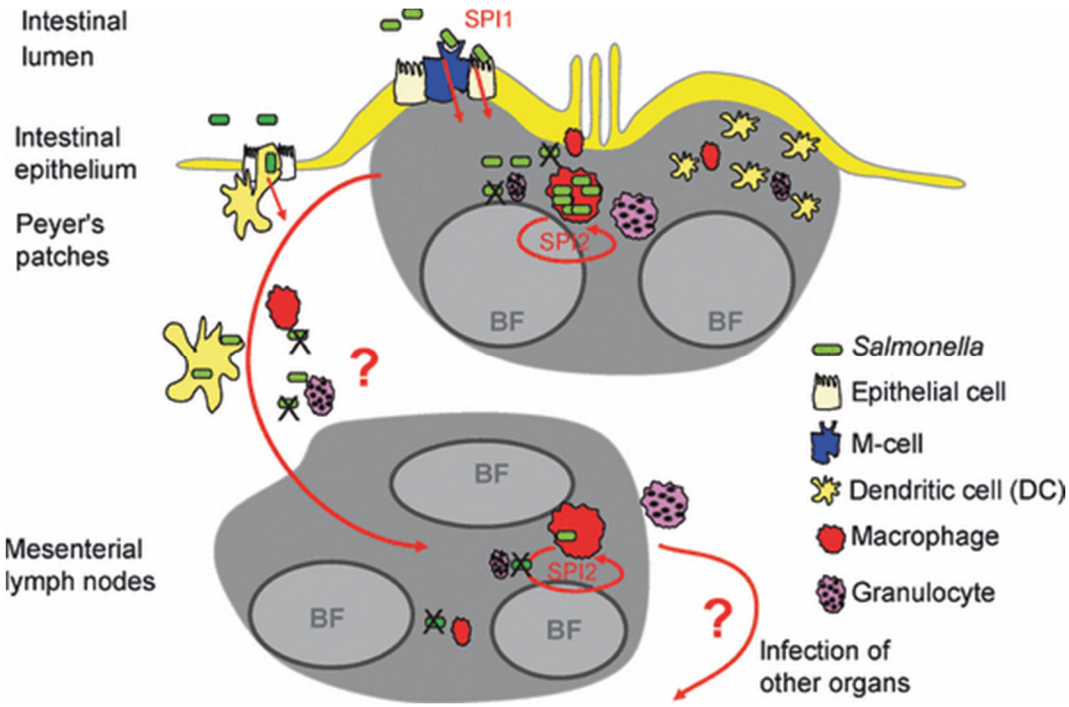


**Figure L.2.** Routes of infection by *Salmonella enterica*, host barriers, and immune defense mechanisms in various tissues. Adapted from Jonathan Jantsch, Deepak Chikkaballi, and Michael Hensel (2011)

# 2. *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum

## 2-1. Serologic and biochemical properties

There are over 2,500 *Salmonella enterica* serovars and belong to 6 subspecies (Table L.1) (Fierer and Guiney, 2001; Ochman and Groisman, 1994). *Salmonella* Gallinarum (SG) belongs to the most numerous subspecies, *enterica*, and H antigen does not exist due to the absence of flagella. SG belongs to the serogroup D according to the Kauffman White scheme and has 1, 9, and 12 O antigens, which are the same as *Salmonella* Enteritidis (SE) (Shivaprasad and Barrow, 2017; Shivaprasad, 2000). The biochemical properties of SG is highly similar to *Salmonella* Pullorum (SP). Both bacteria can ferment arabinose, dextrose, galactose, mannitol, rhamnose, and xylose. The differences between the two organisms appeared in using citrate, D(2) sorbitol, L(2) fucose, D(2) tartrate, and cysteine hydrochloride gelatin (Trabulsi and Edwards, 1962). However, as some strains may show variation, these differences are difficult to be used for differentiation.

**Table L.1. Present number of serovars in each species and subspecies**

| ***Salmonella enterica*** | **2,557** |
|---|---|
| *S. enterica* subsp. *enterica* | 1,586 |
| *S. enterica* subsp. *salamae* | 522 |
| *S. enterica* subsp. *arizonae* | 102 |
| *S. enterica* subsp.*diarizonae* | 338 |
| *S. enterica* subsp.*houtenae* | 76 |
| *S. enterica* subsp.*indica* | 13 |
| ***Salmonella bongori*** | 22 |
| **Total (genus *Salmonella*)** | **2,659** |

Adapted from Issenhuth-Jeanjean, Sylvie, et al. (2014)

## 2-2. Virulence factors

### A. Flagella

SG is a non-motile bacteria without flagella, H antigen. Flagellin, the core component of flagella, is an agonist of toll-like receptor 5, which recognize pathogen and activate the innate immunity (Gewirtz et al., 2001). The absence of flagella prevents stimulating initial immune response and facilitates the development of systemic infections (Chappell et al., 2009). The previous studies demonstrated that when flagella is restored to SG, its pathogenicity is significantly reduced, and on the contrary, if flagella was removed from SE, it developed more easily into a systemic infection. (de Freitas Neto et al., 2013; Iqbal et al., 2005). The non-motile SE by removing *fliC* and *flhD*, respectively, did not proliferate in organs when inoculated with SC, unlike wild type. However, when the strain without *flhD* was inoculated orally, invasiveness was rather enhanced (Parker and Guard-Petter, 2001). Therefore, in addition to immune evasion due to the absence of flagellin, the absence of *flhD* seems to increase the pathogenicity of chicken adapted *Salmonella*. The presence of flagella is also related to proliferation in eggs. Bacteria should reach yolk as there is no iron available in albumen, which is impossible to non-motile *Salmonella* (Cogan et al., 2004).

### B. *Salmonella* pathogenicity island

There are two pathogenicity islands, *Salmonella* pathogenicity island 1 (SPI-1) and *Salmonella* pathogenicity island 2 (SPI-2) which encodes the respective type III

secretion system (Hueck, 1998). Pathogenicity islands are critical for survival of *Salmonella* and retained by both species, *S.bongori* and *S.enterica* (Lostroh and Lee, 2001; Ochman and Groisman, 1996). SPI-1 mainly confer penetrating ability, while SPI-2, 3, and 4 act in bacterial survival within the host (Marcus et al., 2000). SPI encodes type III secretion system (TTSS) which can directly transfer bacterial virulence proteins in to host cell cytoplasm (Hueck, 1998). Transferred bacterial proteins orchestrate the host immunity to be advantageous for the bacteria. Although SPI-1 affected invasion into avian cells in vitro, SG requires a TTSS by SPI-2 to develop systemic infection. SPI-1 mutant hardly affect the virulence of the SG, SPI-2 mutant showed decreased viability in macrophage and faster clearance by host immunity (Jones et al., 2001). Furthermore, the type VI secretion system (T6SS), encoded by SPI-19, has emerged as a new SG and SE mechanism of colonization in organs (Blondel et al., 2010). Also, although SPI-19 and T6SS do not involve cytotoxicity of infected cells, they are required for survival within the macrophages (Blondel et al., 2013).

## C. Virulence plasmid

*Salmonella* has a large plasmid that plays a large role in pathogenicity, and even though the different serotypes have the various size of the virulence plasmid from 50 kb to 90 kb, it has a fairly conserved virulence sequence which is known as *spv* (*Salmonella* plasmid virulence) (Fig L.3) (Baird et al., 1985; Guiney and Fierer, 2011; Rotger and Casadesus, 1999). The *spv* locus consists of five genes of *spvRABCD*. SpvR is presumed to be a regulatory protein, and SpvB has a similar sequence to the Vibrio cholerae toxin

(Rotger and Casadesus, 1999). The *spvB* gene plays a large part in the virulence of the *spv* locus by encoding an enzyme that destabilizes the cytoskeleton of eukaryotic cells (Tezcan-Merdol et al., 2001). The structure of SpvB is different between N-terminal and C-terminal, and a polyproline linker connects each structure. If the length of the linker extended, the virulence of the toxin increased (Kim et al., 2019; Pust et al., 2007). The contribution of large plasmid to virulence was also substantial in SG, resulting in no mortality in plasmid cured SG (Barrow et al., 1987). In addition to the *spv* locus, the SG plasmid ORFs, homologous with faeH and faeI of enterotoxigenic *E.coli*, confer the ability of survival, proliferation in the reticuloendothelial system, and tissue invasion, which were not associated in *S.typhimurium* or *S.dublin* (Rychlik et al., 1998*)*. Complement-mediated killing resistance by virulence plasmid is controversial, but *traT*, *rck*, and *rsk* are presumed to be involved. SG can proliferate in serum regardless of the plasmid, suggesting that the plasmid's effect on the serum resistance in SG seems low (Barrow et al., 1987).

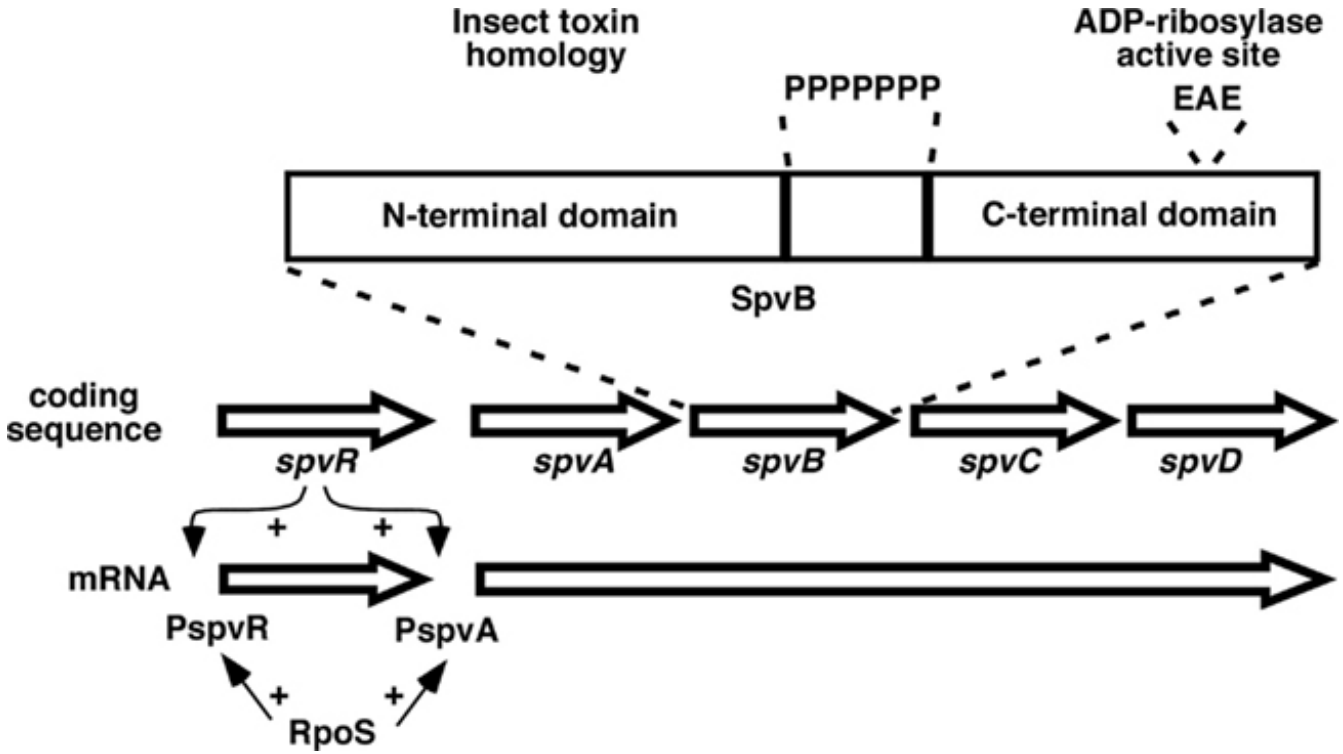


**Figure L.3.** Map of the *spv* region found on virulence plasmids in subspecies 1 strains, not drawn to scale. Adapted from Donald G. Guiney and Joshua Fierer (2011)

## D. Endotoxin

Lipopolysaccharides (LPS) consist of a lipid and a polysaccharide chain (O antigen) composed of O-antigen. It presents in the outer membrane of gram-negative bacteria and is divided into three parts: lipid A, core oligosaccharides, and O antigen (Fig L.4). O antigen is very diverse by strain, and the presence or absence of full-length O antigen determines smooth or rough strain (Raetz and Whitfield, 2002). The characteristics of each strain are very different, and in general, as rough strains with short LPS are susceptible to complement-mediated killing, the smooth strains are more pathogenic (Pluschke et al., 1983).

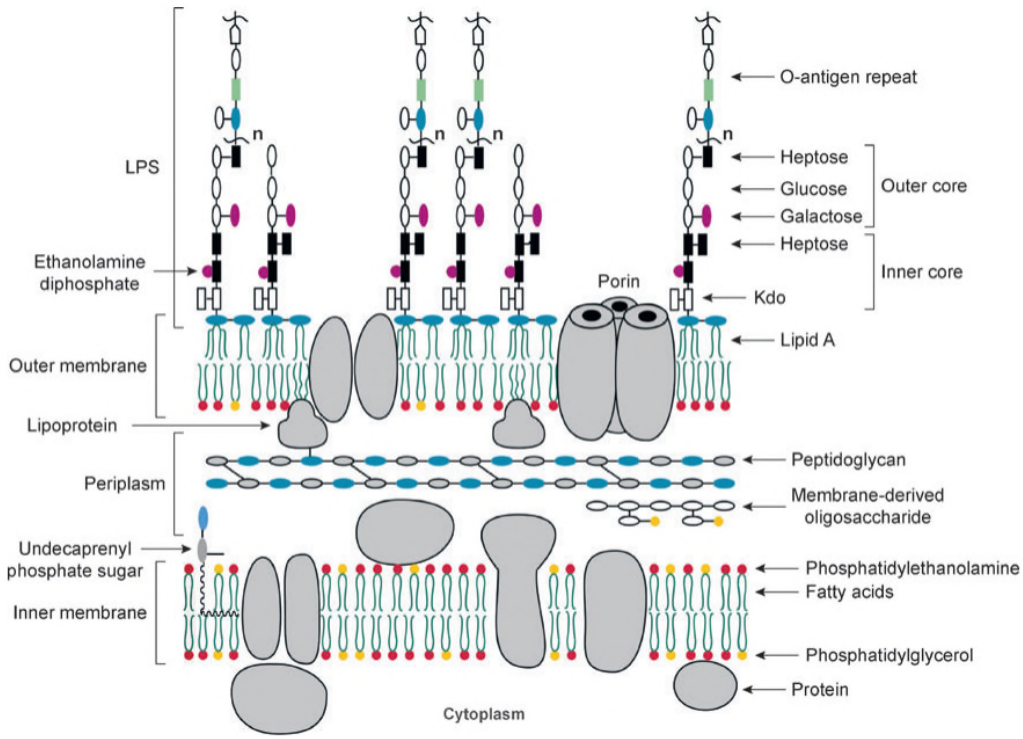


**Figure L.4.** Schematic structure of the *E.coli* K-12 cell envelope. Adapted from Raetz, Christian RH (2007)

Lipid A is biosynthesized by the Raetz pathway and is very important in toxicity to gram-negative bacteria (Raetz et al., 2007) (Fig L.5). Nine enzymes mainly act in the pathway, and the first step is initiated by LpxA catalyzing fatty acid acylation in UDP-GlcNAc (Anderson et al., 1985). LpxC deacetylate the UDP-3-*O*-(actl)-GlcNAc and second acyl chain (*R*-3-hydroxymyristate chian) is added by LpxD (KELLY et al., 1993; Young et al., 1995). The structure of LpxD is similar to LpxA, which has a similar role of adding the acyl chains, but LpxC showed no similarity to other enzymes (Buetow et al., 2007). The lipid X is made by the LpxH which cleaves pyrophosphate linkage and combined with the UDP-diacyl-GlcN by LpxB (Babinski et al., 2002; Ray et al., 1984). Lipid $IV_A$ was generated by phosphorylating enzyme LpxK, and KdtA enzyme which adds two Kdo residues (Anderson and Raetz, 1987; Belunis et al., 1995). Finally, lauroyl and myristoyl residues are added by LpxL and LpxM (Clementz et al., 1997). When *lpxL* and *lpxM* are deleted, the pathogenicity of the bacteria is significantly reduced (Feodorova et al., 2007; Fisseha et al., 2005). In particular, the *lpxL* mutant became sensitive to temperature, unable to proliferate above 32.5℃, and susceptible to phagocyte-mediated killing (Karow and Georgopoulos, 1992; Mills et al., 2017). Although *lpxM* is not associated with phagocyte resistance, the pathogenicity of its mutant is substantially weakened, anticipated as a vaccine candidate in many studies (Clements et al., 2007). LPS is modified according to changes in growth conditions, one of which is *pagP* and *pagL*, which are regulated by *phoP/phoQ* (Golubeva and Slauch, 2006). PagP adds palmitate, and PagL removes the acyl chain, and this adjustment occurs according to conditions such as pH, $Fe^{3+}$, and $Mg^{2+}$ (Bader et al., 2005).

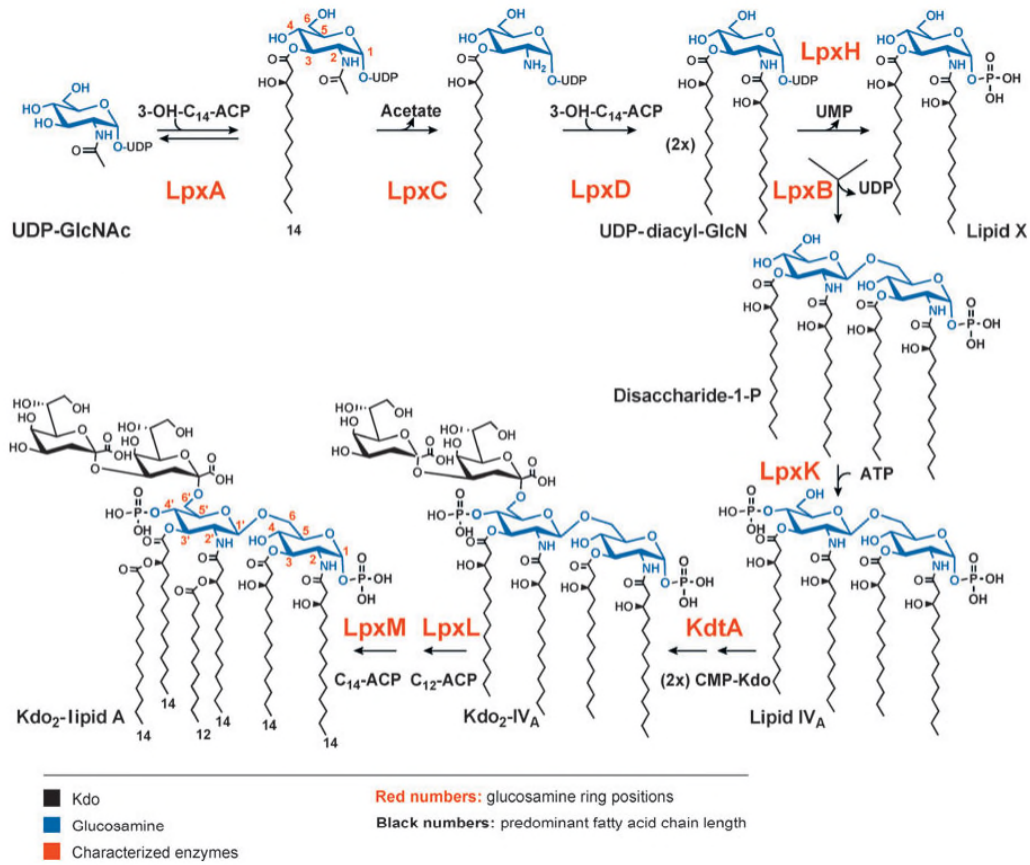


**Figure L.5.** Constitutive pathway for biosynthesis of the Kdo2-lipid A portion of LPS in *E. coli* K-12. Each enzyme of the constitutive Kdo2-lipid A pathway is encoded by a single structural gene. Adapted from Raetz, Christian RH (2007)

## 2-3. Genetic analysis

*Salmonella* Gallinarum (SG) is predicted to share recent common ancestries with serovars Choleraesuis, Paratyphi C, Enteritidis and Typhimurium. Among serovars, SG is assumed to have evolved from *Salmonella enterica* serovar Enteritidis based on shared genes and pseudogenes (Fig L.6). Intracellular antigens tend to harbor abundant pseudogenes, and narrow host specificity also contributes to the increase of pseudogenes. Therefore, SG has more pseudogenes compared to other broad hosts *Salmonella* ssp. and its extensive degradation is speculated to remove unnecessary functional traits during host restriction (Thomson et al., 2008). The genetic structure of SG and SP were very similar. Considering the electrophoretic typing result and mutations of flagella coding genes, SG and SP are monophyletic and might share a common ancestor of non-motile *Salmonella* (Li et al., 1993). Pseudogene analysis between SG and SP demonstrated that one fourth of pseudogenes were the same while they were intact in SE, suggesting that the common ancestor of non-motile *Salmonella* in bird may have already been restricted to poultry (Feng et al., 2013). As the most SG are genetically homogenous, it is difficult to discriminate variations among SG strains by traditional methods like multilocus sequence typing, multiple locus variable number tandem-repeat analysis, and pulsed-field gel electrophoresis (Xie et al., 2017). Therefore, methods such as pseudogene profiling or CRISPR sequence subtyping can be used for epidemiologic studies between SG strains. As a result of analyzing SG isolated in Korea, they have evolved by accumulating more pseudogenes and enhancing their pathogenicity (Kim et al., 2020a; Kim et al., 2019).

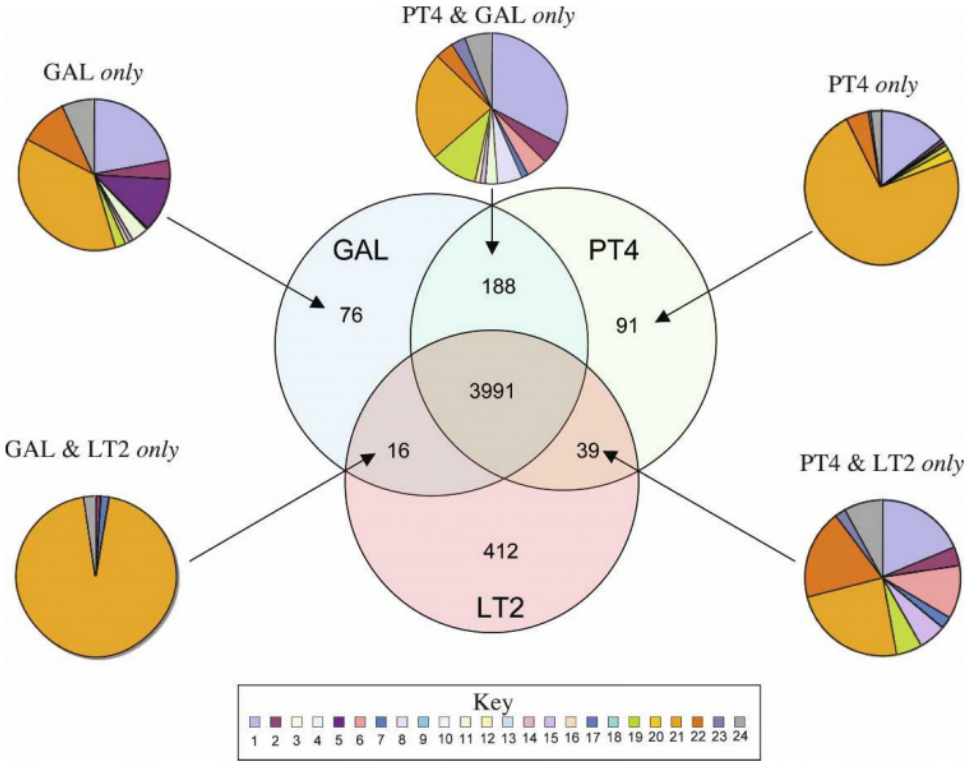


**Figure L.6.** Distribution of orthologous CDS in *S*. Enteritidis PT4, *S*. Typhimurium LT2, and *S*. Gallinarum 287/91. Adapted from Thomson, Nicholas R (2008)

# 3. Disease control

Brown laying hens are more susceptible to SG than white laying hens. This is due to the different survival of SG in chicken macrophages. However, brown eggs dominate about 60% worldwide, which is one of the reasons that makes the eradication of SG difficult. In addition, poultry red mite, *Dermanyssus gallinae*, is a major ectoparasite of poultry and acts as a vector of SG transmission (Cocciolo et al., 2020).

Fowl typhoid free countries often suffer from food borne disease caused by SE. In Korea, excessive diagnostic tests are being performed to monitor SE. SG competitively excludes SE, resulting in a low isolation rate of SE in countries with fowl typhoid (Rabsch et al., 2000). Even if SG is completely eradicated in Korea, preparation for SE control is necessary.

## 3-1. Diagnosis

Fowl typhoid can be diagnosed by bacterial isolation, immune response including serum agglutination test, whole blood agglutination test, rapid slide agglutination test, and enzyme-linked immunosorbent assay (ELISA), and other alternative methods like polymerase chain reaction (PCR). Serological methods mainly have been used to diagnose SG. The plate agglutination test using SG antigen can rapidly diagnose SG, but it has a disadvantage occurring non-specific reactions. The ELISA coated with *Salmonella* D group O antigen can measure the titer of the antibody, but it cannot differentially diagnose infection with SE, SP, and SG. Particularly, as the SG and SP are closely related genetically,

techniques which could differentiate between two biovars has been developed via polymerase chain reaction. In the past, unique restriction enzyme sites in the *rfbS* gene were used to differentiate SG and SP by restriction fragment length polymorphism (Park et al., 2001). Recently, the multiplex quantitative PCR provided advanced and more rapid differentiation, and loop-mediated isothermal amplification targeting the *sefA* gene was also developed for rapid diagnosis in the field (Gong et al., 2016; Rubio et al., 2017). *Salmonella* D group antibody could be applied to distinguish the isolated SG into rough vaccine strain and field strain.

## 3-2. Vaccine

For *Salmonella* Typhi, a live attenuated vaccine, Ty21a, and Vi capsular polysaccharide vaccine (subunit vaccine) are widely used (Mastroeni et al., 1993). Ty21a is a live vaccine and cannot be applied to children, but the subunit vaccine can safely boost immunity in children between the ages of 2 and 5 (Lin et al., 2001).

In Poultry, live vaccines targeting *Salmonella* Enteritidis or Typhimurium were also successful in reducing the re-isolation of the challenge strain (Cooper et al., 1994; Hassan and Curtiss, 1994). In case of the fowl typhoid, the most widely used vaccine for fowl typhoid is SG9R, which is also a live attenuated vaccine. This vaccine is highly pathogenic and can cause death when vaccinated at 1 day of age, and lesions appear when immunity is compromised even at a later age (Kwon and Cho, 2011). To replace SG9R several live attenuated vaccines have been developed. For example, modified SG9R, which

secretes heat-labile enterotoxin B subunit protein as an adjuvant, or virulence gene eliminated mutants (Cheng et al., 2016; Nandre and Lee, 2014). As live attenuated vaccine sufficiently stimulates innate immunity, it is advantageous to obtain a protective efficacy. Still, it always poses a risk that it may be transferred to humans due to latent infection and contamination of the environment. Researchers are trying to stimulate cellular immunity with inactivated vaccines in pathogens where innate immunity is important, like *Salmonella*. A recent study developed the *Salmonella* Gallinarum ghost vaccine, which lost cytoplasmic contents by transformed plasmid containing the bacteriophage PhiX174 lysis gene *E* (Jawale et al., 2014). The vaccine is inactivated when *E*-mediated lysis is induced at 42°C, which is similar to the body temperature of chickens. Therefore, SG is inactivated within 24 hours of vaccination, stimulating both innate immunity and humoral immunity, and can safely disappear. This method was also applied to *Salmonella* Pullorum to induce both types of immunity (Guo et al., 2016).

# Chapter I

# Molecular evolution of *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum in the field

# Abstract

*Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum (SG) causes fowl typhoid (FT) and substantial economic loss in Korea due to egg drop syndrome and mortality. Despite the extensive use of vaccines, FT still occurs in the field. Therefore, the emergence of more pathogenic SG or the recovered pathogenicity of a vaccine strain has been suspected. SpvB, an ADP-ribosyl transferase, is a major pathogenesis determinant, and the length of the polyproline linker (PPL) of SpvB affects pathogenic potency. SG strains accumulate pseudogenes in their genomes during host adaptation, and pseudogene profiling may provide evolutionary information. In this study, it was found that the PPL length of Korean SG isolates varied from 11 to 21 prolines and was longer than that of a live vaccine strain, SG 9R (9 prolines). According to growth competition in chickens, the growth of an SG isolate with a PPL length of 17 prolines exceeded that of an SG isolate with a PPL length of 15 prolines. I investigated the pseudogenes of the field isolates, SG 9R and reference strains in GenBank by resequencing and comparative genomics. The pseudogene profiles of the field isolates were notably different from those of the foreign SG strains, and they were subdivided into 7 pseudogene subgroups. Collectively, the field isolates had gradually evolved by changing PPL length and acquiring additional pseudogenes. Thus, the characterization of PPL length and pseudogene profiling may be useful to understand the molecular evolution of SG and the epidemiology of FT.



***Keywords*:** *Salmonella* serovar Gallinarum; *spvB*, polyproline linker; pseudogene; molecular epidemiology.

# 1.1. Introduction

*Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum (SG) causes fowl typhoid in poultry and is analogous to *Salmonella* serovar Typhi in humans (Thomson et al., 2008). Since the first outbreak of fowl typhoid in 1992, it has become enzootic in Korea (Lee et al., 2003). Although live attenuated vaccines have been extensively used in the field, virulent isolates continue to be isolated. For this reason, the appearance of novel and more virulent SG or the reversion mutation of a vaccine strain has been suspected (Kwon and Cho, 2011).

The large virulence plasmid of SG increases the $LD_{50}$ of SG by approximately $10^6$-fold, and the *Salmonella* plasmid virulence genes *spvB* and *spvC* can replace the virulence of the entire large virulence plasmid of pathogenic *Salmonella* serovars (Matsui et al., 2001). The *spvB* gene encodes an actin-ADP-ribosylating toxin, which is one of the most important virulence factors enhancing the intracellular proliferation of pathogenic *Salmonella* serovars (Otto et al., 2000). The length of the polyproline linker (PPL) connecting the N- and C-terminal domains of SpvB determines the pathogenic activity of SpvB and is variable among *Salmonella* serovars (Kwon and Cho, 2011; Pust et al., 2007). In particular, SG has a longer PPL than other serovars; moreover, SG isolated in Korea generally showed a much longer PPL length than the other SG strains deposited in GenBank (Kwon and Cho, 2011). Considering the wide range of PPL lengths that have a crucial impact on pathogenicity, PPL length could be an indicator of the evolution of SG pathogenicity in Korea.

*Salmonella* serovar Enteritidis (SE), *Salmonella* serovar Gallinarum biovar Pullorum (SP) and SG are classified into the same O-antigen group (O-1, 9, 12) and may share a common ancestor (Langridge et al., 2015). In contrast to SE, SG and SP have accumulated many more pseudogenes that have lost their original function by nonsense or frame shift mutations during host adaptation. Pseudogenes may occur randomly, but pseudogenes with beneficial or neutral effects on phenotypes may have accumulated during evolution (Kuo and Ochman, 2010). The accumulated pseudogenes may be related to the host specificity and virulence of SG, and pseudogene profiling may be useful to understand the evolutionary status of SG and the molecular epidemiology of FT.

In this study, the PPL lengths of Korean field isolates, virulent reference strains, a vaccine strain (SG 9R) of SG and other *Salmonella* serovars were investigated. Additionally, the pseudogenes were identified by resequencing the genomes of five field isolates and SG 9R and comparative genomics with reference strains in the GenBank database. The identified pseudogenes were verified in an additional 23 randomly selected SG field isolates, and they were classified into 7 subgroups based on pseudogene profiles. By combining the results of PPL length and pseudogene studies, this study aimed to unravel the molecular evolution of SG in the field and the molecular epidemiology of FT in Korea.

# 1.2. Materials and Methods

## Experimental birds

Ten commercial Lohmann brown layer-hens that were not administered SG vaccines were purchased from a farm (Duki farm, Korea), and the anti-D group antibody was confirmed using an ELISA kit (IDEXX Co., Westbrook, USA). Animal experimentation was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (Permission Number SNU-181122-3).

## Bacterial strains

Of the 75 *Salmonella* isolates in Korea, 44 were from BioPOA Inc. (Korea), 8 were from Green Cross Inc. (Korea), 2 were from Korea Centers for Disease Control and Prevention (KCDC), 1 was from Animal and Plant Quarantine Agency (QIA) and 20 were from the Laboratory of Avian Diseases, College of Veterinary Medicine, Seoul National University (SNU). Five reference strains of SG [SG287/91 (NC011274.1), SG9184 (CP019035.1), SG_ST572 (NZ_LHST00000000.1), SG_BR_RS12 (NZ_LNON00000000.1), and SG_NCTC10532 (NZ_MWLV00000000.1)] and SE [SE92-0392 (CP018657.1), SE_EC20120916 (CP007332.2), SE_CFSAN063790 (NZ_QVVR00000000.1), SE_NCTR380 (NZ_NQWN 00000000.1), and SE_BCW_4356 (NZ_MYTC00000000.1)], two reference strains of SP [SP_RKS5078 (CP003047.1) and SP_S06004 (CP006575.1)] and SD [SD_ST_02021853 (CP001144.1) and SD_pOU1115 (DQ115388.2)], and one

reference strain of ST [ST_DT104 (CQO10863)] were selected in the GenBank database for pseudogene profiling (Table 1.1). Additionally, various non-D group serovars, including Agona (1), Blockley (1), Brandenburg (1), Derby (1), Hadar (1), Infantis (1), Montevideo (1), Newport (1), Senftenberg (1), Schwarzengrund (1), Thompson (1), Typhimurium (2), and Virchow (1), which had been reported in a previous work, were tested for the presence of *spvB* (Seong et al., 2012).

## PCR, sequencing and sequence analysis

The *Salmonella* Gallinarum strains were grown overnight in LB broth at 37°C, and genomic DNA was extracted with a G-spin Genomic DNA Extraction Kit for bacteria (iNtRON Biotechnology Co., Seongnam-si, Korea). The new primer sets to amplify the PPLs and pseudogenes are listed in Table 1.2. PCR was conducted by using the following conditions: 3 μl of 10 × buffer, 3 μl of dNTPs (5 mM), 0.5 μl of each forward and reverse primer (10 pmol/μl), Taq DNA polymerase (5 units/μl; MGMed Co., Seoul, Korea) and 1 μl of template DNA (50 ng/μl). The final volume of the PCR was 30 μl. The PCR cycles were 95°C for 5 min; 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 2 min; and 72°C for 5 min. PCR amplicon purification was carried out using a PCR/Gel purification kit (MGMed Co.), and sequencing was performed with ABI3711 automatic sequencer (Cosmogenetech Co., Seoul, Korea). Nucleotide sequences were translated and compared using the BioEdit program (ver. 7.2.5).

## Resequencing and whole-genome analysis

Each sample was prepared according to Illumina protocols. The HiSeq™ 2000 platform (Illumina, San Diego, USA) was employed to sequence the field isolates and vaccine strain of SG. Filtered data were mapped using BWA (v0.7.12) to *Salmonella enterica* serovar Gallinarum str. 287/91 (GenBank Accession Number NC_011274.1) from the NCBI RefSeq database (Li and Durbin, 2010). Captured variants were annotated with SnpEff (v.3.2) to predict annotation type, putative impact and amino acid change information (Cingolani et al., 2012). To select the variants that had a genetically significant effect, the genes whose putative impact was high and analyzed them were sorted.

## *In silico* pseudogene profiling and phylogenetic network analysis

The genome-wide pseudogene profiles of the reference strains of SG, SP, and SE in the GenBank database were compared by MAUVE (Darling et al., 2010). Using MAUVE, the genes corresponding to the selected pseudogenes were confirmed to be pseudogenes or intact genes. Some non-annotated genome sequences of reference strains were annotated with the DFAST program, and then the pseudogenes were profiled (Tanizawa et al., 2016). When some regions of the genomes were not correctly aligned by MAUVE, the region sequences were directly compared with the BioEdit program. The collected pseudogene information was analyzed by median joining analysis with Network 5.0.0.3 (Huson and Bryant, 2006).

## Growth competition test in chickens

Two field SG isolates representing 15-proline (SNU16035) and 17-proline (BP-64) PPLs were selected for the growth competition test in chickens. Each strain was cultured in LB broth (Duchefa Biochemie, Groot Bijgaarden, Belgium) at 250 rpm at 37°C overnight. The overnight culture of each SG isolate was diluted to an optical density at 600 nm ($OD_{600}$) of 0.2. The diluted SG isolate (100 µl/chicken) was mixed together (50:50) and challenged per os to ten brown laying hens. Dead birds were immediately autopsied and surviving birds were fasted at 15 dpi for 3 days with drinking water supplied as previously described (Cho et al., 2015). The liver was taken aseptically and homogenized with 10 ml of 2% Triton X-100 in PBS with an autoclaved mortar and pestle. The homogenized samples (adjusted to 15 ml with 2% Triton X-100) were transferred to 50 ml conical tubes and centrifuged at 1,000 rpm for 10 min. The supernatants (5 ml) were transferred to 15 ml conical tubes and centrifuged at 3,000 rpm for 20 min. The pellets were resuspended in 2 ml of PBS and spread on MacConkey agar (BD, New Jersey, USA) after 10-fold dilution. Bile juice was collected aseptically with a 1 ml syringe and spread on MacConkey agar (BD) for bacterial culture. Single colonies were used for PCR to amplify PPLs, and the amplicons were sequenced as above to identify PPL length.

## 1.3. Results

### Distribution and characterization of *spvB* in *S.* enterica subsp. enterica (*S.* enterica) serovars

The presence of *spvB* in 16 *S. enterica* serotypes other than SG was tested by PCR, and only SD, SE, SP, and ST showed a targeted *spvB*-specific amplicon. The PPL length was determined by sequencing the amplicons, and the PPL lengths in reference strains in GenBank were identified from the *spvB* genes in the database. All serotypes except some SD strains (11 prolines) possessed the shortest PPL length, 7 prolines (Table 1.1).

### Variation in the PPL length of SpvB among field isolates of SG

The PPL length of 61 field isolates of SG and SG 9R were determined by PCR and sequencing (Table 1.1). The PPL length of 287/91 was determined from the *spvB* gene in the GenBank database. The PPL length varied from 9 to 21 prolines, and the PPLs with 15 prolines (76.2%, 48/63) were the most prevalent, followed by 13 (11.1%, 7/63), 17 (4.8%, 3/63), 11 and 9 (6.3%, 4/63), and 21 (1.6%, 1/63).

SG 9R and a field-isolated vaccine strain, GC-SG132, possessed 9-proline PPLs. Generally, SG showed longer PPL lengths than other serovars, and the PPL lengths of Korean field isolates were especially variable and elongated (11, 13, 15, 17, and 21). Interestingly, the PPL length varied by 2 prolines.

## Identification of specific pseudogenes in Korean SG isolates

Of 61 SG field isolates, five isolates with different PPL lengths (BP-SG002, SNU16035, BP-SG177, BP-SG221, and BP-SG52) and SG 9R were selected for resequencing to identify pseudogenes. Fifty-five pseudogenes (data not shown) different from a reference strain, 287/91, were found by resequencing the 5 field isolates and SG 9R. Among them, fifty genes shared by all isolates and strains of SG, SP, and SE were selected for pseudogene analysis. The gene names, functions, and distributions in the SG isolates and strains are summarized in Table 1.3. Within the 50 genes, 13 pseudogenes were common, but 15, 9 and 7 pseudogenes were only found in Korean field isolates, 287/91, and SG 9R, respectively. However, 6 pseudogenes (*gspE*, *rfbP*, *SG_RS06090*, *terC, ybdH* and *ygiD*) were variably found only in Korean field isolates (Table 1.3). Therefore, the Korean isolates 287/91 and SG 9R had 28-31, 22 and 24 pseudogenes, respectively (Table 1.3 and 1.4), and the strains could be differentiated by their pseudogene profiles.

## Pseudogene-based phylogenetic analysis of SG, SP and SE

To understand the evolutionary relationships of SG, SP and SE, network analysis was carried out with pseudogenes. In addition to the 50 pseudogenes of SG, an additional 76 pseudogenes shared by SP strains were used for median joining network analysis (Fig. 1.1, Table 1.5) (Feng et al., 2013). As a result, SE was more closely related to SG than SP, and the reference strains of SG (287/91, SG9184, ST572, and BR_RS12) and SG 9R were

more closely related to SE than the Korean SG isolates. The Korean isolates formed a unique cluster different from 287/91 or SG 9R, and all tested field isolates were linked to BP-SG002, the second earliest isolate from Korea that was isolated in 1993 (Fig. 1.1).

## Subgrouping of Korean SG isolates by variable pseudogenes

To verify the pseudogene profiles of the Korean field isolates, primers for 37 pseudogenes, excluding the 13 common SG pseudogenes in Table 1.3 were synthesized and PCR and sequencing were performed with an additional twenty-three SG field isolates (Table 1.4). The additional SG isolates except GC-SG132 shared the same pseudogene profile with the five original isolates except for the six variable genes. GC-SG132 showed the same pseudogene profile as SG 9R, as expected. Based on the six variable pseudogenes, the Korean field isolates were subdivided into 7 subgroups (Fig. 1.2, Table 1.4). Subgroup 1 (S1) had no pseudogenes, but it was subdivided into S1-1, S1-2, and S1-3 based on different PPL lengths, with 15, 17 and 13 prolines, respectively. S2 and S3 had 1 (*gspE*) and 2 (*gspE* and *terC*) pseudogenes, respectively, with the same PPL length (15 prolines). S4 and S5 had 1 (*SG_RS06090*) and 2 (*SG_RS06090* and *rfbP*) pseudogenes, and S4 was subdivided into S4-1 and S4-2 based on different PPL lengths, with 17 and 21 prolines, respectively. S6 had 1 pseudogene (*ygiD*) and a 13-proline PPL, but S7 had two additional pseudogenes (*SG_RS06090*, *rfbP*, and *ygiD*) and an 11-proline PPL. The frequency of S1-1 (53.6%, 15/28) was the highest, followed by S6 (17.9%, 5/28).

## Growth competition of SG isolates with different PPL lengths in chickens

After inoculation with the mixed SG isolates (SNU16035 and BP-64), 2, 3, 1, and 1 chickens died at 7, 8, 9, and 10 dpi, respectively, and all dead chickens showed necrotic foci in the liver. At 7 and 8 dpi, the frequency of 15-proline PPLs was slightly higher than that of 17-proline PPLs in the liver, but 17-proline PPLs became dominant with no 15-proline PPLs identified at 9 and 10 dpi in the liver and bile juice. Of 3 surviving and fasting chickens, 1 died, and only 17-proline PPLs were identified in the liver and bile juice (Table 1.6).

# 1.4. Discussion

The *spv* locus is present in the large virulence plasmids, but it is also hybridized in the chromosomes of *Salmonella enterica*. An *spv* locus was not identified in the less pathogenic *Salmonella* serovars as previously reported, but horizontal transfer of the large virulence plasmid may justify monitoring the *spv* genes (Boyd and Hartl, 1998; Geisler and Chmielewski, 2007).

According to the pseudogene profiles and the lengths of PPLs, the molecular epidemiology of FT in Korea can be speculated. The early isolates from 1992-1994, BP1, BP-SG002, and BP5, were grouped into S1-1, and the pseudogene profiles and PPL lengths were conserved among 2016 isolates, SNU16003 and SNU16009. S1-1 may have evolved into S1-2 and S1-3 by changing a 15-proline PPL into a 17-proline PPL (GC-128) or a 13-proline PPL (16-p16) without changing the pseudogene profile. In contrast, S1-1 may have evolved into S2 (GC634) and then S3 (16035) by acquiring cumulative pseudogenes, *gspE* and *terC*, respectively, without changing PPL length. Subgroup S4-1 may have evolved from S1-1 by acquiring a single pseudogene (*SG_RS06090*) and a two-proline-elongated PPL (17-proline PPL) and then evolved further into S4-2 by elongating the PPL length to 21 prolines. Therefore, the predominant subgroup S1-1 may have evolved into S1-2, S1-3, S2, S3, S4-1, and S4-2 by acquiring pseudogenes and/or changing PPL length during vertical and horizontal transmission among chickens. The S6 subgroup possesses the *ygiD* pseudogene and a 13-proline PPL and is clearly different from S1-1. The S7 subgroup possesses 3 pseudogenes and an 11-proline PPL. The lower step requirement for a change

in PPL length from a 13-proline to an 11-proline PPL than for a 15-proline to an 11-proline PPL may support S6 to S7 evolution with the acquisition of two additional pseudogenes (*SG_RS06090* and *ybdH*) rather than S4-1 to S7 evolution. Considering the different pseudogene profiles and PPL lengths of BP-SG1 (1992) and BP-SG002 (1993) from those of BP-SG8 (1994), the early outbreaks of FT in Korea may have been caused by at least two different but closely related subgroups, which evolved into several subgroups in the field.

The PPL length varies among *Salmonella* serovars Dublin, Enteritidis, Gallinarum, Pullorum, and Typhimurium. Serovars Dublin and Gallinarum extended their PPL lengths from 7 to 11 and from 9 to 21 prolines, respectively. The dramatically elongated PPL length of the Korean field isolates in comparison with the PPL length of the SG 9R strain that was established in 1956 may reflect the selection of more competent bacterial progeny during additional host infection and adaptation. The ADP-ribosyl transferase activity is in the C-terminal domain of SpvB and plays a role in F-actin depolymerization, probably resulting in the suppression of the effector mechanisms of innate and acquired immunity and autophagy (Chu et al., 2016; Jo et al., 2013). The functions of the N-terminal domain and the PPL may be related to type III secretion system (TTSS)-independent secretion and translocation of SpvB into the cytosol of infected host cells, respectively (Barth and Aktories, 2011; Geisler and Chmielewski, 2007; Gotoh et al., 2003; Pust et al., 2007). Therefore, the PPL length may affect the efficiency of SpvB translocation into the cytosol, and a longer PPL length may confer enhanced virulence activity to SpvB. Indeed, in this study, our observation of the predominant presence of SG isolates with longer PPLs (17-

proline vs. 15-proline PPLs) in the late and persistent stages of infection in chickens may also support the *in vitro* results of previous reports. Therefore, the presence of a shortened PPL length from 15 to 13 prolines or 13 to 11 prolines was unexpected. Considering the continued presence of chicken red mites in Korean layer farms and the long-term isolation of SG from red mites further study to demonstrate a more preferable selection of SG isolates with shorter PPLs in red mites may be interesting (Sigognault Flochlay et al., 2017).

Pseudogene analyses have been used to understand the evolutionary difference between invasive (systemic) and enteropathogenic *Salmonella* serovars and define important common genes involved in host adaptation (Matthews et al., 2015; Thomson et al., 2008). As previous reports, multiple virulence, membrane/surface structure, and central/intermediary metabolism genes were inactivated in SG, SP and SE (Feng et al., 2013; Langridge et al., 2015; Thomson et al., 2008). The pseudogene-based network analysis in this study revealed an evolutionary relationship of SG, SP and SE similar to previous reports but also revealed the ongoing evolution of SG isolates and strains (Thomson et al., 2008). In comparison with SG 9R and 287/91, the Korean SG isolates possessed more pseudogenes, and they evolved into different subgroups by acquiring additional pseudogenes and changing PPL length. Among the Korean SG isolate-specific pseudogenes, some virulence-related genes, such as *araA*, *lpfC*, *murP*, *sfbA* and *sirB1,* were found. (Baumler et al., 1997; López-Garrido et al., 2015; Pattery et al., 1999; Rakeman et al., 1999). Given the frequent infection and passage of the Korean SG isolates through laying hens, more pseudogenes and the inactivation of virulence-related genes

can be expected. However, most laying hens that produce table eggs are brown layers in Korea, which are more susceptible to SG than white layers (Smith, 1956; Wigley et al., 2002). Therefore, the pseudogene profile of the Korean SG isolates may be the result of interactions with less competent macrophage functions in brown layers and may be different from SG in other countries.

Although Korean SG-specific pseudogenes (15), which are common in all Korean isolates, may have been acquired before transmission into Korea, the four variable pseudogenes except *ygiD* may have been acquired in Korea. BP-SG52 possesses the *rfbP* pseudogene, which may be unable to synthesize an O-antigen ligase substrate and shows a rough phenotype (data not shown). Due to weak resistance to complement in serum, BP-SG52 may be able to survive for a short period of time with reduced virulence (Rowley, 1968). Cell-to-cell transfer of *Salmonella* via *Salmonella*-containing vacuoles may help the survival of the aberrant BP-SG52 and SG 9R strains (Steele-Mortimer, 2008; Szeto et al., 2009). However, further study on the effect of the extended PPL (17 prolines) of BP-SG52 on pathogenicity may be interesting. To date, the origin and route of transmission of the first SG strain were unclear, but the same nonsense mutation at codon 495 of the *fliC* gene was also shared by 3 strains isolated in the Middle East out of 56 compared SG strains from around the world (Li et al., 1993). Therefore, pseudogene profiling, including Korean SG isolate-specific pseudogenes, may be useful to unravel future questions.

Thus, two different Korean SG field isolates with similar genetic backgrounds caused early and long-lasting outbreaks of FT in Korea, and they evolved gradually by changing PPL length and acquiring pseudogenes. The conversion of SG 9R to a

pathogenic strain was not observed, but the appearance of more pathogenic SG can be demonstrated by testing the pathogenicity of PPL-extended field isolates.

**Table 1.1 *Salmonella* strains used in this study and the length of their polyproline linker**

| Strain | Length of PPL | Sero type | Year of isolation | Strain | Length of PPL | Sero type | Year of isolation |
|---|---|---|---|---|---|---|---|
| SG 9R | 9 | SG[a] | 1956 | BP-SG195 | 15 | SG | - |
| GC-132 | 9 | SG | - | BP-SG197 | 15 | SG | - |
| BP-SG221 | 11 | SG | - | BP-SG198 | 15 | SG | - |
| SG287/91 | 11 | SG | | BP-SG199 | 15 | SG | - |
| BP-SG8 | 13 | SG | 1994 | BP-SG201 | 15 | SG | - |
| BP-SG176 | 13 | SG | - | BP-SG207 | 15 | SG | - |
| BP-SG177 | 13 | SG | - | BP-SG208 | 15 | SG | - |
| BP-SG202 | 13 | SG | - | BP-SG209 | 15 | SG | - |
| BP-SG224 | 13 | SG | - | BP-SG212 | 15 | SG | - |
| BP-SG226 | 13 | SG | - | BP-SG222 | 15 | SG | - |
| GC-16-p16 | 13 | SG | 2016 | GC-002 | 15 | SG | - |
| BP-SG1 | 15 | SG | 1992 | GC-149 | 15 | SG | - |
| BP-SG002 | 15 | SG | 1993 | GC-634 | 15 | SG | - |
| BP-SG5 | 15 | SG | 1994 | BP-SG52 | 17 | SG | 2000 |
| BP-SG47 | 15 | SG | 2000 | BP-SG64 | 17 | SG | 2001 |
| BP-SG49 | 15 | SG | 2000 | GC-128 | 17 | SG | 2015 |
| BP-SG50 | 15 | SG | 2000 | SNU16037 | 21 | SG | 2016 |
| BP-SG51 | 15 | SG | 2000 | SG9184 | na | SG | - |
| BP-SG56 | 15 | SG | 2001 | SG_ST572 | na | SG | 2009 |
| BP-SG59 | 15 | SG | 2001 | SG_BR_RS12 | na | SG | 2014 |
| BP-SG60 | 15 | SG | 2001 | SG_NCTC10532 | na | SG | - |
| BP-SG63 | 15 | SG | 2001 | KCDC_21 | 7 | SD[b] | - |
| BP-SG67 | 15 | SG | 2001 | SD_CT020218513 | 11 | SD | - |
| BP-SG86 | 15 | SG | 2001 | SD_pOU1115 | 11 | SD | - |
| BP-SG88 | 15 | SG | 2001 | SNU1093 | 7 | SE[c] | 2010 |
| BP-SG96 | 15 | SG | 2001 | SNU1076 | 7 | SE | 2010 |
| SNU9125 | 15 | SG | 2009 | SNU1091 | 7 | SE | 2010 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| SNU1070 | 15 | SG | 2010 | SNU12016 | 7 | SE | 2012 |
| SNU11066 | 15 | SG | 2011 | SNU12028 | 7 | SE | 2012 |
| SNU1111 | 15 | SG | 2011 | QIA_SE | 7 | SE | - |
| SNU14032 | 15 | SG | 2014 | SE_92-0392 | 7 | SE | 1992 |
| SNU14035 | 15 | SG | 2014 | SE_EC20120916 | na | SE | - |
| SNU14042 | 15 | SG | 2014 | SE_CFSAN063790 | na | SE | 1998 |
| SNU14057 | 15 | SG | 2014 | SE_NCTR380 | na | SE | 2005 |
| GC-15-241 | 15 | SG | 2015 | SE_BCW_4356 | na | SE | 2007 |
| SNU16003 | 15 | SG | 2016 | BP_SP1 | 7 | SP[d] | - |
| SNU16004 | 15 | SG | 2016 | BP_SP5 | 7 | SP | - |
| SNU16009 | 15 | SG | 2016 | BP_SP8 | 7 | SP | - |
| SNU16035 | 15 | SG | 2016 | BP_SP11 | 7 | SP | - |
| SNU16049 | 15 | SG | 2016 | BP_SP12 | 7 | SP | - |
| GC-16-p65 | 15 | SG | 2016 | SP_SNU | 7 | SP | - |
| BP-SG178 | 15 | SG | - | SP_RKS5078 | 7 | SP | - |
| BP-SG179 | 15 | SG | - | SP_S06004 | na | SP | 2006 |
| BP-SG182 | 15 | SG | - | KCDC_4 | 7 | ST[e] | - |
| BP-SG183 | 15 | SG | - | ST_DT104 | 7 | ST | - |
| BP-SG187 | 15 | SG | - | - | - | - | - |

[a]*Salmonella* serotype Gallinarum biovar Gallinarum
[b]*Salmonella* serotype Dublin
[c]*Salmonella* serotype Enteritidis
[d]*Salmonella* serotype Gallinarum biovar Pullorum
[e]*Salmonella* serotype Typhimurium

1 **Table 1.2. Primers used in this study**

| Primer | Sequence (5' to 3') | Usage |
|---|---|---|
| PPL-F | GCA CTT TTG AAC AGG CCG TAG | PPL analysis |
| PPL-R | AGC TAG GCC GCT CAT ACC AC | |
| aidB-F | CGC TAC GAT TCG CAT TTG CT | Pseudogene analysis |
| aidB-R | GGT AAA TGG CGA CTG AAA AC | |
| aphA-F | GGC GTT TAC CTG ATT GCC AG | |
| aphA-R | CGG TGT CGT CAA TAT CAA AG | |
| araA-F | CCG CTC TGC TTC GCA TTA TG | |
| araA-R | GCA TAT CGT TCA GAT CCA GC | |
| asnA-F | CCA GGG CTA ACG CCA AAT TG | |
| asnA-R | CAC TGA TCC ACA TAG ACC GA | |
| bgiA-F | CCT ATG TGT TGA ACG AGT GG | |
| bgiA-R | GGT AAG ACC TGG TAA GAC TC | |
| btuB-F | CGT TAA CGG TCC GGT ATC GA | |
| btuB-R | TGG ACC AAC AGT GTG GTC G | |
| cueO-F | CTG CCG TTA TTA ATG GGC GA | |
| cueO-R | CAA CGC GTA CCG TAT CCT TC | |
| fadI-F | CCT GCG TTA ATC CGT TCA TTC | |
| fadI-R | CGC TAT AGG CAT CGG TAT GA | |
| fliC-F | CAG CTT CTG GCG TAA GTA CA | |
| fliC-R | CAG CCC TTA CAA CAG TGA TC | |
| fliK-F | GCT CTC TTT TAG CGA GCA TG | |
| fliK-R | CTG ATA CTG CTT TGT CCC AAC | |
| foxA-F | CAC AGT TCG TTG GTG GTT GA | |
| foxA-R | CCA TAT TTT CAC GGC GAT CG | |
| gspE-F | GGC ACA ACT TAC CGC TTT TA | |
| gspE-R | ATG TAG CGT TGA AAG TAC CA | |
| lamB-F | GAT TAC TCT GCG CAA ACT CC | |
| lamB-R | CTG CTG AAG GTG TAA GAG CC | |

| | |
|---|---|
| lpfC-F | CAC CCT GGA GTT TAA GGC AG |
| lpfC-R | CAC AAT GGC ACG GCT TAG AG |
| mdlA-F | CTG ACC AGC ATC CGT ATG AT |
| mdlA-R | TGC GGA TAG CAA AAC TCA CG |
| rfaJ-F | TCC AGT CGA TGC TGA TAC TG |
| rfaJ-R | GTA AAC CCT TCT CGC CGA AC |
| rfbP-F | GTA TTG TGC ATT TGC GCG CT |
| rfbP-R | CAA AAC ACC CAG ACA TAG CG |
| sfbA-F | CGA TCC GAC CAA AGC CTC TG |
| sfbA-R | TCC ATT CCG GTT TCT GGA AG |
| SG-RS06090-F | GAG AGG AGG TAG TTG TCT TC |
| SG-RS06090-R | ACT GTC ATG TCA TCA CGG C |
| SG-RS13085-F | CTT GGC GGC TGG ATT TCA GA |
| SG-RS13085-R | CGC CAA TAA ATG GCT TAC CC |
| SG-RS17135-F | CGA TTT CAC CCG TCA CCT CG |
| SG-RS17135-R | GCG ATC AGC TCC TAC CTG CT |
| SG-RS24305-F | GTA TCC AGG CCT TCA ATG AG |
| SG-RS24305-R | GCC ATC GGC ATA TAG GAA TG |
| terC-F | GAA ACG CCG GAA ATC GTT G |
| terC-R | GCC GAT GCC CAT TAG AGA AT |
| tsr-F | GAC CAG AGC AAT ATT GGC AG |
| tsr-R | GTT ACG TAC ATC GCC AAC GG |
| ybdH-F | CAT TGT AGC GAA CGC CAT GT |
| ybdH-R | CTG CTG TTT ATC CGC TAA TG |
| ybiR-F | GCG GCT ATT TTG ATG TGC TG |
| ybiR-R | CCA TAA ATA CCA GCA GCA GC |
| ybgE-F | GGA AAA TGT TCG GTC GTA TC |
| ybgE-R | TGG ATT ATG CCT GCG CAA AC |
| ychA-F | CTG TGT GAT GGC ATG ATC CT |
| ychA-R | CAT TTG CCG TTC CTC CAT CA |
| ydfI-F | CGT ATC CTC TAT CAC CCA CTG |

| | |
|---|---|
| ydfI-R | ACC TCT ATA CCG TGG CGG AG |
| yfiP-F | CGG TAA AGT CCG TTT CGT CA |
| yfiP-R | GTA TTG CTG GGT TTC ATC GG |
| ygiD-F | CTG AGT TCT TCC TCT GTG AAG |
| ygiD-R | CCA CGT CAG ATT GGC TTT CA |
| yhbW-F | GAA AAA CCG TAC GCG ATG GT |
| yhbW-R | GCC CAA CAA CTC TTC CTT AAC |
| yhhJ-F | CTC GGC GTT AAA GAA CTG CG |
| yhhJ-R | CAT CAT GAT CTC AAA GGG CG |
| ytcJ-F | CAG GGT TTT CTT CCT CAG AG |
| ytcJ-R | CGT GTA TAT AAC GCT AGC GC |

1

**Table 1.3. Variable or common pseudogenes among the SG isolates, SG 287/91, SG9R and SP**

| Gene | Function | Description |
|---|---|---|
| *araA* | L-arabinose isomerase | Pseudogenes only common in Korean isolates (15) |
| *aphA* | acid phosphatase | |
| *fliC* | flagellin | |
| *fliK* | flagellar hook length control protein | |
| *lamB* | porin involved in the transport of maltose and maltodextrins | |
| *lpfC* | outer membrane usher protein | |
| *murP* | PTS sugar transporter subunit IIC | |
| *phsA* | thiosulfate reductase | |
| *sfbA* | metal ABC transporter substrate-binding protein | |
| *sirB1* | putative regulator | |
| *tsr* | methyl-accepting chemotaxis protein | |
| *ytcJ* | amidohydrolase | |
| *yfiP* | DTW domain-containing protein | |
| *yhbW* | LLM class flavin-dependent oxidoreductase | |
| *yhhJ* | ABC transporter permease | |
| *gspE* | type II secretion system protein | Isolate-specific pseudogenes in Korean isolates (6) |
| *rfbP* | undecaprenyl-phosphate galactose phosphotransferase | |
| *SG_RS06090* | putative phage protein | |
| *terC* | integral membrane protein | |
| *ybdH* | oxidoreductase | |
| *ygiD* | 4,5-DOPA dioxygenase extradiol | |
| *aidB* | probable acyl Co-A dehydrogenase | 287/91-specific pseudogenes (9) |
| *asnA* | aspartate--ammonia ligase | |
| *dcuA* | anaerobic C4-dicarboxylate transporter | |
| *fadI* | acetyl-CoA C-acyltransferase | |
| *foxA* | TonB-dependent siderophore receptor | |
| *mdlA* | multidrug ABC transporter permease/ATP-binding protein | |
| *SG_RS20965* | fumarate hydratase | |
| *ybgE* | cyd operon protein | |
| *yjcC* | environmental sensor c-di-GMP phosphodiesterase | |
| *bgiA* | 6-phospho-beta-glucosidase | |

| | | |
|---|---|---|
| *btuB* | vitamin B12 transporter | SG 9R-specific pseudogenes (7) |
| *cueO* | multicopper oxidase | |
| *brkB* | virulence factor BrkB protein | |
| *rfaJ* | lipopolysaccharide glucosyltransferase | |
| *ybiR* | anion transporter | |
| *ydfI* | putative D-mannonate oxidoreductase | |
| *dinG* | probable ATP-dependent helicase DinG | Pseudogenes common in Korean isolates, 287/91, SG 9R and SP(13) |
| *emrB* | multidrug resistance protein B | |
| *gabT* | 4-aminobutyrate aminotransferase | |
| *kefB* | glutathione-regulated potassium-efflux system protein (K(+)/H(+) antiporter) | |
| *phoE* | outer membrane pore protein E precursor | |
| *SPUL_1682* | transposase | |
| *SPUL_2451* | outer membrane protein | |
| *SPUL_2756* | large repetitive protein | |
| *SPUL_3734* | putative membrane transport protein | |
| *sspH2* | secreted effector protein | |
| *ydiQ* | putative electron transfer flavoprotein subunit | |
| *yjeH* | putative permease | |
| *ynhG* | LysM peptidoglycan-binding domain-containing protein | |

1

**Table 1.4. Subgroup of Korean field isolates of SG (n=28) based on pseudogenes and PPL length**

| Strain | PPL length | Year of isolation | Subgroup | *gspE* | *rfbP* | *SG_RS06090* | *terC* | *ybdH* | *ygiD* | Frequency (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| BP1 | 15 | 1992 | S1-1 | - | - | - | - | - | - | 15/28 (53.6%) |
| BP-SG002 | 15 | 1993 | S1-1 | - | - | - | - | - | - | |
| BP5 | 15 | 1994 | S1-1 | - | - | - | - | - | - | |
| BP47 | 15 | 2000 | S1-1 | - | - | - | - | - | - | |
| BP49 | 15 | 2000 | S1-1 | - | - | - | - | - | - | |
| BP50 | 15 | 2000 | S1-1 | - | - | - | - | - | - | |
| BP51 | 15 | 2000 | S1-1 | - | - | - | - | - | - | |
| BP59 | 15 | 2001 | S1-1 | - | - | - | - | - | - | |
| BP60 | 15 | 2001 | S1-1 | - | - | - | - | - | - | |
| BP63 | 15 | 2001 | S1-1 | - | - | - | - | - | - | |
| SNU1111 | 15 | 2011 | S1-1 | - | - | - | - | - | - | |
| SNU16003 | 15 | 2016 | S1-1 | - | - | - | - | - | - | |
| SNU16009 | 15 | 2016 | S1-1 | - | - | - | - | - | - | |
| BP-SG197 | 15 | - | S1-1 | - | - | - | - | - | - | |
| GC-002 | 15 | - | S1-1 | - | - | - | - | - | - | |
| GC-128 | 17 | 2015 | S1-2 | - | - | - | - | - | - | 1/28 (3.6%) |
| 16-p16 | 13 | 2016 | S1-3 | - | - | - | - | - | - | 1/28 (3.6%) |
| GC634 | 15 | - | S2 | P | - | - | - | - | - | 1/28 (3.6%) |
| 16035 | 15 | 2016 | S3 | P | - | - | P | - | - | 1/28 (3.6%) |
| BPSG64 | 17 | - | S4-1 | - | - | P | - | - | - | 1/28 (3.6%) |
| SNU16037 | 21 | 2016 | S4-2 | - | - | P | - | - | - | 1/28 (3.6%) |
| BPSG52 | 17 | - | S5 | - | P | P | - | - | - | 1/28 (3.6%) |
| BP8 | 13 | 1994 | S6 | - | - | - | - | - | P | 5/28 (17.9%) |
| BP176 | 13 | - | S6 | - | - | - | - | - | P | |
| BP202 | 13 | - | S6 | - | - | - | - | - | P | |
| BP226 | 13 | - | S6 | - | - | - | - | - | P | |
| BP177 | 13 | - | S6 | - | - | - | - | - | P | |
| BPSG221 | 11 | - | S7 | - | - | P | - | P | P | 1/28 (3.6%) |

**Table 1.5. Pseudogenes known to be shared by *S.* Pullorum and its confirmation in other strains**

| genes | functions |
|---|---|
| *allD* | ureidoglycolate dehydrogenase |
| *citE2* | citrate lyase beta chain |
| *cysU* | sulfate transport system permease protein CysT |
| *ddlB* | D-alanine:D-alanine ligase B |
| *dmsA* | anaerobic dimethyl sulfoxide reductase chain A precursor |
| *elaC* | ribonuclease Z |
| *fhuE* | outer membrane receptor for Fe(iii)-coprogen, Fe(iii)-ferrioxamine b and Fe(iii)-rhodotrulic acid |
| *hemF* | coproporphyrinogen III oxidase, aerobic |
| *hisJ* | histidine-binding periplasmic protein |
| *idnO* | gluconate 5-dehydrogenase |
| *idnT* | gnt-ii system l-idonate transporter (l-ido transporter) - |
| *ilvI* | acetolactate synthase isozyme III large subunit |
| *leuA* | 2-isopropylmalate synthase |
| *lpfE* | fimbrial protein (LpfE) |
| *melB* | melibiose carrier protein |
| *mntH* | manganese transport protein MntH |
| *msyB* | acidic protein MsyB; multicopy suppressor of SecY |
| *mutL* | DNA mismatch repair protein |
| *orf32* | putative hydrolase |
| *pgtP* | phosphoglycerate transporter protein |
| *pps* | phosphoenolpyruvate synthase |
| *proW* | glycine betaine/L-proline transport system permease protein P |
| *res* | type III restriction-modification system enzyme |
| *SPUL_0041* | possible sulfatase |
| *SPUL_0374* | sensor domain-containing phosphodiesterase |
| *SPUL_0402* | penicillin binding protein PBP4B |
| *SPUL_0405* | hypothetical protein |
| *SPUL_0618* | putative MR-MLE-family protein |
| *SPUL_1056* | putative cytochrome |
| *SPUL_1368* | putative hydrolase |
| *SPUL_1417* | putative benzoate membrane transport protein |

| | |
|---|---|
| *SPUL_1469* | putative multidrug transporter |
| *SPUL_1486* | putative oxidoreductase |
| *SPUL_2212* | LysR-family transcriptional regulator |
| *SPUL_2624* | putative outer membrane efflux lipoprotein |
| *SPUL_2643* | 3-isopropylmalate dehydratase small subunit 2 |
| *SPUL_2799* | putative transcriptional regulator |
| *SPUL_3174* | possible membrane transport protein |
| *SPUL_3658* | hypothetical protein |
| *SPUL_3794* | putative transferase |
| *SPUL_3866* | probable glucarate transporter |
| *SPUL_4306* | putative GerE family regulatory protein |
| *SPUL_4330* | entericidin A precursor |
| *SPUL_4530* | PTS transport system, IIC component |
| *stfC* | outer membrane usher protein StfC |
| *trxC* | thioredoxin 2 |
| *yacH* | DUF3300 domain-containing protein |
| *ybbY* | putative permease protein |
| *ybdN* | phosphoadenosine phosphosulfate reductase |
| *ybfM* | putative outer membrane protein |
| *ycaQ* | hypothetical protein – winged helix-turn-helix domain-containing protein |
| *ychK* | patatin-like phospholipase RssA |
| *yciI* | Ycil family protein |
| *ydeV* | putative sugar kinase |
| *ydiS* | putative electron transfer flavoprotein-quinone oxidoreductase |
| *yecD* | putative hydrolase |
| *yeeO* | putative membrane transport protein |
| *yeiU* | inner membrane protein |
| *yfcC* | putative basic amino acid antiporter |
| *yfeR* | putative transcriptional regulator |
| *yfiK* | cysteine/O-acetylserine efflux protein |
| *ygbE* | putative cytochrome oxidase subunit |
| *ygbF* | type I-E CRISPR-associated endoribonuclease Cas2 |
| *ygcA* | putative RNA methyltransferase |
| *ygeA* | Aspartate/glutamate racemase |
| *ygfX* | toxin CptA |
| *yhaO* | putative membrane transport protein |

| | |
|---|---|
| *yhiH** | hypothetical ABC transporter ATP-binding protein |
| *yhjH* | Cyclic-guanylate-specific phosphodiesterase |
| *yjaD* | NAD(+) diphosphatase |
| *yjfJ* | PspA/IM30 family protein |
| *yjhP* | SAM-dependent methyltransferase |
| *yjiE* | probable transcriptional activator |
| *ylaC* | DNA directed RNA polymerase sigma-70 factor |
| *ynfI* | Tat proofreading chaperone DmsD |
| *yqjF* | DoxX family protein |

*Also found in Korean isolates

**Table 1.6. Growth competition of SG field isolates with 15-proline and 17-proline PPLs in chickens**

| | 7 dpi [a] | 8 dpi | 9 dpi | 10 dpi | 15 dpi[b] |
|---|---|---|---|---|---|
| No. of dead chickens | 2 | 3 | 1 | 1 | 1 |
| No. of identified SG isolates in the liver (15/17) [c] | 12/7 | 13/9 | 0/10 | 0/10 | 0/20 |
| No. of identified SG isolates in bile juice (15/17) [c] | nt [d] | nt | 0/7 | 0/7 | 0/10 |

[a]dpi: day post inoculation.

[b]Surviving chickens (3) were fasted at 15 dpi for 3 days with drinking water supplied.

[c]PPL length was confirmed by sequencing analysis.

[c]nt: not tested.

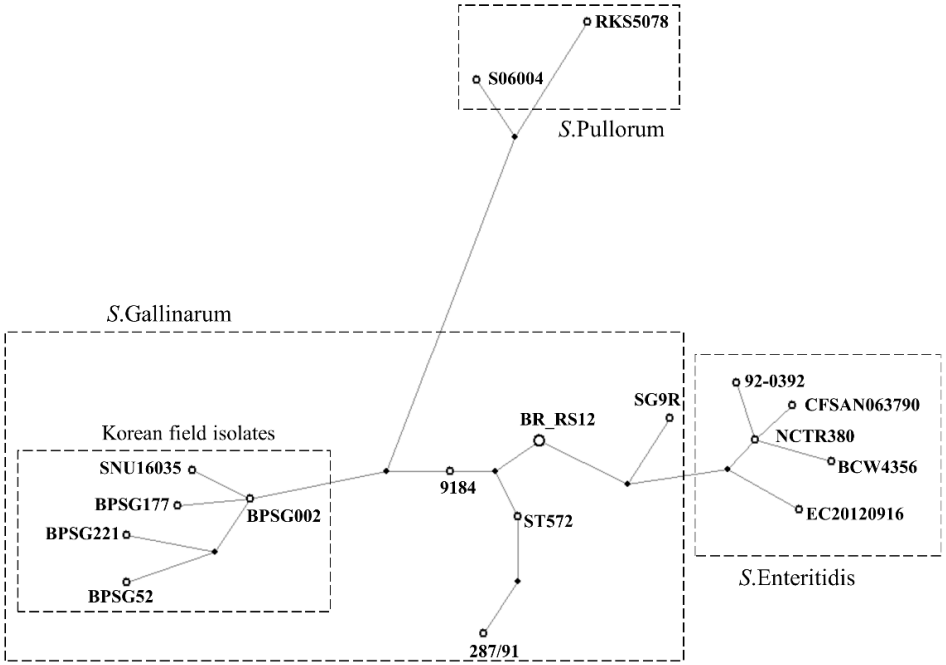


**Figure 1.1. Evolutionary relationship of SE, SG, and SP. Seventeen strains and isolates of SE, SG, and SP were analyzed with Network software.** A total of 122 genes were entered as 0 (intact gene) and 1 (pseudogene), and these binary data were used for

median joining network analysi

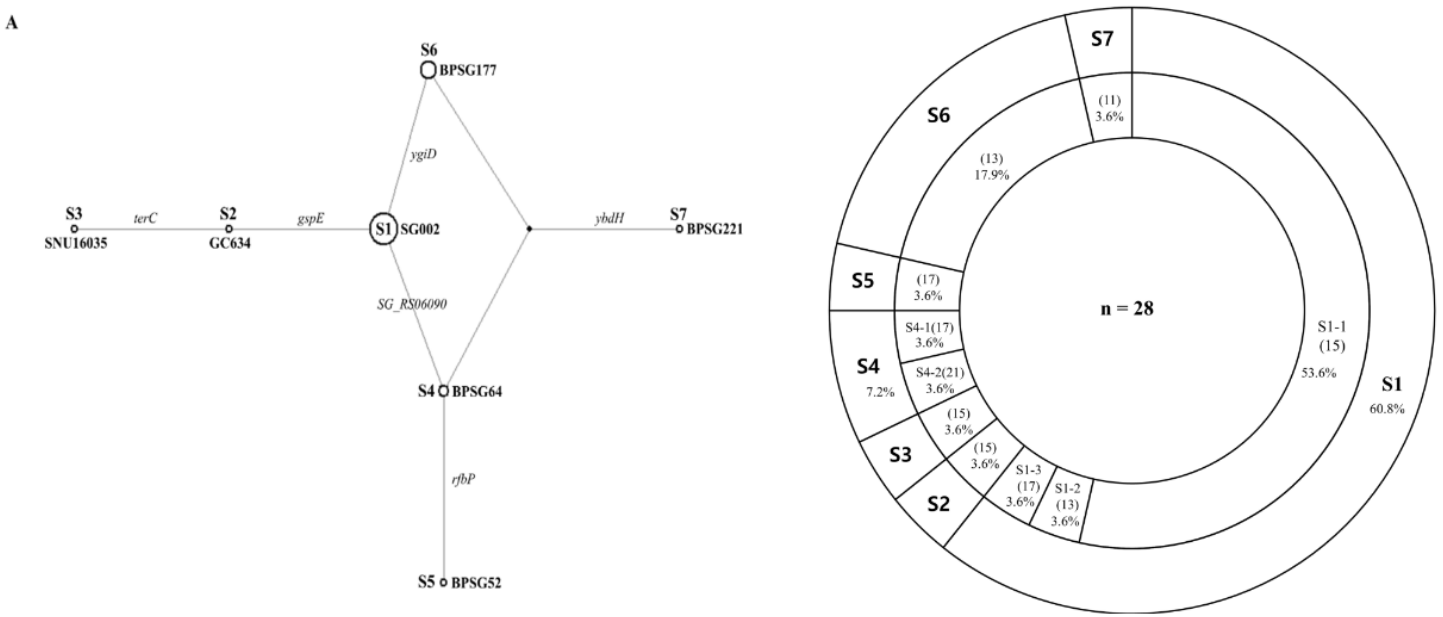


**Figure 1.2. Subgrouping of SG field isolates in Korea by pseudogene profiles and PPL lengths.** Twenty-eight Korean field isolates were divided into 10 subgroups by six isolate-specific pseudogenes (*gspE*, *rfbP*, *SG_RS06090*, *terC*, *ybdH*, and *ygiD*) and PPL lengths. (**A**) Schematic view of pseudogene accumulation in the SG field isolates. The network was generated using median joining network

analysis. (**B**) The ratio of subgroups among the field isolates of SG. The numbers in the inner circle are PPL lengths.

# Chapter II

# Comparison of humoral immune responses to different forms of *Salmonella enterica* serovar Gallinarum biovar Gallinarum

# Abstract

Fowl typhoid is caused by *Salmonella enterica* serovar Gallinarum biovar Gallinarum (SG), and live attenuated, rough vaccine strains have been used. Both humoral and cellular immune responses are involved in protection, but the humoral responses to different forms of SG antigens are unclear. In this study, humoral responses to a killed oil-emulsion (OE) smooth vaccine (SG002) and its rough mutant vaccine (SR2-N6) strains were compared using proteomic analysis. Two immunogenic outer membrane proteins (OmpA and OmpX) were identified, and the selected linear epitopes were successfully applied in peptide-ELISA. The peptide- and total OMP-ELISAs were used to compare the temporal humoral responses to various SG antigens: OE SG002 and SR2-N6; live, killed [PBS-suspension (PS) and OE)] and mixed (live and PS) formulations of another rough vaccine strain (SG 9R); and orally challenge with a field strain. Serum antibodies to the linear epitopes of OmpA and OmpX lasted only for the first 2 weeks, but serum antibodies against OMPs increased over time. The rough strain (SR2-N6) and mixed SG 9R induced higher serum antibody titers than the smooth strain (SG002) and single SG 9R (OE, live and PS SG 9R), respectively. Infection with the field strain delayed the serum antibody response by approximately 2 weeks. Mucosal immunity was not induced by any formulation, except for infection with the field strain after SG 9R vaccination. Thus, our results may be useful to understand humoral immunity against various SG antigens and to improve vaccine programs and serological diagnosis in the field.

***Keywords:*** *Salmonella* serovar Gallinarum biovar Gallinarum, humoral immunity, vaccines, natural infection, peptide-ELISA

# 2.1. Introduction

*Salmonella enterica* serovar Gallinarum biovar Gallinarum (SG) is a pathogen causing fatal and persistent infection, fowl typhoid (FT) (Pomeroy and Nagaraja, 1991; Shivaprasad, 2000). Both humoral and cell-mediated immune responses are required to prevent mortality and achieve bacterial clearance (Mastroeni et al., 2000). A live vaccine strain, SG 9R, mimics infection of pathogenic field strains and has been used to prevent FT worldwide (Lee et al., 2007).

The potent immunostimulatory effect of lipopolysaccharide (LPS) is mediated by O-Ag and lipid A, which induce T cell-independent humoral and TLR4-mediated innate immune responses, respectively (Poltorak et al., 1998). Although LPS induces a strong humoral immune response to concomitantly inoculated antigens, LPS on the surface of bacteria may also shield or compete with outer membrane proteins (OMPs), resulting in decreased immunogenicity of OMP (Johnson et al., 1956; Seppala and Makela, 1984). Therefore, while SG 9R is a rough strain with defective outer-core and O-antigen regions (O-Ag) of lipopolysaccharide (LPS), it may induce a different humoral immune response from field strains against OMP (Kwon and Cho, 2011). The protective efficacy of OMP vaccines has already been established, and protective OMPs of *S.enterica* serovars have been identified for vaccine development (Gil-Cruz et al., 2009; Yang et al., 2018).

Although SG 9R has been commonly used in the field, it displays potential pathogenicity and may cause mortality and gross lesions in the liver under immunosuppressive conditions (Kwon and Cho, 2011). Therefore, SG 9R was not

recommended for use in chicks under 6 weeks old (w-o) who are most susceptible and may become carriers (Feberwee et al., 2001; Lee et al., 2007). For this reason, killed vaccines, if possible, need to be considered, but basic data on the differences in humoral immune responses to different forms of SG antigens (oil-emulsion, killed, smooth vs. rough SG; live vs. killed with or without oil adjuvant vs. a mixture of live and killed SG 9R; or field strain) are insufficient. In addition, humoral immunity against natural infection with field strains is unclear. Humoral immunity to live or killed bacteria is the sum of antibodies directed to multiple antigens and their epitopes. Therefore, investigations of a single epitope-specific antibody in the antiserum against different antigens using single peptide epitopes may provide more insights into the kinetics of humoral immunity. The humoral immune responses to smooth and rough SG strains were compared and immunogenic OMPs and their linear epitopes were identified. This study developed linear epitope-based peptide-ELISAs to compare humoral immune responses to different forms of SG antigens, and the results were compared with data from the OMP-ELISA.

## 2.2. Materials & Methods

### Bacteria, serum samples and experimental birds

A commercial rough vaccine strain, SR2-N6 (DAE SUNG Microbiological Lab., Uiwang-si, Korea), and its parent strain SG002 were used to compare the effect of LPS on humoral immunity, and a commercial rough vaccine strain, SG 9R, was purchased from the manufacturer (Nobilis; Intervet International, Boxmeer, the Netherlands) (Cho et al., 2015). SG0197, a virulent strain isolated from commercial chickens in 2001, was used to observe the immune response of challenged chickens (Cho et al., 2015). The strains were cultured in Luria-Bertani broth (Duchefa Biochemie, Groot Bijgaarden, Belgium) with shaking at 37°C overnight.

One d-o male Hy-Line brown layer chicks without SG vaccination were purchased from a farm (Yangji Farm, Pyeongtaek-si, Korea) and reared for animal experiments to compare humoral immune responses to different forms of SG antigens. Feed and water were supplied *ad libitum*.

Fifty-six field serum samples obtained for serological tests from 6 layer and breeder farms were used to determine humoral immunity to SG in the field. In detail, L1D included 10 samples from 1-day-old (d-o) layer chicks, L12W included 10 samples from 12-w-o layer chickens vaccinated at 10 w-o, L19W included 10 samples from 19-w-o layer chickens vaccinated twice at 8 and 15 w-o, and L41W included 10 samples from 41-w-o layer chickens vaccinated twice at 7 and 16 w-o. PS18W and PS23W included 10 and 6 samples from 18 and 23-w-o parent stocks, respectively.

## 2D-gel electrophoresis, immunoblotting and LC-MS/MS

Total bacterial proteins were extracted via cell lysis with 7 M urea, 2 M thiourea, 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), and 2.5% dithiothreitol (DTT) and quantified with the Bradford protein assay. OMPs were extracted using the previously described sodium lauroyl sarcosine (SLS) method (Baik et al., 2004), with some modifications. Briefly, the cultured bacteria were centrifuged and the pellet was washed with 50 mM Tris-HCl. After centrifugation, lysis buffer (50 mM Tris-HCl and 150 mM NaCl) was added, and the cells were lysed by ultrasonication. The supernatant was ultracentrifuged at 100,000 ×g for 1 hour. Pellets were resuspended in 2% SLS and 50 mM Tris-HCl, and then incubated at room temperature for 40 min. After another ultracentrifugation step, pellets were stored by adding 1% Triton X-100 to the lysis buffer.

2D-gel electrophoresis of total proteins or OMPs was performed using isoelectric focusing (pH 3-10 for whole bacteria or pH 4-7 for OMPs) and 14% SDS-PAGE gels, and separated proteins were electrotransferred to nitrocellulose membranes for western blotting (ProteomeTech, Seoul, Korea) (Park et al., 2004). Membranes were incubated with anti-SR-N6 (1:10,000 dilution) and anti-SG002 (1:5,000 dilution) serum samples. LC-MS/MS was performed as described below. The analysis was performed using a nano ACQUITY UPLC and LTQ-Orbitrap-mass spectrometer (Thermo Electron, San Jose, CA). One of the mobile phases for LC separation was 0.1% formic acid in deionized water, and the other was 0.1% formic acid in acetonitrile. The flow rate was 0.5 μl/min, and the transfer tube temperature was set to 160°C. The MS/MS data were interpreted using SEQUEST software

(Thermo Quest, San Jose, CA, USA), and the generated peak lists were compared using the MASCOT program (Matrix Science Ltd., London, UK).

## B cell epitope prediction and peptide synthesis

B cell epitopes were predicted by the IEDB B cell epitope prediction program (http://tools.iedb.org/bcell/), and they were located on the 3D structure files of corresponding proteins generated with PyMOL 2.2 (Schrodinger, New York, USA). Selected peptides were synthesized with a modification of the N-terminus by adding aminocaproic acid for better performance of the peptide-ELISA (Cosmogenetech, Seoul, Korea).

## ELISA

Synthesized peptides (1 μl/ml) or SG 9R OMP extracts (105 ng/ml) in 100 mM sodium bicarbonate/carbonate coating buffer (pH 9.6) were used to coat an immunoplate (SPL Life Science, Pocheon-si, Korea) at 4°C overnight. Antigen-coated wells were washed twice with PBST (PBS containing 0.5% Tween 20) and blocked with 1% bovine serum albumin (BSA) (GenDEPOT, Katy, USA) at room temperature for 2 hours. After washing the plates as described above, the primary antibody, which was serum or bile juice (1:300 in PBST containing 1% BSA), was added, incubated for 30 min, and then the plate was washed 4 times with PBST. The secondary antibody, an HRP-conjugated goat-anti chicken IgG or IgA antibody (Bethyl, Laboratories, Montgomery, USA; diluted 1:10,000 in PBST containing 1% BSA), was added for 30 min, and the plate was washed as described

above. TMB substrate (SurModics, Eden Prairie, USA) was added for 10 min, and the OD was measured at 450 nm after the addition of stop solution. A commercial *Salmonella* D group ELISA kit was used to test the anti-O-Ag antibody according to the manufacturer's recommendation (BioChek BV., Reeuwijk, the Netherlands).

## Inactivation and preparation of oil-emulsion (OE) SG

Cultured bacteria were centrifuged and washed once with PBS. Bacteria were inactivated at 65°C for 2 hours in a water bath and cooled gradually to room temperature. The inactivation was confirmed by culture on Mueller Hinton Agar (Duchefa Biochemie, Groot Bijgaarden, Belgium). The live and heat-inactivated bacteria were diluted to $1x10^7$ cfu/100 µl and $1x10^9$ cfu/100 µl in PBS, respectively. The live and killed mixture was prepared by mixing the same volume of both preparations of bacteria to obtain 200 µl. The OE bacteria were prepared by emulsifying heat-inactivated bacteria with oil adjuvant (Montanide ISA 70, Seppic Co., Courbevoie, France) at a ratio of 3 to 7 (approximately $3.3x10^8$ cfu/100 µl of OE) (Table 2.1).

## Animal experiments

Fifteen (5 chickens in each group) 3-w-o male brown layer chickens were divided into SR2-N6, SG002 and negative control groups to compare the humoral immune responses to smooth (SG002) and rough (SR2-N6) strains, respectively. OE SR2-N6 and OE SG002 were inoculated via the intramuscular route (100 µl/chicken), and serum samples were collected weekly for up to 3 weeks postinoculation (wpi). Bile juice samples

were collected from the gall bladder at 3 wpi with a 1 ml syringe. Specific antibodies in serum (IgG) and bile juice (IgA) samples were measured using the ELISA.

Forty 3-w-o male brown layer chickens were assigned to OE SG 9R (10), live SG 9R (10), mixed SG 9R (10), PS (PBS –suspension) SG 9R (5) and negative control groups (5) to compare the humoral immune responses to different forms of SG 9R. All groups were inoculated via the intramuscular route (100 µl/chicken), and serum samples were collected weekly for up to 3 wpi. Bile juice samples were collected at 3 wpi as described above. Specific antibodies in serum and bile juice samples were measured using the ELISA.

Fifteen 4-w-o chickens were infected with the field strain (SG0197, $1x10^6$ cfu/0.1 ml/chicken) *per os,* and serum samples were collected from the surviving chickens weekly for 4 wpi. Bile juice samples were collected after 3 days of starvation at 4 wpi, as described above. Specific antibodies in serum and bile juice samples were measured using the ELISA.

Twenty 6-w-o chickens were divided into SG 9R vaccine and no vaccine groups to compare the mucosal immune responses. The SG 9R vaccine group was vaccinated with SG 9R ($1x10^7$ cfu/100 µl/chicken) via the intramuscular route, and both groups were challenged with SG0197 at 2 wpi (8 w-o). After 2 wpi, SG0197 bile juice samples were collected as described above, and specific antibodies were measured using the ELISA.

All animal experiments were approved by the Institutional Animal Care and Use Committee of BioPOA Co. (permission number BP-2019-C31-1).

## Statistical analysis

Analyses were performed with SPSS Statistics version 26.0 (SPSS, Chicago, IL, USA).

One-way ANOVA was used to analyze significant differences between the groups, followed by the Bonferroni post hoc test (Figs. 2.2C 1 wpi and 3 wpi, 2.2D 1 wpi; and 2.6A and C). When unequal variance was observed, the Welch test was used for the analysis, and the Games-Howel test was performed as the post hoc test (Figs. 2.2B 2 wpi, 2.2C 2 wpi, 2.2D 2 wpi and 3wpi; 2.3A 2 wpi, 2.3B 2wpi, 2.3C 1 wpi, and 2.3D 1-3 wpi). Data with a non-normal distribution were subjected to the Kruskal-Wallis H test, and the Bonferroni correction was used as the post hoc test (Figs. 2.2A 1-3 wpi, 2.2B 1 wpi, and 3 wpi; 2.3A 1 wpi and 3 wpi, 2.3B 1 wpi and 3 wpi, 2.3C 2 wpi and 3 wpi; 2.4; 2.5; 2.6B and D; and 2.7). If only two groups were analyzed, the significance was determined with the t-test for data with a normal distribution (Fig. 2.6E, F, and G), and the Mann-Whitney U test was used for data with a non-normal distribution (Fig. 2.6H and I). Statistical significance was considered when the p-value was less than 0.05.

## 2.3. Results

### Comparison of humoral immune responses to smooth (SG002) and rough (SR2-N6) strains

In contrast to anti-SR2-N6 serum samples, anti-SG002 serum samples showed a strong antibody reaction to O-Ag (at least two distinct ladders at different isoelectric points) (Fig. 2.1A, black dotted rectangle). The different spots recognized by the anti-SR2-N6 serum sample were analyzed with LC-MS/MS. Interestingly, most of the spots were not OMP and included translation elongation factor G, GroEL, phosphoglycerate kinase, elongation factor Tu, electron transfer flavoprotein subunit beta, *etc*. (Table 2.2). To identify immunogenic OMPs, 2D-gel electrophoresis and immunoblotting were performed with OMPs of SR2-N6 and anti-SR2-N6 serum samples to identify major antigens. The three major antigen spots were identified to be OmpA (spots a and b) and OmpX (spot c) (Fig. 2.1C and Table 2.3). This study selected candidate peptides for the peptide-ELISA according to the amino acid sequences of OmpA (CAR36850) and OmpX (CAR36706) (Table 2.4). A pilot study with the synthesized peptides (OmpA-N-L1, OmpA-N-L2, OmpA-N-L3, OmpA-N-L4, OmpX-L1, and OmpX-L2) and anti-SG002 and anti-SR2-N6 serum samples revealed that the reactivity of OmpA-N-L1, OmpA-N-L2, and OmpX-L1 was too low to differentiate responses from anti-SG002 and anti-SR2-N6 serum samples. OmpA-N-L3, OmpA-N-L4, and OmpX-L2 were selected for the peptide-ELISA. According to the results, the anti-SR2-N6 antibody titer was significantly higher than the anti-SG002 antibody titer in the OmpX-L2 and OMP-ELISA at 1 week postinoculation

(wpi) (Fig. 2.2C-D). All the anti-SR2-N6 and anti-SG002 serum samples showed significantly higher OD values than the negative control only for the first 2 weeks using peptide-ELISAs, except for OmpX_L2. However, in the OMP-ELISA, significantly higher OD values were observed than the negative control, with a gradual increase during the observation period.

## Comparison of humoral immune responses to live, killed and mixture of live and killed rough vaccine strains (SG 9R)

The OD values of anti-OE SG 9R, anti-live SG 9R and anti-mixed SG 9R serum samples were not significantly different from each other, and produced higher OD values than the anti-PS SG 9R serum samples and negative control samples at 1 and 2 wpi in the peptide-ELISAs (Fig. 2.3). Interestingly, anti-mixed SG 9R showed significantly higher OD values than the negative control in the OmpA-N-L3 and OmpA-N-L4 peptide-ELISAs at 3 wpi. According to the results of the OMP-ELISA, anti-OE SG 9R, anti-SG 9R and anti-mixed SG 9R serum samples showed significantly higher OD values than the negative control samples with a gradual increase over time. Anti-PS SG 9R serum samples did not show significantly higher OD values than the negative control in either peptide- or OMP-ELISAs.

This study tested two additional peptides (OmpA-C-L1 and OmpA-C-L2) in the C-terminal domain of OmpA using the peptide-ELISA (Table 2.4). The anti-OE SG 9R serum samples showed significantly higher OD values than the negative control at 1 and 2 wpi, but not at 3 wpi (Fig. 2.4).

## Humoral immunity against natural infection with a field strain (SG0197)

SG0197 infection caused 86.7% (13/15) mortality within 4 weeks (7/15 at 2 wpi, 5/15 at 3 wpi and 1/15 at 4 wpi); therefore, the numbers of serum samples were 8 at 2 wpi, 3 at 3 wpi, and 2 at 4 wpi as the number of surviving chickens decreased. The serum samples from the challenged group only showed significantly higher OD values than the negative control group at 3 wpi using peptide-ELISAs (Fig. 2.5C) but significantly higher OD values at 3 and 4 wpi than the negative control group using the OMP-ELISAs (Fig. 2.5D).

## Comparison of mucosal immunity against various SG antigens

The anti-OE SG 9R, anti-SG 9R, anti-mixed SG 9R, anti-PS SG 9R, anti-OE SR2-N6, and anti-OE SG002 IgA levels in bile juice samples were not significantly different from the negative control using peptide- and OMP-ELISAs (Fig. 2.6A-D).

The inoculation of SG0197 in 8-w-o male brown layer chickens did not cause mortality in either the SG 9R vaccine or no vaccine (CONT) group at 2 wpi. The SG 9R vaccine and no vaccine groups did not display different OD values for the peptide-ELISA (Fig. 2.6E-G). However, the SG 9R vaccine group showed significantly higher OD values than the no vaccine group using the OMP-ELISA ($P<0.05$) and O-Ag-ELISA (Fig. 2.6H-I).

## Humoral immunity against SG in the field

When the field serum samples were tested using peptide-ELISAs, L12W showed higher OD values than the other samples, although the differences were not significant. The OMP-ELISA revealed significantly higher OD values in the L12W, L19W and L41W groups than in the negative control (Fig. 2.7D).

## 2.4. Discussion

Fowl typhoid vaccines are categorized into live attenuated and inactivated vaccines, and live attenuated vaccine strains are subdivided into rough and smooth strains (Barrow, 1990; Chaudhari et al., 2012; de Paiva et al., 2009). As O-Ag of LPS hides OMPs and induces strong activation of specific B cells, the immunogenicity of OMPs of smooth strains may be less than rough strains (Muotiala et al., 1989). Our western blotting results from 2D-gel electrophoresis with whole bacterial lysates revealed predominant humoral immunity to O-Ag, and the results of the peptide- and OMP-ELISAs of SG002 and SR2-N6 supported the hypothesis that OMPs of the rough strain are more immunogenic than OMPs of the smooth strain (Figs. 2.1 and 2.2). The increased immunogenicity of OMPs may be due to unrestricted exposure to B cells without shielding by O-Ag and the absence of a competing strong immunogen monopolizing most of the resources of humoral immunity. Considering the already improved protective efficacy of OMP vaccines and antigenic conservation among Gram-negative bacteria, the potential value of rough strains to become universal vaccines needs to be demonstrated in future studies (Liu et al., 2016).

SG 9R has been used worldwide due to its better protective efficacy, but the humoral immune responses to live, killed and a mixture of live and killed SG 9R have never been compared. The significantly lower immunogenicity of PS SG 9R than SG 9R was unexpected because a killed rough strain of *Salmonella* serovar Typhimurium generated a higher antibody titer than the live rough strain (Muotiala et al., 1989). The bacteria was killed at 65°C for 2 h, while the authors of the previous used 100°C for 30 min and subsequent treatment with 1% human serum albumin and 0.16% formaldehyde. Therefore, the additional treatment with albumin and formaldehyde may have resulted in different

results. Interestingly, a synergistic effect of PS SG 9R and live SG 9R was apparent and may reflect cooperative stimulation of humoral immunity by dead and live bacteria. Therefore, this new formulation without the use of the carcinogen formaldehyde may be useful to improve the protective efficacy of conventional live vaccines against infection with virulence variants recently detected in the field (Kim et al., 2019).

The oil adjuvant significantly increased the immunogenicity of OE SG 9R compared with PS SG 9R and generated similar serum antibody titers to SG 9R. Because of its potential pathogenicity, SG 9R vaccination of chickens aged less than 6 w-o is not recommended (Kwon and Cho, 2011; Van Immerseel et al., 2013; Wigley et al., 2005). Because the highest susceptibility and likelihood of infection are observed during the prevaccination ages (1 d-o to 6 w-o or age before vaccination), clinical measures, including early inoculation with adjuvanted killed vaccines, can be considered to protect chicks from vertical and/or horizontal transmission of SG.

As invasive *Salmonella enterica* serovars penetrate the cell as an intracellular pathogen, researchers assumed that live bacteria might not produce sufficiently high titers of specific antibodies in the bloodstream. SG did not stimulate the initial immune response via proinflammatory cytokines or chemokines due to the absence of flagella, and the ability of SG to evade the immune system was very remarkable, even when the systemic infection had progressed (de Freitas Neto et al., 2013; Seo et al., 2002). Consistent with previous reports, infection with a field strain might delay humoral immunity by approximately two weeks compared with SG 9R inoculation (Fig. 2.5) and might result in insignificant mucosal antibody levels compared with the negative control (Fig. 2.6). However, their different routes of infections should be considered. The observation that surviving chickens

mounted antibodies against OmpX peptides and OMPs may support the importance of humoral immunity in survival. Additionally, a significant increase in the IgA titer in the bile juice of SG 9R-vaccinated chickens may support the importance of humoral immunity induced by SG 9R vaccination (Fig. 2.6). Because most commercial layer farms inoculate animals with SG 9R vaccines, testing IgA levels in bile juice, intestinal washes and feces may be useful for the differential diagnosis of FT and an estimation of the risk of SG exposure.

Considering temporary increases in OD values obtained from peptide-ELISAs during the first two weeks after SG vaccine inoculations, the higher OD value of L12W may be related to the SG 9R vaccination at 10 weeks of age (Fig. 2.7). Thus, the peptide-ELISAs were able to detect specific antibodies induced by recent SG 9R vaccination under both experimental and field conditions. However, the OMP-ELISA revealed significantly higher antibody levels in vaccinated flocks than in unvaccinated flocks. All field samples were tested using the O-Ag ELISA, and no positive sample indicating a field strain or SE infection was examined (data not shown). Therefore, these assays may be useful to verify the efficacy of the inoculated vaccine and monitor unlawful vaccination with parent stocks in the field in combination with the O-Ag ELISA.

The immunogenic OMPs of *Salmonella* enterica have been reported, and OmpA and OmpX are known to be protective antigens (Erova et al., 2013; Pore et al., 2011; Song et al., 2020). Although this study selected OmpA and OmpX due to their immunodominance, the rapid but short-lived antibody responses induced by these antigens were unexpected and have not been reported. Additionally, this study is the first to investigate the kinetics of the production of specific antibodies against linear epitopes of

OmpA and OmpX compared with OMPs. In summary, with the gradual increase in the titers of antibodies against OMPs over time, there may be other OMPs inducing long-lasting antibody responses. Although the immune dominance of the C-terminus of OmpA was reported previously, any difference between the results of C- and N-terminal peptide-ELISAs could not be found (Fig. 2.4) (Singh et al., 2003).

In conclusion, rough strains are better than smooth strains in terms of the immunogenicity of OMPs, and a mixture of a live and killed rough vaccine strains may potentiate the efficacy of the conventional live vaccine. The evasion of humoral immunity by the field strain was demonstrated again, but SG 9R may be useful to prime mucosal immunity against infection with a field strain. Additionally, combined serological tests with peptide, OMP, and O-Ag ELISAs may be useful for the differential diagnosis of FT in the field.

**Table 2.1. Vaccines and field strain used in this experiment**

| Sample | Solvent | Dose (cfu/chicken) | Inoculation Route |
|---|---|---|---|
| SR2-N6 | PBS with the ISA 70 adjuvant | $3x10^8$ | IM |
| SG002 | PBS with the ISA 70 adjuvant | $3x10^8$ | IM |
| SG9R | PBS | $1x10^7$ | IM |
| OE SG9R | PBS with the ISA 70 adjuvant | $3x10^8$ | IM |
| PS SG9R | PBS | $1x10^9$ | IM |
| MX SG9R | PBS | $1x10^7$(SG9R)+$1x10^9$(PS SG9R) | IM |
| SG197 | PBS | $1x10^6$ | *per os* |

**Table 2.2. 2DE western blotting and LC-MS / MS analysis were performed using whole bacteria as antigen**

| Band label | NCBI BLAST | Protein name | Score | Mass |
|---|---|---|---|---|
| **A** | gi\|1028875858 | translation elongation factor G [*Salmonella* enterica] | 568 | 77,788 |
| **B** | gi\|446651785 | molecular chaperone GroEL [*Salmonella* enterica] | 1,342 | 57,382 |
| **C** | gi\|513039272 | phosphoglycerate kinase [*Salmonella* enterica] | 601 | 41,310 |
| **D** | gi\|823641791 | elongation factor Tu [*Escherichia coli*] | 311 | 43,486 |
| **E** | gi\|446079918 | electron transfer flavoprotein subunit beta [*Salmonella* enterica] | 419 | 33,608 |
| **F** | gi\|972719165 | elongation factor TU [Erwinia gerundensis] | 222 | 45,118 |
| **G** | gi\|321222631 | 3~(2~),5~-bisphosphate nucleotidase [*Salmonella* enterica subsp. enterica serovar Typhimurium str. TN061786] | 185 | 25,890 |
| **H** | gi\|353561131 | Endoribonuclease L-PSP [*Salmonella* enterica subsp. enterica serovar Gaminara str. A4-567] | 90 | 14,141 |
| **I** | gi\|518056966 | MULTISPECIES: cold-shock protein [Firmicutes] | 83 | 7,257 |

**Table 2.3. Proteins predicted by the LC-MS/MS analysis**

| Spot label | NCBI BLAST | Protein name | Score | Mass |
|---|---|---|---|---|
| **a** | WP_065702086.1 | porin OmpA [*Salmonella enterica*] | 4,039 | 37,640 |
| **b** | WP_065702086.1 | porin OmpA [*Salmonella enterica*] | 1,735 | 37,640 |
| **c** | WP_058343733.1 | outer membrane protein OmpX [*Salmonella enterica*] | 3,409 | 17,570 |

**Table 2.4. B cell epitopes of OmpA and OmpX tested in the peptide-ELISA**

| Protein/location | Peptide name | Sequence (N- to C-terminus) |
|---|---|---|
| OmpA/N-terminus | OmpA-N-L3 | TKSNV PGGPS |
| | OmpA-N-L4 | TNNIG DANTI GTR |
| OmpA/C-terminus | OmpA-C-L1 | QLYSQ LSNLD PKDGS |
| | OmpA-C-L2 | GESNP VTGNT CDNVK |
| OmpX | OmpX-L2 | GKFQT TDYPT YKHDT |

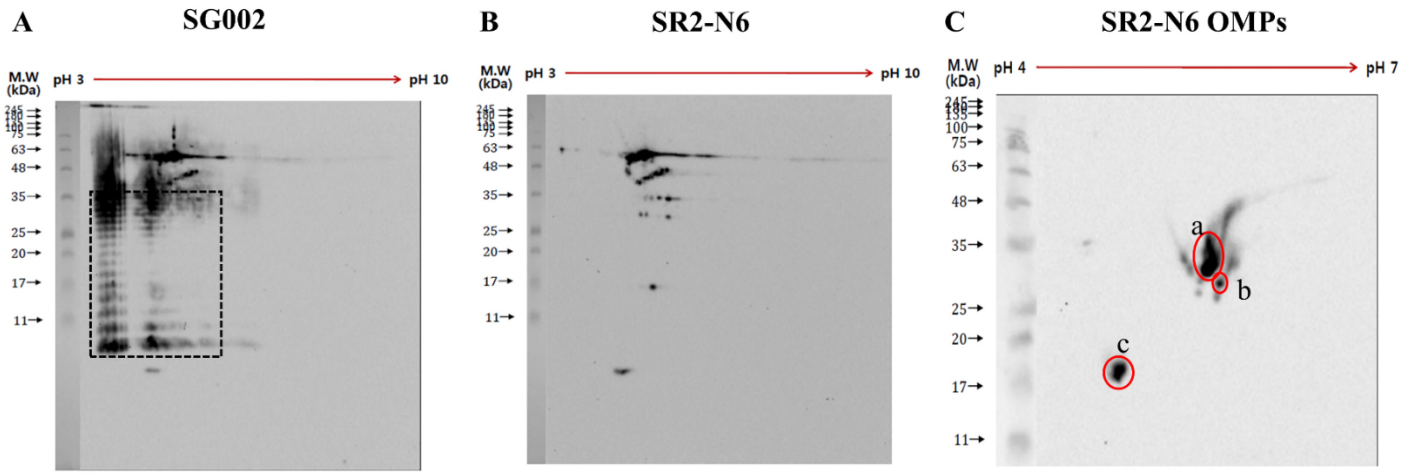


**Figure. 2.1. Different 2DE results of smooth strain (SG002) and rough strain (SR2-N6) and 2DE results of SR2-N6 OMP extracts against SR2-N6 antiserum.** Antigens of SG002 were tested against antiserum of SG002 (A) and SR2-N6 (B) using 2DE western blotting. Different blots obtained in (A) and (B) were analyzed using LC-MS/MS (Table 2.2). Distinct ladders at different isoelectric points represented the O antigen (black dotted rectangle). Omp extracts of SR2-N6 were also tested against the antiserum of SR2-N6 (C) using 2DE western blotting. Three spots (a, b, and c) were analyzed with LC-MS/MS (Table 2.3).

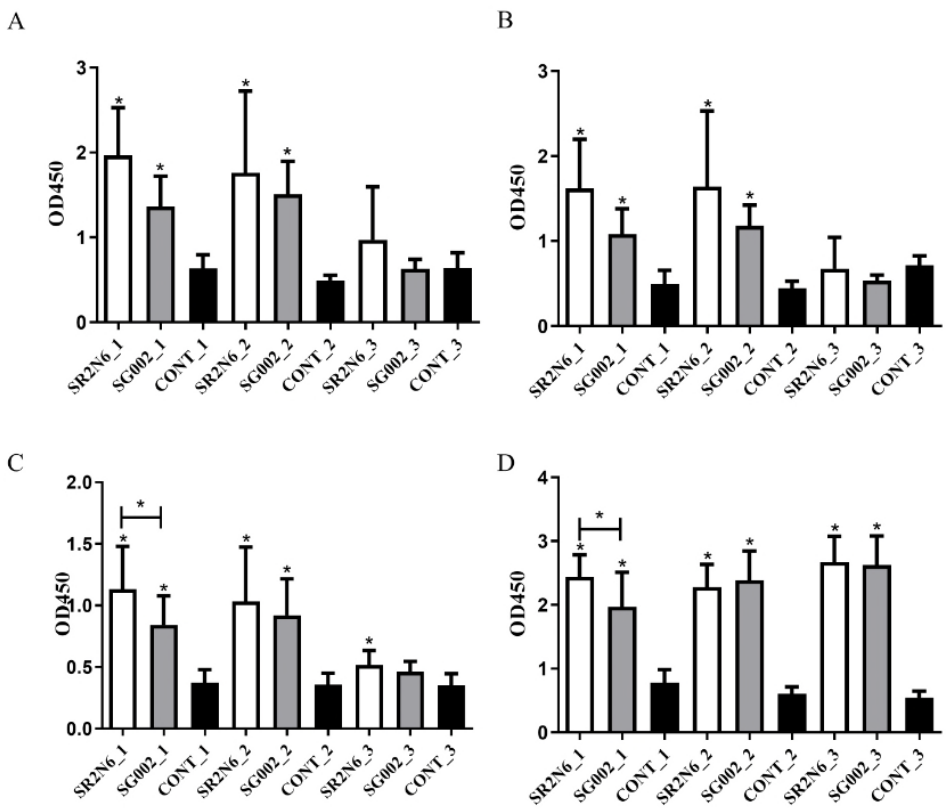


**Figure. 2.2. Humoral immune responses to the smooth strain (SG002) and rough strain (SR2-N6) measured using ELISAs (mean with SD): OmpA-N-L3 (A), OmpA-N-L4 (B), OmpX-L2 (C) and OMP ELISAs (D).** *Indicates a significant difference (*P* values - A: SR2-N6 vs. CONT at 1 wpi (0.000) and 2 wpi (0.001); SG002 vs. CONT at 1 wpi (0.008) and 2 wpi (0.000); B: SR2-N6 vs. CONT at 1 wpi (0.000) and 2 wpi (0.005); SG002 vs. CONT at 1 wpi (0.01) and 2 wpi (0.000); C: SR2-N6 vs. SG002 at 1 wpi (0.046); SR2-N6 vs. CONT at 1 wpi (0.000), 2 wpi (0.002), and 3 wpi (0.003); SG002 vs. CONT at 1 wpi (0.001) and 2 wpi (0.000); D: SR2-N6 vs. SG002 at 1 wpi (0.035); SR2-N6 vs. CONT at 1 wpi (0.000), 2 wpi (0.000), and 3 wpi (0.000), SG002 vs. CONT at 1 wpi (0.000), 2 wpi (0.000), and 3 wpi (0.000)).

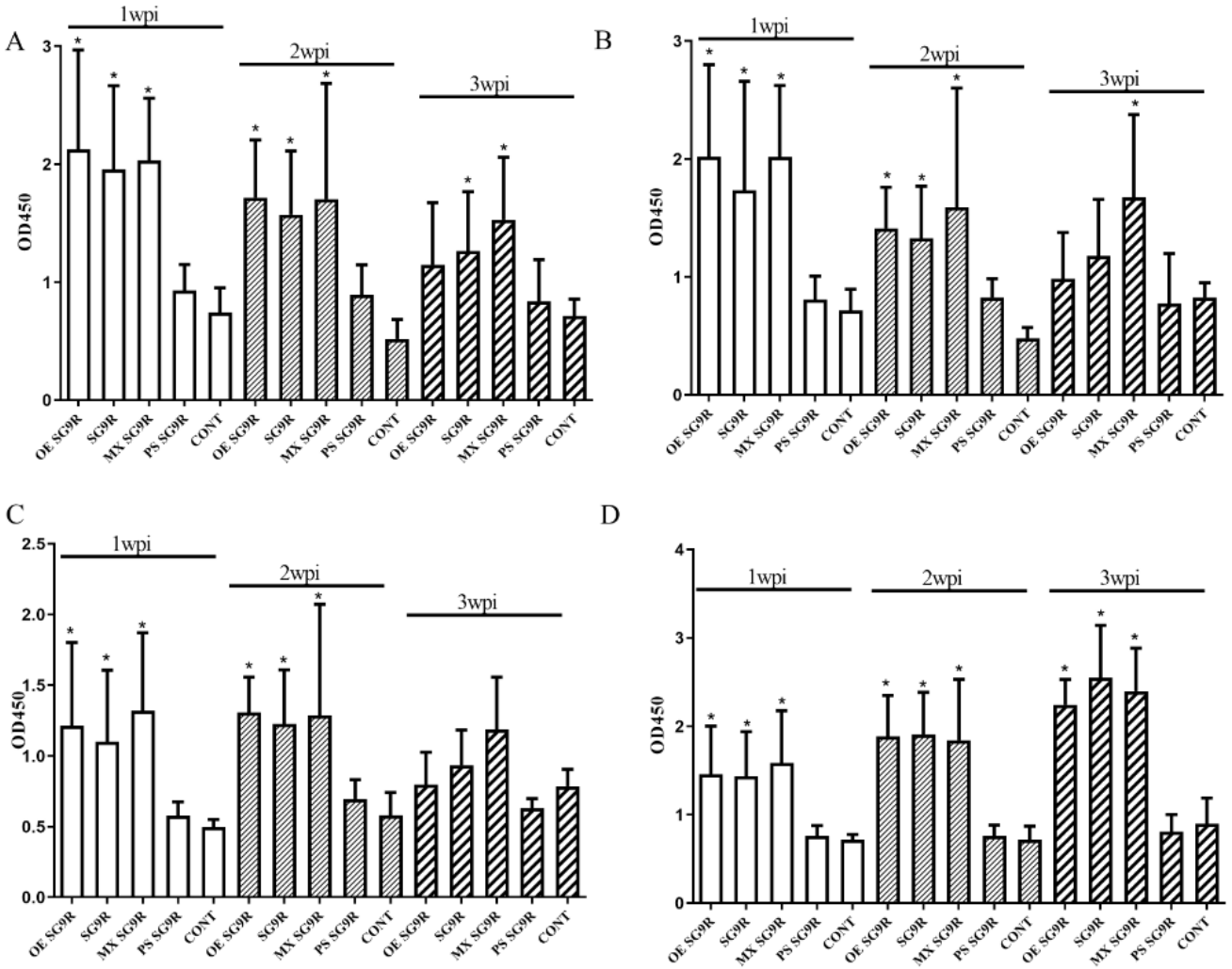
A
B
C
D
1wpi
2wpi
3wpi
OD450
OE SG9R
SG9R
MX SG9R
PS SG9R
CONT

**Figure. 2.3. Humoral immune responses measured using ELISAs under various conditions (mean with SD): OmpA-N-L3 (A), OmpA-N-L4 (B), OmpX-L2 (C) and OMP-ELISA (D).** SG 9R was inoculated as killed adjuvanted (OE), live, mixed with live and killed (MX), or killed bacteria in a PBS suspension (PS) and compared with the negative control (CONT). *Indicates a significant difference compared with CONT (*P* values - A: OE SG 9R at 1 wpi (0.000) and 2 wpi (0.000); SG 9R at 1 wpi (0.000), 2 wpi (0.000), and 3 wpi (0.048); MX SG 9R at 1 wpi (0.000), 2 wpi (0.000), and 3 wpi (0.000); B: OE SG 9R at 1 wpi (0.001) and 2 wpi (0.000); SG 9R at 1 wpi (0.019) and 2 wpi (0.000); MX SG 9R at 1 wpi (0.000), 2 wpi (0.001), and 3 wpi (0.022); C: OE SG 9R at 1 wpi (0.000) and 2 wpi (0.000); SG 9R at 1wpi (0.000) and 2 wpi (0.000); MX SG 9R at 1 wpi (0.000) and 2 wpi (0.006); D: OE SG 9R at 1wpi (0.013), 2 wpi (0.000), and 3 wpi (0.000); SG 9R at 1 wpi (0.009), 2 wpi (0.000), 3 wpi (0.000); MX SG 9R at 1 wpi (0.008), 2 wpi (0.004), and 3 wpi (0.000)).

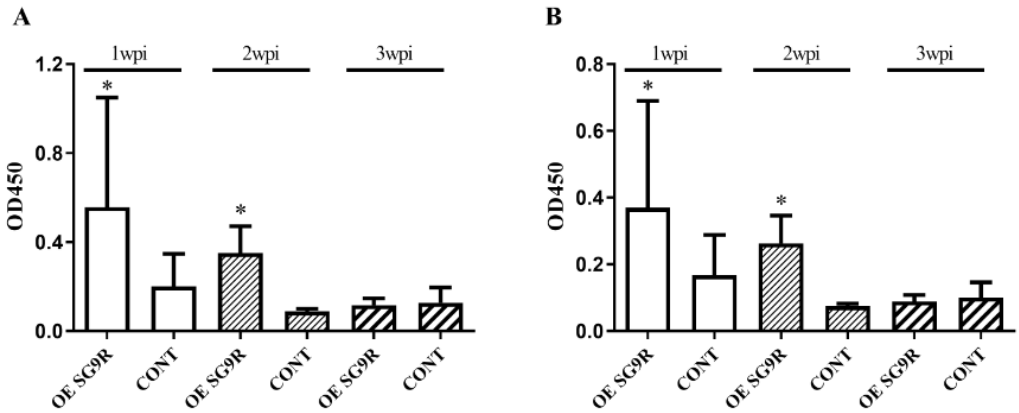


**Figure. 2.4. Humoral immunity evaluation with C-terminal epitope ELISA (mean with SD): OmpA-C-L1 (A) OmpA-C-L2 (B).** SG9R was inoculated with killed adjuvanted (OE) and serum samples were colledcted weekly for 3 weeks. *Indicates a significant difference compared with CONT. (P values – A: 1wpi (0.007) and 2wpi (0.000); B: 1wpi(0.015) and 2wpi(0.000)).

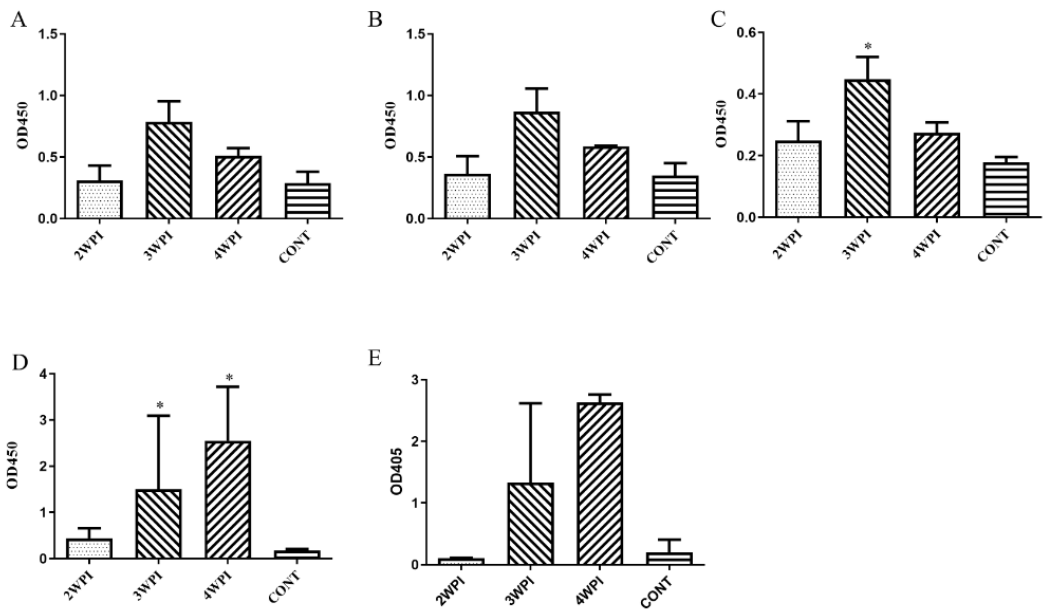


**Figure. 2.5. Evaluation of humoral immunity in response to challenge with a field strain (mean with SD) using the OmpA-N-L3 (A), OmpA-N-L4 (B), OmpX-L2 (C), OMP-ELISA (D), and *Salmonella* D group O-Ag ELISA (E).** The field strain SG0197 was infected *per os* at 4 weeks of age, and serum samples were collected weekly for 4 weeks. *Indicates a significant difference compared with CONT (*P* values - C: 3 wpi (0.016); D: 3 wpi (0.036) and 4 wpi (0.017)).

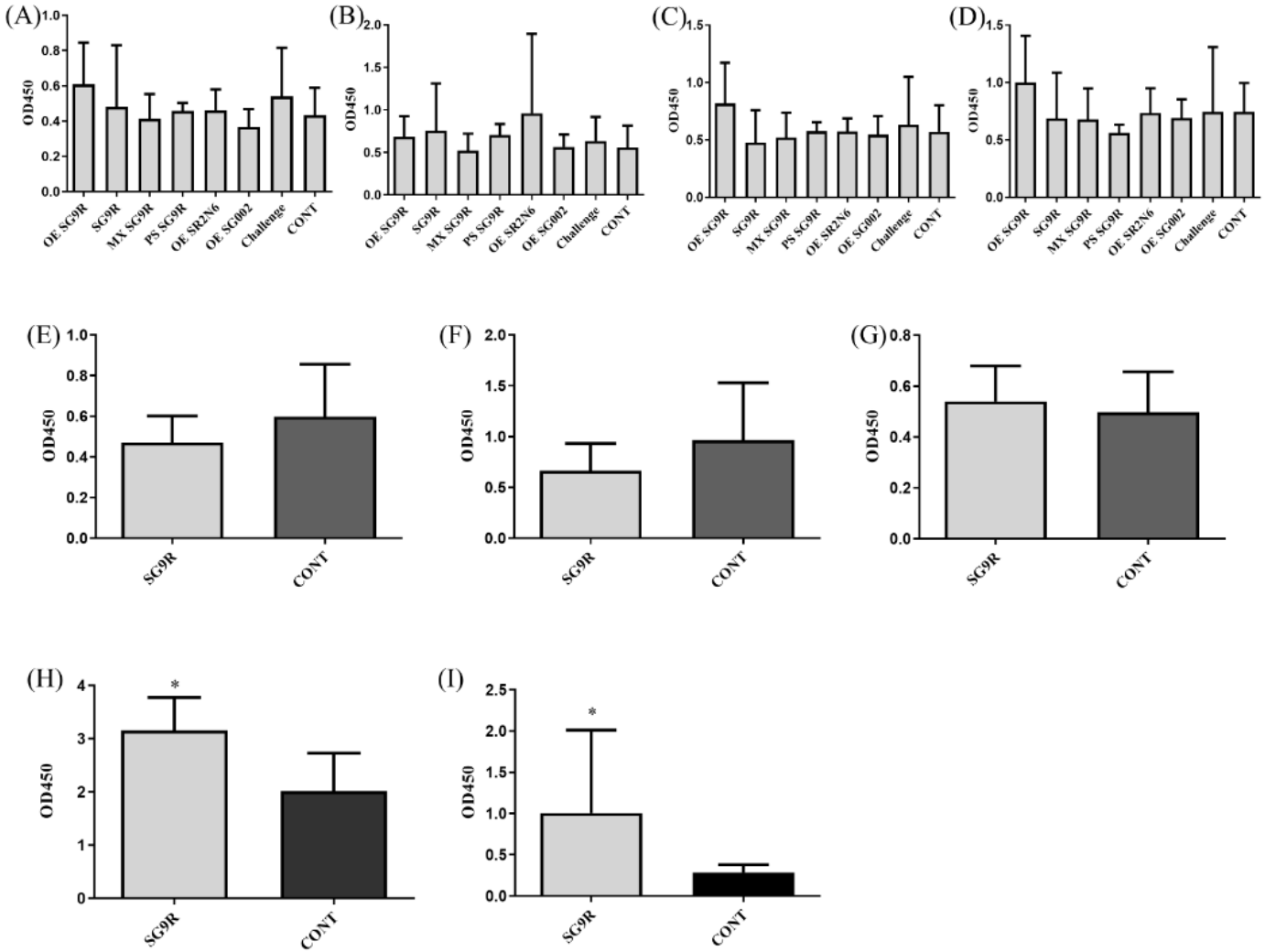
(A)
(B)
(C)
(D)
OD450
OE SG9R
SG9R
MX SG9R
PS SG9R
OE SR2N6
OE SG002
Challenge
CONT
(E)
(F)
(G)
SG9R
CONT
(H)
(I)
*

**Figure. 2.6. The mucosal immunity of bile was measured with IgA secondary antibody with the same ELISA (mean with SD): OmpA-N-L3 (A and E), OmpA-N-L4 (B and F), OmpX-L2 (C and G), OMPs (D and H), and O-Ag IgA ELISA (I).** Birds were inoculated with the vaccine at 3 weeks of age, and bile juice was collected at 3 wpi (A-D). Eight-week-old brown layer chickens with or without SG 9R vaccination were challenged with SG0197 at 6 weeks of age, and bile juice samples were collected at 10 weeks of age (E-I). *Indicates a significant difference compared with CONT (*P* values - H: (0.001) and I: (0.001)).

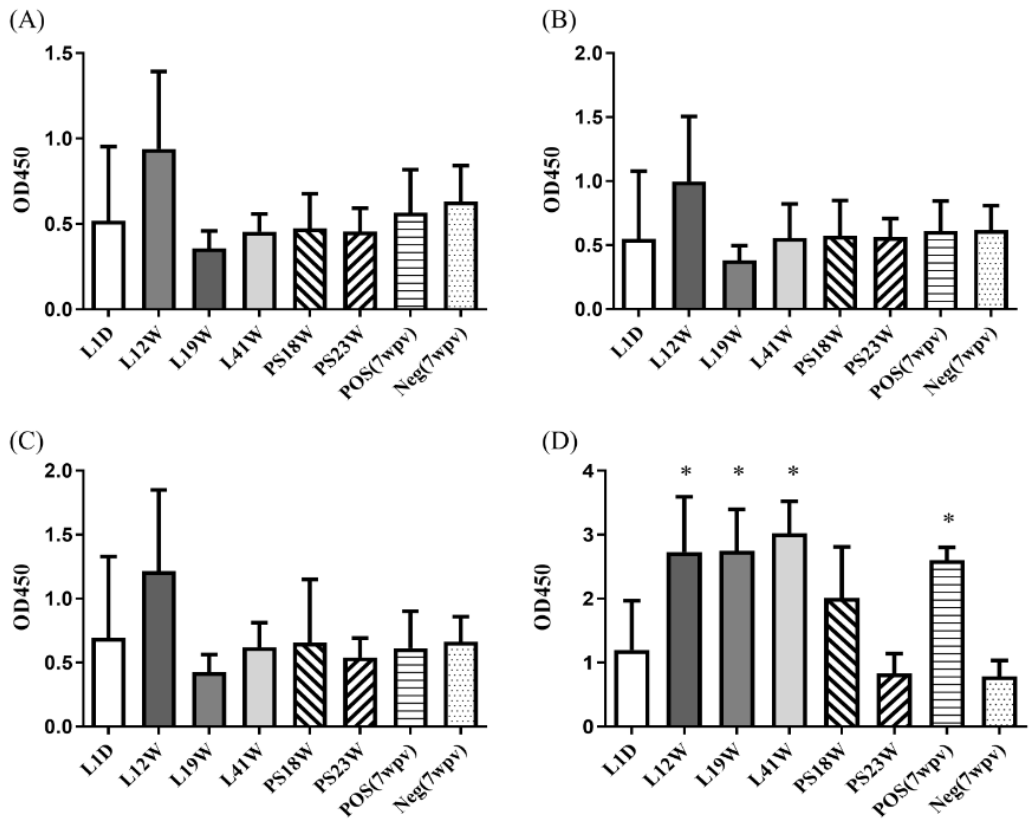


**Figure. 2.7. Humoral immunity against SG in the field (mean with SD): OmpA-N-L3 (A), OmpA-N-L4 (B), OmpX-L2 (C), and OMP-ELISA (D).** The field serum samples from the L1D (1 d-o), L12W (12 w-o, vaccination at 10 weeks of age), L19W (19 w-o, vaccination at 8 and 15 weeks of age), and L41W (41 w-o, vaccination at 7 and 16 weeks of age) groups were collected from laying hens, and the PS18W (18 w-o) and PS23W (23 w-o) samples were collected from parent stocks. *Indicates a significant difference compared with Neg. (*P* values - D: L12W (0.002), L19W (0.002), L41W (0.000), and POS (0.028))

# Chapter III

# Genetic and proteomic characterization of a live vaccine strain (SR2-N6) against fowl typhoid

# Abstract

A rough vaccine strain, SR2-N6, is used in the field against fowl typhoid and paratyphoid, but the genetic and proteomic background of attenuation has never been reported. The aim of this study is to investigate the genetic and proteomic differences between SR2-N6 and its parent strain SG002. Comparative genomics and proteomics studies were conducted between SR2-N6 and SG002. Based on the results, a minimum inhibitory concentration (MIC) and cold shock susceptibility test were performed. In addition, *ΔrfaJ* mutants were prepared and tested to confirm the positive results of the O antigen ELISA occurring in SG9R inoculated chickens and compared to SR2-N6. SR2-N6 possesses a 13-nucleotide deletion of *rfaL,* resulting in defective O-side chain repeats of LPS. G81C mutation in the quinolone resistance-determining region of *gyrA* makes the strain to be resistant to some antibiotics. Additionally, the key virulence genes (*spvB* and *spvC*) located in a large virulence plasmid were not detected in SR2-N6. SR2-N6 was more susceptible to cold shock compared to SG002, which may be related to the decreased expression of the DNA starvation/stationary phase protection gene (*dps*). Unlike a popular rough vaccine strain SG9R, SR2-N6 did not show a false positive reaction in an O-antigen (O-Ag) ELISA, which was used to monitor D group *Salmonella* serovar infections in poultry. SR2-N6 is safer due to the low chance of reversion of the *rfaL* mutation and the removal of the key virulence genes. Also it may be more compatible with O-Ag ELISA-based monitoring than SG9R.



***Keywords:*** fowl typhoid, live vaccine, comparative genomics, *Salmonella* virulence genes, proteomics

# 3.1. Introduction

Fowl typhoid is an acute septicemic disease of young chicks and laying hens that is characterized by high mortality, hepatomegaly and necrotic foci in the liver (Shivaprasad and Barrow, 2008). This disease causes enormous economic loss in the poultry industry because it is difficult to eradicate. The causative agent, *Salmonella enterica subsp. enterica* serovar Gallinarum *biovar* Gallinarum (SG), is nonmotile and host-adapted (Barrow et al., 1994; Shivaprasad and Barrow, 2008). The lipopolysaccharide (LPS) rough vaccine strain SG9R, which lacks outer core and O-side chain repeats of LPS due to a single nonsense mutation in *rfaJ*, has been used in fowl typhoid prevention (Kwon and Cho, 2011). Recently, impaired expression of the SPI-1 and SPI-2 virulence-associated genes and nonsynonymous mutations in pyruvate dehydrogenase subunit E1 (*aceE*) have been reported to attenuate SG9R (Kang et al., 2012; Van Immerseel et al., 2013). The *spvB* and *spvC Salmonella* virulence plasmid (*spv*) genes (which also include *spvR, A, B, C,* and *D*) on the large virulence plasmids of pathogenic *Salmonella* serovars can replace the virulence of the entire plasmid (Gulig, 1990; Matsui et al., 2001). However, SG9R possesses intact *spvB* and *spvC*, which may play important roles in vertical transmission, SG9R-induced fowl typhoid in the field, and high pathogenicity to chicks in the context of protein-energy malnutrition (PEM) (Kwon and Cho, 2011; Silva et al., 1981).

Recently, a new rough vaccine strain, SR2-N6, against fowl typhoid was established and verified to be as efficacious as SG9R. In contrast to SG9R, SR2-N6 did not cause any mortality or liver lesions and was not reisolated in the context of PEM (Kwon and Cho, 2011). Although SR2-N6 was licensed and commercialized in Korea, the

attenuation mechanism and molecular differential diagnosis method of SR2-N6 have never been reported.

In Korea, efforts to eradicate *Salmonella enterica subsp. enterica* serovar Enteritidis (SE) have involved an O-antigen (O-Ag) ELISA-based monitoring system to detect anti-D group O-Ag antibody in the field. A single point mutation in *rfaJ* of SG9R may recover the intact outer core and O-side chain of LPS and may induce anti-D group O-antigen antibody, which may cause confusion with the monitoring system (Kwon and Cho, 2011; Van Immerseel et al., 2013). Additionally, the inner core of LPS is known to be immunogenic, and SG9R vaccination may induce an anti-inner core antibody reactive to the O-Ag ELISA (Jakel et al., 2008; Kong et al., 2016).

In the present study, we performed a comparative genomics study with SR2-N6 and its parent strain, SG002, and determined the presence of the virulence genes *spvB* and *spvC*. The differential gene expression of SR2-N6 was compared with that of SG002 by a proteomic analysis. Furthermore, the ability to induce antibody directed to the anti-D group and inner core antigens was compared by O-Ag ELISA after inoculation into chickens with SR2-N6, SG9R, and SE.

## 3.2. Materials & Methods

### Bacteria

The rough vaccine strains SR2-N6 (Daesung Microbiology Lab., Korea), SG9R (Intervet, Boxmeer, The Netherlands) and Safe 9R; the virulent parent strain SR2-N6 (SG002); and *Salmonella* Enteritidis (SE6) from commercial chickens were cultured on MacConkey agar plates (Difco, Detroit, MI) and Luria-Bertani (LB) broth (Difco) at 37°C overnight under shaking condition. Cultured bacteria were stored at 4°C until used. Bacteria used in this study were demonstrated in Table 3.1.

### Experimental chickens

Six-week-old (w-o) chickens without anti-D group antibodies (VALO BioMedia, Adel, IA, USA) were purchased from BioPOA Co. (Yongin, Korea) and Yangi Farm (Pyeongtaek-si, Korea). All chickens were used for the LPS immunogenicity tests.

### PCR, RT-PCR and real-time PCR

The total DNA of bacteria was extracted with the G-spin for bacteria kit (iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's instructions. To detect the mRNA of target genes, total bacterial RNA was extracted with the easyBlue reagent (iNtRON Biotechnology) according to the manufacturer's instructions with slight modifications. Bacteria were resuspended in 1 mL of easyBlue reagent and centrifuged to separate the aqueous and organic layers. Two hundred µl from the aqueous layer was harvested and added to 1 mL of easyBlue reagent, and RNA was extracted following the

manufacturer's instructions. For amplification of *rpoB*, *spvB*, *spvC,* and *invA* and for amplification and sequencing of LPS biosynthesis, primers from previous studies were used (Galan et al., 1992; Kwon et al., 2001; Kwon and Cho, 2011). Primers were designed using nucleotide sequences of the *Salmonella* Gallinarum 287/91 strain (NC_011274.1). The primers for amplification and sequencing of *rfaL* are listed in Table 3.2. RT-PCR was conducted using a one-step RT-PCR kit (iNtRON Biotechnology) following the manufacturer's instructions. The PCR solution consisted of 10× buffer (2 μl), dNTPs (2.5 mM, 0.4 μl), forward and reverse primers (10 pmol/μl, 0.5 μl each), Taq DNA polymerase (5 U/μl, iNtRON Biotechnology; 0.2 μl), distilled water (15.4 μl), and template DNA (50 ng/μl, 1 μl). Cycling conditions were 94°C at 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2.5 min, with a final extension step at 72°C for 5 min. The amplicons were analyzed via electrophoresis on 1.0% agarose gels, and a 100 bp ladder was used as the molecular size marker (iNtRON Biotechnology).

To compare the proteomics results at the mRNA level, real-time PCR was conducted by synthesizing cDNA. The mRNA was extracted from the overnight culture of SG002 and SR2-N6 cells using the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany). Each RNA was synthesized to cDNA using the amfiRivert cDNA synthesis kit (genDEPOT, Katy, USA), and the concentration of DNA was determined with a Multiskan Go instrument (Thermo, Waltham, USA). qPCR was performed with the AMPIGENE qPCR Green Mix (Enzo, Farmingdale, USA), and the primers used for amplification of each gene are listed in Table 3.2.

## Analysis of LPS biosynthesis genes and next generation sequencing

The amplicons of the LPS biosynthesis gene cluster (13,786 nucleotides [nt] covering *rfaD*, *rfaF*, *rfaC*, *rfaL*, *rfaK*, *rfaZ*, *rfaY*, *rfaJ*, *rfaI*, *rfaB*, *rfaP*, *rfaG*, and *rfaQ*) were purified using a PCR purification kit (iNtRON Biotechnology) according to the manufacturer's instructions and were sequenced with an ABI3711 automatic sequencer (Macrogen, Seoul, Korea). Sequences were analyzed with the BioEdit program (T.A. Hall Software, version 5.0.9.1) and were compared to the GenBank database of the National Center for Biotechnology Information by BLAST search (Altschul and Lipman, 1990). Next-generation sequencing (NGS) was performed as described in a previous study (Kim et al., 2019). The resequencing data of SG002 and SR2-N6 were compared.

## Minimal inhibitory concentration (MIC)

MIC was performed to confirm the effect of the *gyrA* quinolone resistance domain region (QRDR) missense mutation. SR2-N6 and SG002 were tested for the QRDR mutation of *gyrA*, and SG9R and ATCC29213 were used as reference strains. The amount of each bacterium was adjusted to an OD600 of 0.2 in LB broth and then diluted 100-fold in MHB. The quinolone antibiotics used were enrofloxacin (Sigma-Aldrich, Saint Louis, USA) and ofloxacin (Sigma-Aldrich, Saint Louis, USA), which were diluted in a 2-fold series of dilutions, starting with an initial concentration of 256 μg/mL. The 96-well microplate was incubated overnight at 37°C.

## 2D-gel electrophoresis (2-DE), MALDI-TOF MS and identification of proteins

Sample preparation and 2-DE were performed as previously described (Matsui et

al., 2001). The procedure for in-gel digestion of protein spots from Coomassie Blue-stained gels was carried out as previously described (Park et al., 2004). Mass measurements of tryptic peptides were carried out with a Voyager-DE STR mass spectrometer (PerSepctive Biosystems) in reflectron positive ion mode as previously described (Barrow et al., 1994). The proteins were identified by using ProFound (http://129.85.19.192/profound_bin/WebProFound.exe, Rockefeller University, Version 4.10.5), MASCOT (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF), or MS-Fit (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm, University of California, San Francisco, Version 4.0.5).

## Cold-shock susceptibility test

The cfu reduction was measured by applying temperature stress. Overnight cultures of SG002 and SR2-N6 were adjusted to an optical density at 600 nm (OD600) of 0.2, further diluted to $2\times10^2$ cfu/100 μL and stored at 4°C. The viability of each strain was measured at approximately one-week intervals by spreading 100 μL onto Mueller Hinton agar (BD, Franklin Lakes, USA).

## Generation of the *rfaJ* knockout mutant strain (Safe 9R)

Safe 9R was generated by the Red/ET recombination kit (Gene Bridges, Heidelberg, Germany) with the following modification (Zhang et al., 1998). The linear cassette for homologous recombination was prepared by attaching the homology arms for deletion, which are shown in Table 3.2. PCR amplification was carried out using MGMax polymerase (MGMED, Seoul, Korea) in a total reaction volume of 50 μL with 40 cycles at

an annealing temperature of 55°C. SG9R was transformed with the Red/ET plasmid and the cassette by an electroporator (Biorad, Hercules, USA) at 2500 V, 10 μF, and 600 ohms with 1 mm slit electroporation cuvettes (Biorad, Hercules, USA). Each transformation was plated on LB Tetra and LB Kan plates. The deleted region was 1005 nt (10~1014), which was the locus after the nonsense mutation in SG9R, but there were 22 and 23 nt residues remaining in the cassette that were used for recombination upstream and downstream of the removed area, respectively.

## D group O-Ag ELISA

To compare the immunogenicity of LPS from SR2-N6, live SR2-N6 ($1\times10^7$cfu/chick, ck), SG9R ($7.0\times10^7$ cfu/ck), or Safe 9R ($1\times10^7$ cfu/ck) were inoculated into six-week-old (wo) chickens via the intramuscular route. The oil emulsion vaccine was prepared by mixing the same volume of complete Freund's adjuvant with heat-inactivated ($1.0\times10^{10}$ cfu/ck; 65°C for 120 min) SE6. Twenty 6-wo SPF chickens were inoculated twice via the intramuscular route at three-week intervals. The serum from chickens was collected three weeks after inoculation. Field samples were collected from layer flocks that had been vaccinated twice with SG9R (SG9R vac.) or not vaccinated (SG9R no vac.). Anti-D group O-antigen antibody was measured using an ELISA kit following the manufacturer's recommendation (Biochek, Foster City, CA).

## Animal experiments

All animal experimentation was approved by the Institutional Animal Care and Use Committee of Seoul National University (permission number SNU-200324-7).

## Statistical analysis

Analyses were performed with SPSS Statistics version 26 (SPSS, Chicago, USA). The normality was confirmed with the Shapiro-Wilk test, significance was confirmed with a t-test. Statistical significance was considered when the p-value was less than 0.05.

## 3.3. Result

### Genetic characterization and molecular differential diagnosis of SR2-N6

The 13,786 nucleotide sequence of SR2-N6 that contained the LPS biosynthesis genes was determined and deposited in GenBank (accession no. KF250354). The *rfaL* gene encodes an O-antigen ligase, which links O-side chain repeats to the outer core of LPS. SR2-N6 was found to possess a 13-nucleotide deletion in the *rfaL* gene, which could be easily detected by PCR (amplicon size decreased from 158 bp to 145 bp) (Table 3.3; Fig. 3.1). The 13-nucleotide deletion causes a reading frame shift and results in the truncation of the O-antigen ligase. This was also confirmed in the analysis result through NGS. The amplicons of *spvB* and *spvC* from the total DNA and *spvB* from the RNA of SR2-N6 were not detected (Table 3.3; Fig. 3.2).

There were a total of 23 SNPs between SG002 and SR2-N6. We confirmed by sequencing that the SNPs with low analysis quality were not accurate; therefore, we excluded them, along with silent mutations and pseudogenes. As a result, 3 mutations in the coding region were identified (Table 3.3). The variants in the coding region were flagellin protein (*flgD*), DNA gyrase subunit A (*gyrA*) and O-antigen ligase (*rfaL*). Several additional SNPs existed in the resequencing results with high quality, but were excluded as they differ from the direct sequencing analysis.

### Quinolone resistance of SR2-N6

The 81, 83 and 87 amino acids of the *gyrA* gene are known to be related to one of

the QRDRs (Hamouda and Amyes, 2004; Walker et al., 2003). There were mutations different from reference strain 287/91 in SR2-N6 at 81 aa (G81C) and in both SG002 and SR2-N6 at 83 aa (F83S). MIC assay results showed that the quinolone resistance of SR2-N6, compared to SG002, increased approximately 32- and 8-fold on enrofloxacin and ofloxacin, respectively. The quinolone resistance of ATCC29213 was in the reference range (Table 3.4).

## Proteomics

The whole proteins of SR2-N6 and SG002 were separated by 2-DE, and the stained spots were compared (Fig. 3.3). We selected several decreased and increased spots from SR2-N6 for MALDI-TOF MS and protein identification, and finalized two decreased and two increased proteins by excluding identified proteins that did not match the molecular weight or pI values. The decreased spots were identified as acetyl-CoA hydrolase (2-fold) and DNA starvation/stationary phase protection protein (Dps) (2-fold). The increased spots were identified as cysteine synthase A (268-fold) and chaperonin GroEL (372-fold) (Table 3.5).

## Cold-shock susceptibility of SR2-N6

SR2-N6 significantly decreased cell viability from the 3rd week compared to SG002 (Fig. 3.4). The difference was greatest at 3 weeks and slightly decreased at 4 weeks, but both data showed significantly different results. At week 3, SG002 showed a concentration of $1.71\times10^3$ cfu/mL, but SR2-N6 showed a much lower concentration of $1.36\times10^3$ cfu/mL. The following week, SG002 showed a concentration of $1.22\times10^3$ cfu/mL,

and SR2-N6 showed a concentration of $1.06\times10^3$ cfu/mL.

## Analysis of antibodies against SG D group O-antigen

The antibodies against the D group O-antigen was measured in sera collected from chickens inoculated with SR2-N6, SG9R, Safe 9R or heat-inactivated SE6 by ELISA. Serum samples from SPF chickens, unvaccinated commercial chickens, and SR2-N6-vaccinated chickens were negative, but some serum samples from SG9R, Safe 9R and SE6-vaccinated chickens were partially positive for anti-D group O-antigen antibody. In addition, a positivity rate of 3.3% was found in the SG9R-vaccinated field samples (Table 3.6).

To verify that the positive reaction of antiserum samples collected from SG9R-vaccinated chickens was not caused by the antibody response to D group O-Ag produced by the reverse mutant of SG9R, we generated the *rfaJ* (918 bp) knockout mutant, Safe 9R, to remove the possibility of the appearance of the reverse mutant (Fig. 3.5). Interestingly, the antiserum samples collected from Safe-9R-vaccinated chickens also showed a partial positive reaction (2/10) in the O-Ag ELISA (Table 3.6).

## 3.4. Discussion

SR2-N6 and SG9R are rough vaccine strains in which the LPS O-side chains are deleted. In contrast to SG9R, which can be reverted with only one nonsense mutation of *rfaJ*, the 13-nucleotide deletion in the *rfaL* gene of SR2-N6 makes it virtually impossible to reverse to a smooth strain (LeClerc et al., 1996; Van Immerseel et al., 2013). Furthermore, amplification of the *rfaL* gene containing the 13-nucleotide deletion region allows differentiation of SR2-N6 from the wild-type and SG9R strains.

SG9R possesses *spvB* and *spvC* genes that inhibit the phagocyte function of macrophages and innate immunity, respectively, via mitogen-activated protein kinase (MAPK) inactivation (Arbibe et al., 2007; Lesnick et al., 2001; Li et al., 2007; Tezcan-Merdol et al., 2001). However, SR2-N6 may have lost *spvB* and *spvC* during the selection of attenuated strains (Cho et al., 2015). A previous study revealed that the *spv* virulence locus expedited the reproduction and dissemination of *Salmonella* by inhibiting the actions of neutrophils and macrophages (Barrow et al., 1987). In contrast to SG9R, SR2-N6 did not cause mortality and persisted in the liver in the PEM model (Cho et al., 2015). Furthermore, SR2-N6 could not disseminate to the liver when inoculated into the trachea and could not induce protective immunity (data not shown) (Bahadur Basnet et al., 2008). Thus, the pathogenicity of SR2-N6 was significantly reduced compared to SG9R, and the absence of *spvB* and *spvC* is also useful for differential diagnosis.

Nalidixic acid has been used for bacterial attenuation and plasmid curing and was used for the establishment of SR2-N6 (Linde et al., 1998; Weisser and Wiedemann, 1985). The G81C mutation in the QRDR of *gyrA* increases resistance to quinolones and may be related to the higher resistance of SR2-N6 to both enrofloxacin and ofloxacin compared to

SG002 and SG9R (Hamouda and Amyes, 2004; Walker et al., 2003).

The proteomics study revealed increased expression of some proteins and decreased expression of other proteins in SR2-N6 compared to those in the parent strain. The DNA starvation/stationary phase protection protein (Dps) is involved in the protection of DNA from various stresses, such as oxidative damage, UV and gamma radiation, and pH/temperature/metal stress during the stationary phase of growth (Almiron et al., 1992; Martinez and Kolter, 1997; Nair and Finkel, 2004). SR2-N6 showed significantly less viability under cold stress in the present study and more susceptibility to acidic stress in our previous study than SG002, and the reduced amount of Dps is the only factor related to these phenotypes (Cho et al., 2015). The increased expression of the chaperone protease GroEL may reflect the production of misfolded or damaged proteins during cultivation, and increased or decreased expression of metabolic enzymes (cysteine synthase A and acetyl-CoA hydrolase) may also reflect interconnection of bacterial metabolism and virulence factor synthesis (Hendrick and Hartl, 1993; Somerville and Proctor, 2009). However, the mismatches between the amount of protein and the copy number of mRNA in the present study were unexpected. To date, correspondence of mRNA and protein levels is still debated. Some researchers have indicated that only approximately 40% of the variation in protein concentration is related to the change in mRNA copy numbers, but others claim this may be as high as 84% (Zapalska-Sozoniuk et al., 2019).

To monitor infection, a *Salmonella* Enteritidis ELISA kit has been used for field detection of anti-D group O-antigen antibody. The positive antibody rate (3.3%) in SG9R-vaccinated flocks was unexpected. *Salmonella* Enteritidis infection or reverse mutation of the *rfaJ* gene in SG9R in the flock are thought to be possible causes of the positive antibody response (Van Immerseel et al., 2013). However, the positive reaction of serum samples

collected from Safe 9R-vaccinated chickens may rule out the appearance of a reverse mutant of SG9R during vaccine preparation. The absence of a positive reaction in serum samples from SR2-N6-vaccinated chickens may be caused by both a lower sample number and different structures of LPS. The inner core of LPS is conserved among related bacteria and is immunogenic for producing direct natural antibodies in serum. For this reason, inner core-based vaccines were developed (Jakel et al., 2008; Kong et al., 2016). The inner core of SG9R is located outermost and is exposed outside freely. However, the inner core of SR2-N6 is linked to the outer core covering the outside. Although the immunogenicity of the outer core is unclear to our knowledge, the different LPS structures of SG9R and SR2-N6 need to be noted for further study.

Fowl typhoid cases caused by the SG9R revertant strain and the demonstration of persistence and residual pathogenicity of SG9R in the PEM model may urge the replacement of SG9R with safer vaccine strains (Cho et al., 2015; Van Immerseel et al., 2013). Therefore, the multigenic attenuation and nonpathogenicity in the PEM model may support priority selection of SR2-N6 for fowl typhoid vaccine strains. In conclusion, SR2-N6 may be a better choice to prevent fowl typhoid and paratyphoid compared to SG9R in regards to both safety and serological differential diagnosis.

**Table 3.1. Bacterial strains used in this study**

| Strains | Description |
|---|---|
| SR2-N6 | *Salmonella* Gallinarum; *rfaL* defect rough strain live attenuated fowl typhoid vaccine |
| SR2-N6-p5 | SR2-N6 passaged 5 times in LB broth |
| SR2-N6-p10 | SR2-N6 passaged 10 times in LB broth |
| SR2-N6-p20 | SR2-N6 passaged 20 times in LB broth |
| SG9R | *Salmonella* Gallinarum; *rfaJ* defect rough strain live attenuated fowl typhoid vaccine |
| Safe-9R | *ΔrfaJ* mutant of SG9R |
| SE6 | An isolate of *Salmonella* Enteritidis |

**Table 3.2. Primers used in the study**

| Primers | 5′ to 3′ | Purpose |
|---|---|---|
| ***rfaJ_deletion_F*** | CTTTAAACGTAAACTTCTTGAATAAAACCCATAG GTGATGTAATGGATTAAATTAACCCTCACTAAA GGGCG | *rfaJ* deletion |
| ***rfaJ_deletion_R*** | AGTTTTTAATCTTTTTTTCAATAATCATAATAGA GATTTAGGCAGGGGAATAATACGACTCACTATA GGGCTC | |
| ***rfaJ_F*** | TCCAGTCGATGCTGATACTG | Safe 9R deletion confirmation |
| ***rfaJ_R*** | GTAAACCCTTCTCGCCGAAC | |
| ***rfaL_F*** | CAGCAGACAGATAGCTCATATCG | SR2-N6 deletion confirmation |
| ***rfaL_R*** | TTAAAGGTCCACGTTGGATAATCG | |
| ***IC1_F**** | GGAAATCTGGGAAGACACCG | Proteomics data confirmation by qPCR |
| ***IC1_R*** | AAGATCGGTTTTACCCTTCG | |
| ***IC2_F*** | GTTGCCTCTAAAGCGAACGA | |
| ***IC2_R*** | CCTTCGGTAATGATGGACTG | |
| ***DC1_F*** | TTCAGAAATTGACGCCATTG | |
| ***DC1_R*** | TAGCAATATCTTCGGCTACG | |
| ***DC2_F*** | CATAACGTGCAGGATCACTT | |
| ***DC2_R*** | GGTAAAGATATCGGCAGTGT | |
| ***16S rRNA_F*** | ACTCCTACGGGAGGCAGCAGT | |
| ***16S rRNA_R*** | TATTACCGCGGCTGCTGGC | |

* IC stands for increase, DC stands for decrease, and numbers refer to the order of genes in Table 3.3.

**Table 3.3. Comparison of genetic variation between SR2-N6 and SG002**

| Genes | Function/location | SR2-N6 | |
|---|---|---|---|
| | | Nucleotide change | Amino acid change |
| *flgD* | Flagellin protein / chromosome | 6 nt-ins. (553-558) | inframe insertion |
| *gyrA* | DNA gyrase subunit A / chromosome | G81C | missense mutation |
| *rfaL* | O-antigen ligase / chromosome | 13 nt-del. (846-858) | protein truncation |
| *spvB* | Mono-ADP-ribosyl transferase / virulence plasmid | - [a] | - |
| *spvC* | MAPK phosphothreonine lyase / virulence plasmid | - | - |

[a] No amplicon after polymerase chain reaction

**Table 3.4. Minimal inhibitory concentration (MIC) against *gyrA* variant**

| | Enrofloxacin(μg/mL) | Ofloxacin(μg/mL) |
|---|---|---|
| **SG9R** | 0.0625 | 0.25 |
| **SG002** | 0.125 | 0.25 |
| **SR2-N6** | 2 | 2 |
| **ATCC29213** | 0.25 | 0.25 |

**Table 3.5. Over- and under- expressed proteins of SR2-N6 compared to the parent strain, SG002**

| Expression | Fold | NCBI BLAST | Protein name | Mass | Score |
|---|---|---|---|---|---|
| Up-expressed | 2 | gi\|16766418 | Acetyl-CoA hydrolase [*Salmonella*Typhimurium LT2] | 48,392 | 157 |
| | 2 | gi\|16759749 | DNA starvation/stationary phase protection protein (Dps) [*Salmonella*Typhi CT18] | 18,706 | 213 |
| Down-expressed | 268 | gi\|213855344 | Cysteine synthase A [*Salmonella*Typhi M223] | 30,738 | 157 |
| | 372 | gi\|213648617 | Chaperonin GroEL [*Salmonella*Typhi J185] | 31,991 | 126 |

**Table 3.6. Detection of anti-D group O-antigen antibody of chickens immunized with different *Salmonella* vaccines**

| Groups | Samples | No. of serum samples | No. of positives[a] (%) |
|---|---|---|---|
| Experimental | SPF | 27 | 0 (0) |
| | SE6[b] | 20 | 16 (80) |
| | SR2-N6[c] | 10 | 0 (0) |
| | SG 9R[d] | 4 | 1 (25) |
| | Safe 9R | 10 | 2 (20) |
| Field | Vaccinated with SG 9R | 90 | 3 (3.3) |
| | Un-vaccinated with SG 9R | 27 | 0 (0) |

[a] Biochek ELISA kit was used for antibody detection (1/500 diluted serum samples).

[b] Oil emulsion vaccine of heat inactivated *Salmonella* serovar Enteritidis (SE6, 1:1 oil emulsion with complete Freund's adjuvant; 65℃ 120min; $1.0x10^{10}$cfu/ck; 2 times inoculation via intramuscular route).

[c] $2.8x10^{7}$cfu/ck was inoculated via subcutaneous route.

[d] $6.3x10^{7}$cfu/ck was inoculated via subcutaneous route.

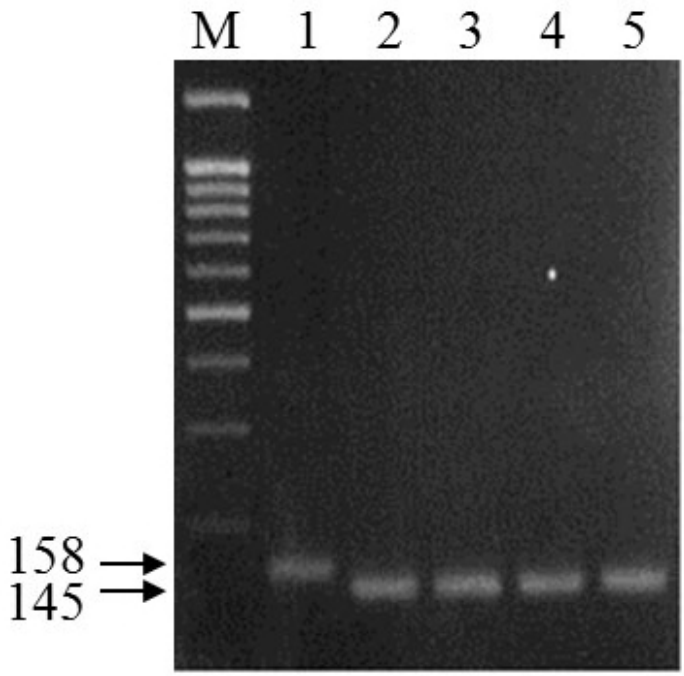


**Figure 3.1. Deletion of *rfaL* of SR2-N6 (1-815).** Lanes: M, 100 bp DNA ladder; 1, SG002; 2, SR2-N6; 3, SR2-N6-p5; 4, SR2-N6-p10; and 5, SR2-N6-p20.

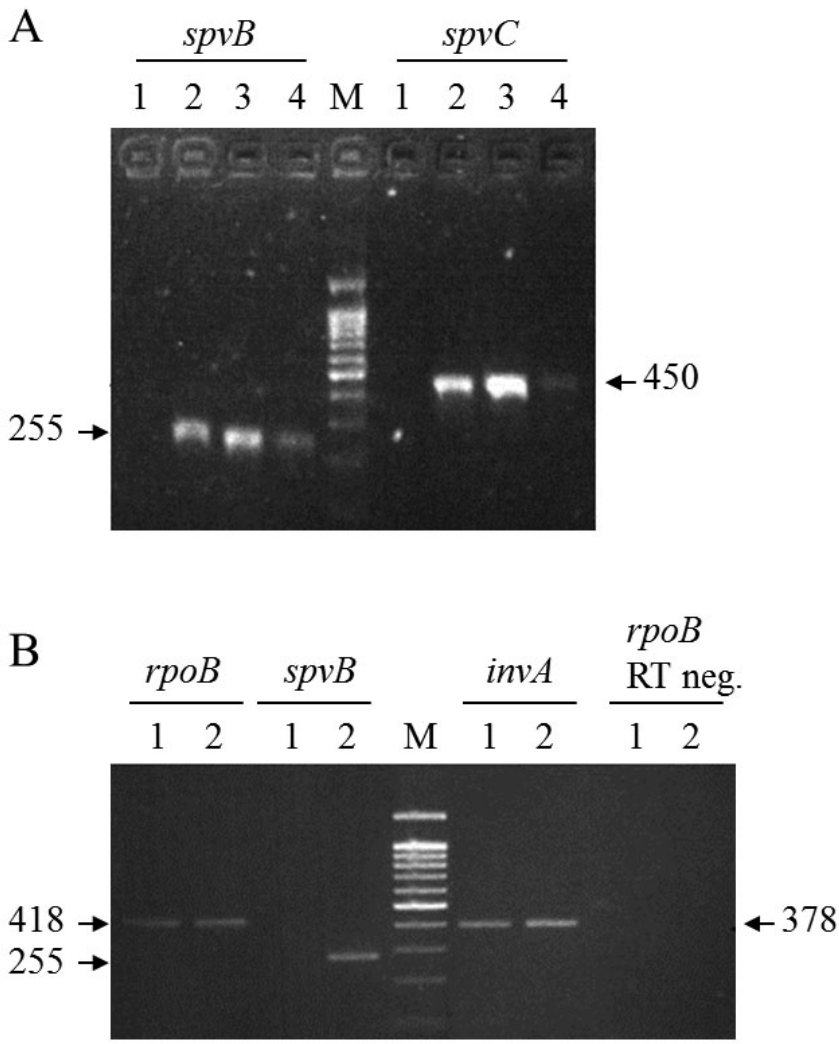


**Figure 3.2. Electrophoretic analysis of virulence gene in SG002 and SR2-N6 after PCR and RT-PCR.** Amplification of the *spvB* and *spvC* genes from genomic DNA (lanes 1, SR2-N6; 2, SG002; 3, SG9R; 4, SE38) (panel A). Amplification of the *rpoB*, *spvB* and *invA* genes from total RNA of SR2-N6 (lane 1) and SG002 (lane 2), along with the control *rpoB* RT neg. (*rpoB* amplification without reverse transcriptase) (panel B).

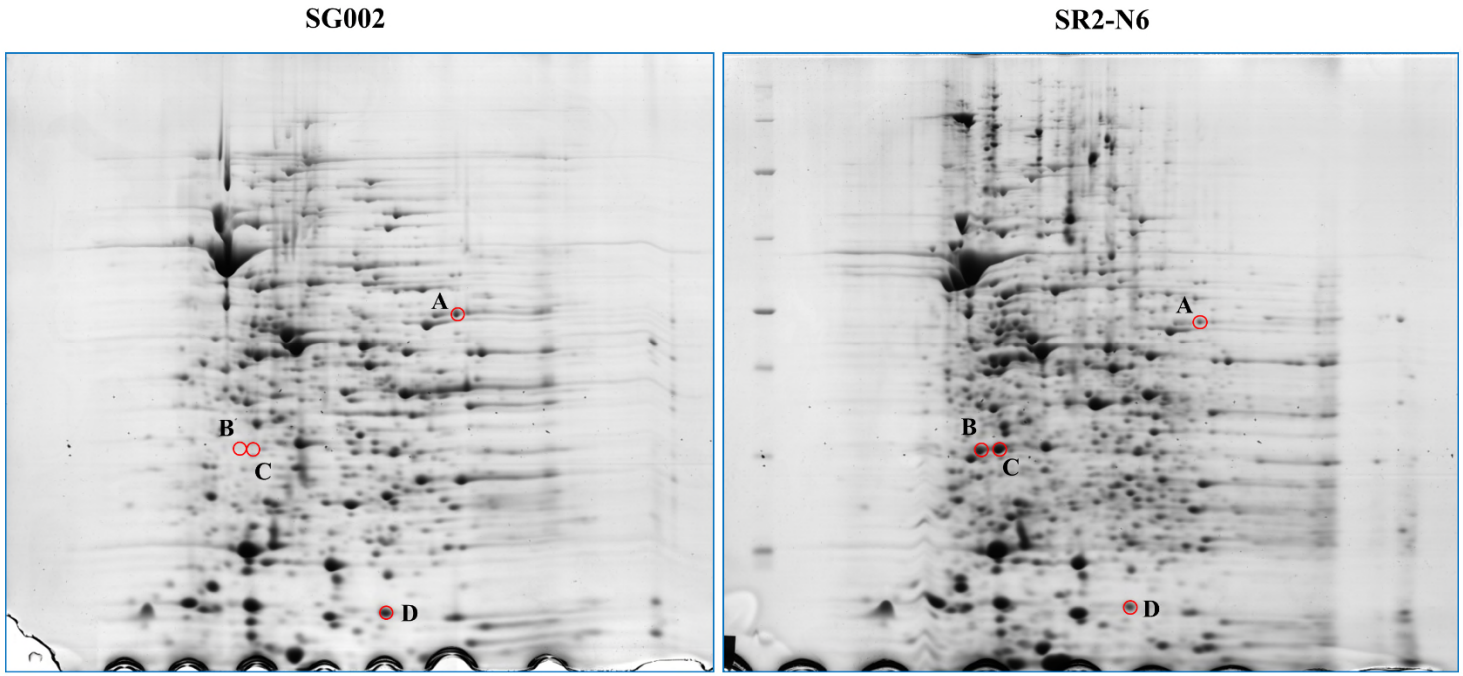


**Figure 3.3. Distinct spots selected in the 2DE electrophoresis.** The spots were analyzed by MALDI TOF and LC-MS/MS. The spots were determined to be (A) acetyl-CoA hydrolase, (B) chaperonin GroEL, (C) cysteine synthase A, and (D) DNA starvation/stationary phase protection protein.

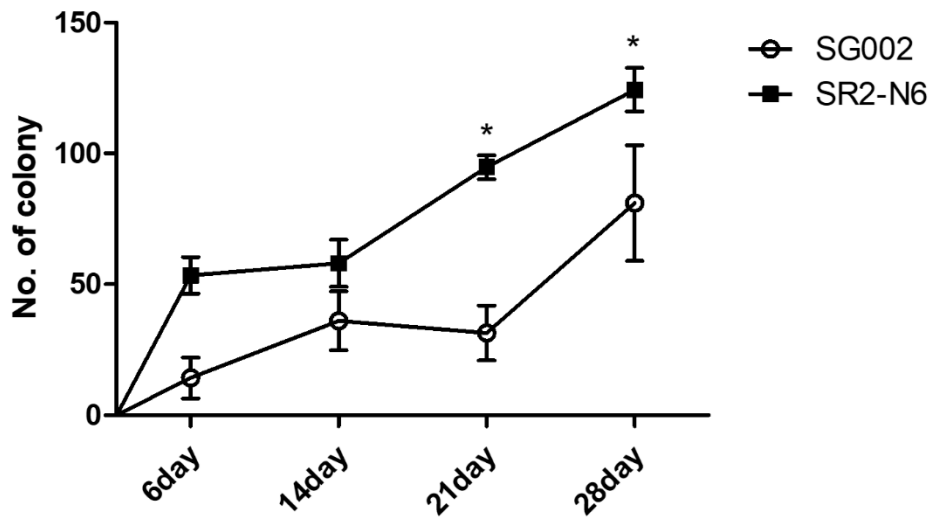


**Figure 3.4. Comparison of cell viability of SG002 and SR2-N6 at 4°C.** The viability of the bacteria stored at 4°C was measured weekly, and it was confirmed that a significant difference appeared after 21 days. After confirming that the data on the 21st and 28th days followed normality with the Shapiro-Wilk test, significance was confirmed with a t-test ($p < 0.001$).

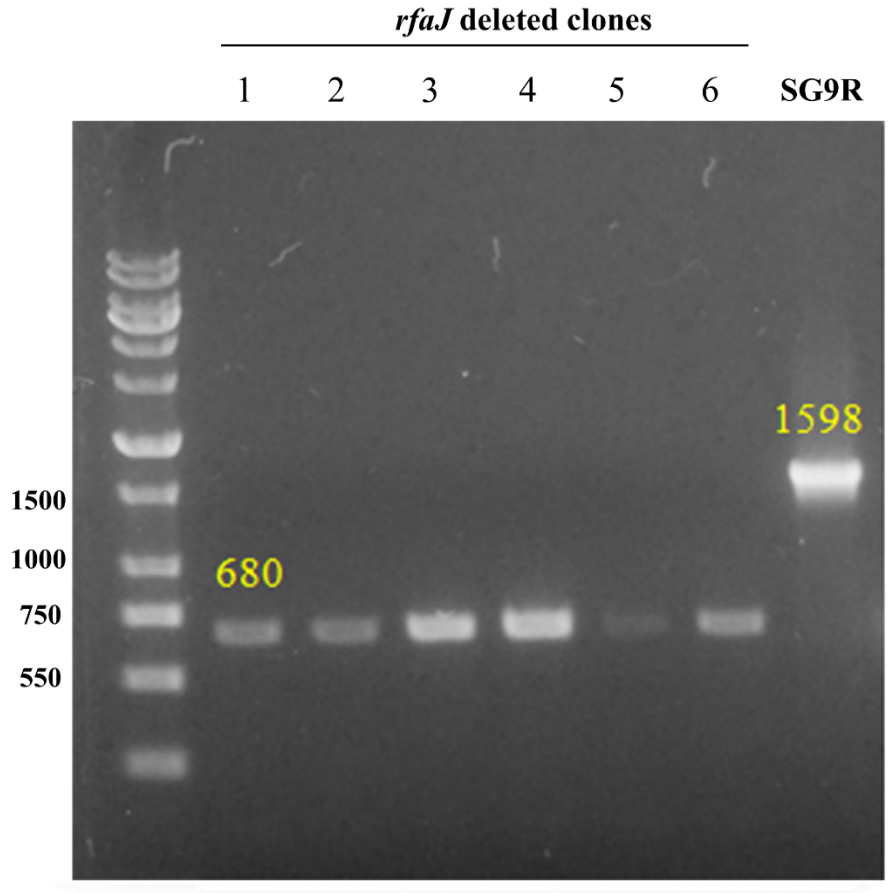


**Figure 3.5. Electrophoretic analysis of the *rfaJ*-deleted mutants.** The deletion of *rfaJ* led to a 918 bp smaller gene size. The PCR primers were designed more broadly to include the entire *rfaJ* gene (Table 3.2).

# Chapter IV

# Optimized detoxification of live attenuated vaccine SG9R to improve vaccine strategy against fowl typhoid

# Abstract

The live attenuated vaccine strain, SG9R, has been used against fowl typhoid worldwide, but it can revert to the pathogenic smooth strain owing to single nucleotide changes such as nonsense mutations in the *rfaJ* gene. As SG9R possesses intact *Salmonella* plasmid with virulence genes, it exhibits dormant pathogenicity and can cause fowl typhoid in young chicks and stressed or immunocompromised brown egg-laying hens. To tackle these issues, we knocked out the *rfaJ* gene of SG9R (named Safe-9R) to eliminate the reversion risk and tested the vaccine efficacy of live and inactivated oil emulsion (OE) Safe-9R vaccines. Live Safe-9R vaccines demonstrated similar protection efficacies to the SG9R vaccine. However, since inoculation of OE Safe-9R caused severe body weight loss in chicks, we generated detoxified strains of Safe-9R by knocking out *lpxL*, *lpxM, pagP,* and *phoP/phoQ* genes. Among the knockout strains, live *ΔlpxL*- (Dtx-9RL) and *ΔlpxM*-9R (Dtx-9RM) strains induced remarkably less expression of inflammatory cytokines in chicken macrophage cells (HD11), and OE Dtx-9RL did not cause body weight loss in chicks. OE of Dtx-9RL and Dtx-9RM induced production of similar levels of specific antibodies to those of Safe-9R and SG9R. Live Dtx-9RM exhibited efficacy against field strain challenge in one week without any bacterial re-isolation, while the non-detoxified strains showed development of severe liver lesions and re-isolation of challenged strains. Thus, *Salmonella enterica* serovar Gallinarum biovar Gallinarum was optimally detoxified by knockout of *lpxL* and *lpxM,* and Dtx-9RL and Dtx-9RM might be applicable as OE and live vaccines, respectively, to prevent fowl typhoid irrespective of the age of chickens.

**Keywords**: *Salmonella* serovar Gallinarum biovar Gallinarum, detoxification, vaccines, lipid A, pro-inflammatory cytokines

# 4.1. Introduction

SG9R has long been used to provide protection against fowl typhoid (FT) caused by *Salmonella enterica* serovar Gallinarum biovar Gallinarum (SG) and food poisoning risk posed by *S. enterica* serovar Enteritidis (SE) infection in egg-laying hens in several countries (Lee et al., 2003; Smith, 1956). SG9R was transformed into a rough strain by a nonsense mutation in the *rfaJ* gene (Kwon and Cho, 2011). However, the fact that the attenuation is attributable to only a single point mutation continues to raise concerns regarding pathogenic reversion. Previous studies have demonstrated that the field-isolated SG exhibits the same DNA fingerprints as SG9R, and several SG9R-like rough strains have been reported (De Carli et al., 2017; Kwon and Cho, 2011; Van Immerseel et al., 2013). SG9R not only has the potential to restore pathogenicity, but also has residual pathogenicity of its own. It can be transmitted vertically, and mortality and re-isolation can occur if there is immune suppression by insufficient nutrition or infection by immunosuppressive pathogens (Kwon and Cho, 2011; Silva et al., 1981). Owing to the negative effects on growth rate, SG9R vaccination has usually been recommended after 6 weeks of age, resulting in the lack of implementation of proper measures to protect against FT during the most susceptible period (Kim et al., 2020b; Lee et al., 2005).

Administration of the rough vaccine strain SR2-N6 defective in *spvB* and *spvC* genes did not cause mortality or lesions even after fasting; thus, the residual pathogenicity of SG9R might be related to the intact *spv* locus of a large *Salmonella* virulence plasmid (Cho et al., 2015). Presence of the *Salmonella* endotoxin decreases body weight in broiler

chickens, and lipid A is the core of the toxic moiety (Blatteis, 1974; Xie et al., 2000). To detoxify lipid A, multiple enzymes involved in the modification of a lipid A precursor, lipid $IV_A$, have been targeted in *Escherichia coli* and *Salmonella* serovar Typhimurium (Lee et al., 2009; Vaara and Nurminen, 1999). When the *lpxL* or *lpxM* gene is removed, the penta-acyl chain is generated, and the double mutant of *lpxL* and *lpxM* harbors tetra-acylated lipid A (Clementz et al., 1996; Clementz et al., 1997; Vaara and Nurminen, 1999). The penta-acylated lipopolysaccharide (LPS) of *lpxL* and *lpxM* mutants express reduced toxicity of LPS, resulting in the attenuation of virulence. These attenuated bacteria showed potential as vaccine candidates (Feodorova et al., 2007; Fisseha et al., 2005). It is challenging to achieve an optimal attenuation of the SG vaccine, for induction of sufficient protective efficacy against the fatal field strain, and to circumvent mortality and persistent infection in young chicks and immunocompromised chickens.

In this study, Safe-9R was developed by knocking out the *rfaJ* gene of SG9R and examined the efficacy and toxicity of its live and killed vaccines. Furthermore, this study generated mutant strains of Safe-9R by knocking out *lpxL*, *lpxM*, *pagP*, and *phoP/phoQ* (*phoP/Q*) genes, which are involved in lipid A biosynthesis. The toxicity was determined by analyzing the expression level of pro-inflammatory cytokines *in vitro* and by comparing the effects of the modified vaccines on body weight loss in young chicks. Additionally, pathogenicity and protective efficacy were assessed in chickens.

## 4.2. Material and Methods

### Bacteria and experimental chickens

A field strain, SG-SNU5161, showing characteristics similar to those of SG9R, was used to generate knockout mutant strains. A virulent field strain, SG0197, was used to test the efficacy of the vaccines (Cho et al., 2015). The bacteria were cultured in Luria Bertani (LB) broth (Duchefa Biochemie, Groot Bijgaarden, Belgium) by incubating under shaking conditions at 37°C overnight. Bacterial strains used in this study were demonstrated in Table 4.1.

One-day-old male brown Hy-Line layer chicks without SG vaccination were obtained from a farm (Yangji Farm, Pyeongtaek-si, Republic of Korea), and had *ad libitum* access to feed and water. All animal experiments were approved by the Institutional Animal Care and Use Committee of BioPOA Co. (permission number BP-2020-001-1).

### Generation of knock-out mutant strains

Safe-9R and detoxified strains were constructed using the Red/ET recombination kit (Gene Bridges, Heidelberg, Germany) according to the manufacturer's instructions (Zhang et al., 1998). Briefly, for homologous recombination, the homology arms were amplified via PCR by attaching oligonucleotides to target the genes (Table 4.2). The Red/ET plasmid and amplified homology arms were transformed to SG9R or Safe-9R using an GenePulser Xcell (Bio-Rad, Hercules, CA, USA) at 2500 V, 10 μF, and 600 Ω

with 1-mm slit Gene Pulser cuvettes (Bio-Rad). Transformed bacteria were selected using LB agar with tetracycline or kanamycin.

## Analysis of mutants

All generated mutants were confirmed via PCR and sequencing. Bacterial genomic DNA was extracted using the G-spin Genomic DNA Extraction Kit for Bacteria (iNtRON Biotechnology, Seongnam-si, Republic of Korea) and PCR was conducted under the following conditions: 1 μL of the template DNA (50 ng/μL), 3 μL of 10X buffer, 3 μL of dNTPs (5 mM), 0.5 μL of each primer (10 pmol/μL), and 0.25 μL of Taq polymerase (MGmed, Seoul, Republic of Korea). The thermal cycling conditions were as follows: 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and 72°C for 5 min. PCR amplicons were purified using a PCR/Gel Purification Kit (MGmed), and sequencing was performed using the ABI 3711 automatic sequencer (Cosmogenetech, Seoul, Republic of Korea). Nucleotide sequences were translated and compared using the BioEdit program version 7.2.5. Primers used in this study are described in Table 4.2.

Safe-9R and SG9R were also subjected to next-generation sequencing, which was performed as per methods described previously (Kim et al., 2019). Briefly, the extracted genomic DNA was sequenced using the HiSeq 2000 platform (Illumina, San Diego, CA, USA) and the filtered data were mapped using BWA version 0.7.12 to *S. enterica* serovar Gallinarum str. 287/91 (GenBank Accession Number NC_011274.1) in the National Center for Biotechnology Information database. The genes that differed between Safe-9R and SG9R were identified.

## Analysis of pro-inflammatory cytokine expression in HD11 cells

The chicken macrophage cell line, HD11, was cultured using the Roswell Park Memorial Institute Medium (RPMI) 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) modified with L-glutamine and phenol red and supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific). Two days before the *Salmonella* infection, aliquots of the HD11 cell suspension ($1 \times 10^6$ cells/mL) were seeded into each well of a 24-well plate (SPL Life Sciences, Pocheon-si, Republic of Korea) at a volume of 500 μL/well and cells were allowed to grow to approximately 85% confluence.

The overnight cultured bacteria were adjusted to an optical density of 0.2 at 600 nm and a tenfold dilution was performed using phosphate-buffered saline (PBS) to obtain multiplicity of infection of 10. The diluted bacterial suspension was centrifuged at 11,000 g for 1 min and resuspended in RPMI 1640. The bacterial suspensions were inoculated after washing the cells twice with the medium and incubated for 2 h at 37℃ in a 5% $CO_2$ incubator. At 2 h post-infection, the cells were washed once and incubated with 150 μL/mL of gentamicin sulfate (Duchefa Biochemie)-supplemented medium for another 2 h. After washing the cells with the medium, total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from equal amounts of total RNA using the amfiRivert cDNA Synthesis Platinum Master Mix (GenDEPOT, Katy, TX, USA).

Quantitative PCR (qPCR) analysis was performed to compare the expression of pro-inflammatory cytokines (interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-18), LPS-recognizing receptor (Toll-like receptor-4 (TLR-4)), and inducible nitric oxide synthase (iNOS). Briefly, 10-μL reaction mixtures contained 5 μL of 2X AMPIGENE

qPCR Green Mix Hi-ROX (Enzo Life Sciences, Farmingdale, NY, USA), 0.5 μL each of the forward and reverse primers, and 1 μL of cDNA. The normalization was performed using glyceraldehyde 3-phosphate dehydrogenase. Primers used in qPCR are listed in Table 4.2 (Han et al., 2017; Rajput et al., 2014; Rychlik et al., 2009). The mRNA expression levels of each pro-inflammatory cytokine were compared using the $2^{-\Delta\Delta Ct}$ method.

## High temperature sensitivity test

The detoxified strains *ΔlpxL* and *ΔlpxM* were evaluated for proliferation at 42°C using Safe-9R as a control. Each vaccine type was inoculated as a single colony on MacConkey agar and in LB broth and examined the growth.

## Protective efficacy and toxicity test of Safe-9R

Live Safe-9R was inoculated at 6 and 18 weeks at $1 \times 10^7$ colony forming units (cfu)/chicken, and 4 weeks after the second vaccination, the pathogenic field strain SG0197 ($1 \times 10^6$ cfu/chicken) was challenged *per os*.

The oil emulsion (OE) Safe-9R vaccine was prepared by heat inactivation of Safe-9R at 65°C for 2 h in a water bath, followed by gradual cooling to room temperature and emulsification of bacteria and oil adjuvant (Montanide ISA 70, Seppic, La Garenne-Colombes, France) at a ratio of 3:7. OE Safe-9R was administered via the intramuscular route to 1-week old brown Hy-Line chicks (approximately $1 \times 10^9$ cfu/100 μL/chick). SG0197 was challenged in vaccinated and negative control groups at 2 and 7 weeks post-

vaccination (wpv), respectively, and the serum samples were collected before the challenge. Mortality was observed for 2 weeks, and the chickens were subjected to fasting conditions for three days to detect persistently infected chickens using the protein-energy malnutrition (PEM) model (Kwon and Cho, 2011).

## Humoral immune response and weight change after administration of OE detoxified strains

The detoxified OE vaccines were prepared as per the methods described for OE Safe-9R. They were diluted to $3 \times 10^9$ cfu/100 μL and mixed at a 3:7 ratio with the oil adjuvant Montanide ISA 70. Mixed vaccines were administered via the intramuscular route to fifteen 1-week-old brown Hy-Line chicks in each group, which were divided into *ΔlpxL, ΔlpxM*, Safe-9R, SG9R, and negative control. Blood samples were collected 2 weeks after vaccination. Antibody titers of OE vaccines were evaluated using OmpA and OmpX peptide ELISA, and outer membrane protein (OMP) extract ELISA as per previously described methods (Kim et al., 2020b). OE vaccines were inoculated into 1- and 2-week-old chicks, and the body weight was measured weekly for 2 weeks after vaccination.

## Efficacy and pathogenicity of live detoxified strains

The detoxified live vaccines were administered to 1-week-old brown Hy-Line chicks (15 per group), and the group was the same as the OE inoculation. They were diluted to $1 \times 10^7$ cfu/100 μL and were administered via the subcutaneous route. One week after

the vaccination, in each group, ten chickens were infected orally with the pathogenic field strain SG0197 at an inoculum of $1 \times 10^6$ cfu/chicken, and the remaining five chickens were tested for the PEM model. Re-isolation of vaccine strains in the group subjected to fasting was performed by scrubbing a sterile cut section of the liver on MacConkey agar (Becton Dickinson, Franklin Lakes, NJ, USA). IgA detection in bile juice was performed as per previously described methods (Kim et al., 2020b).

## FACS

The detoxified live vaccines were administered to 1-day-old brown Hy-Line chicks (10 per group; as mentioned above). Whole blood samples were collected in heparin-containing tubes and pooled by group. Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep (Axis Shield, Dundee, Scotland) and washed with PBS supplemented with 2% FBS (Thermo Fisher Scientific). The number of PBMCs was counted and adjusted to a density of $10^6$ cells/mL. Three-microliter volume of $CD8^+$ T-cell antibody-fluorescein isothiocyanate (LSBio, Seattle, USA) and CD4 T-cell antibody-allophycocyanin (SouthernBio, Bermingham, USA) was inoculated into fifty-microliter aliquot of cells from each group, and another fifty-microliter aliquot was prepared as a control. All aliquots were incubated for 15 min on ice in the dark. After incubation, the cells were washed and resuspended in 300 μL of PBS. Samples were analyzed using FACSCalibur (Becton Dickinson).

## Statistical analysis

Statistical analyses were performed using SPSS Statistics version 26.0 (IBM, Chicago, IL, USA). One-way analysis of variance was used to analyze the significant differences between the groups along with the Bonferroni post-hoc test. The significance of the group that followed the normality but violated equal variance was confirmed using the Games-Howell test. Data that did not follow a normal distribution were analyzed using the Kruskal-Wallis *H* test, and the Bonferroni correction was used as the post-hoc test. *P* value less than 0.05 was considered statistically significant.

## 4.3. Results

### Generation and confirmation of Safe-9R

*rfaJ* gene was knocked out from the SG9R and the knock out was confirmed by genome sequencing after PCR amplification of the gene (Fig 4.1A). The knockout strain was named as Safe-9R and compared its genome sequences with those of SG9R by re-sequencing. There were no differences between the two strains except for the *rfaJ* gene (data not shown).

### Generation and characterization of gene knock-out strains for detoxification

We knocked out target genes (*phoP*/*Q*, *lpxL*, *lpxM*, and *pagP*) by replacing the antibiotic resistance gene and the amplicons of target genes of Safe-9R; as a result, the knockout strains were the same in size (Fig 4.1B). The toxic effects of the knockout strains were compared using reverse transcription quantitative PCR of *IL-1β, IL-18, TNF-α, iNOS,* and *TLR-4* mRNAs. The mRNA levels of *TNF-α* were not significantly different between the knockout strain-infected HD-11 cells and uninfected HD-11 cells. However, the *IL-1β, IL-18,* and *iNOS* mRNA levels of *phoP/Q*- and *pagP*-knockout strains, and Safe-9R were significantly higher than those of *lpxL*- and *lpxM*-knockout strains (named Dtx9RL and Dtx-9RM, respectively), and negative control. Only Safe-9R showed significantly higher *TLR-4* mRNA levels than the negative control (Fig 4.3). Therefore,

live Dtx9RL and Dtx-9RM were successfully detoxified and did not induce the transcription of *IL-1β, IL-18, iNOS*, and *TLR-4* genes.

Dtx-9RL, Dtx-9RM, and Safe-9R proliferated indistinguishably well in broth at 42°C, but Dtx-9RL and Dtx-9RM showed poor proliferation on the surface of MacConkey agar. In contrast to Safe-9R colonies, Dtx-9RL and Dtx-9RM were barely visible on the first day of incubation but were visible on the second day of incubation (Table 4.4).

## Protective efficacy and humoral immune response of Safe-9R

Live Safe-9R vaccines were markedly protective against the fatal challenge of field strain, resulting in no mortality in the vaccinated group in contrast to 100% mortality in the unvaccinated group (10 chickens per group). Although the survival rate of one out of the three experiments confirmed significance, the OE Safe-9R vaccination in 1-week-old chicks protected against mortality in fatal challenge at 2 wpv. However, the OE Safe-9R vaccination did not protect from mortality at 7 wpv and even showed lower survival rates in vaccinated than unvaccinated groups in two (50 versus 90 and 70 versus 90) out of the three experiments (Table 4.3). Interestingly, anti-OmpA and anti-OmpX antibody levels in the vaccinated group at 2 wpv were significantly higher ($P<0.05$) than those of the unvaccinated group, similar to the anti-OMP antibody levels (Fig 4.2A). However, only the anti-OMP antibody level in the vaccinated group at 7 wpv was significantly higher than that of the negative control group (Fig 4.2B).

## In vivo verification of detoxification

OE vaccines Dtx-9RL, Dtx-9RM, and Safe-9R were administered to 2-week-old chicks and their body weights were measured every week for 2 weeks. Only Safe-9R-vaccinated chicks showed significantly lower body weight than the negative control ($P < 0.05$; Fig 4.4B). To differentiate body weight changes between detoxified strains, we performed the same experiment using 1-week-old chicks and including SG9R. The body weight of Dtx-9RL-vaccinated chicks was not significantly different from that of the negative control, but body weights of chicks vaccinated with Dtx-9RM, Safe-9R, and SG9R were significantly lesser than those of the negative control. Therefore, detoxification of Dtx-9RL was apparent in the 1-week-old chick body weight model (Fig 4.4A).

## Humoral immunogenicity of OE vaccines of detoxified strains

The humoral immunogenicity of OE vaccines Dtx-9RL and Dtx-9RM was compared with that of Safe-9R and SG9R in 1-week-old chicks. The anti-OmpA, anti-OmpX, and anti-OMP antibody levels in all the vaccinated groups were significantly higher than those in the negative control group at 2 wpv and OE vaccines of the detoxified strains exhibited similar immunogenicity to Safe-9R and SG9R (Fig 4.5).

## Evaluation of residual pathogenicity and protective efficacy of live detoxified strains

The residual pathogenicity of live detoxified and undetoxified strains was compared in the PEM model. No lesions and mild lesions in the liver were observed with the administration of Dtx-9RL and Dtx-9RM, respectively, and were negative for bacterial re-isolation. However, Safe-9R and SG9R caused moderate to severe lesions and were positive for bacterial re-isolation (4/5 and 3/5), respectively (Table 4.5).

The protective efficacies of live detoxified strains were evaluated using the radical challenge model. Field strain challenge at 1 wpv caused 10% and 20% mortality in the Dtx-9RL-vaccinated and negative control groups, respectively. When autopsied, two chickens in the Dtx-9RL-vaccinated group showed severe and mild lesions, but all chickens in the Dtx-9RM-vaccinated group showed only mild lesions. However, Safe-9R and SG9R-vaccinated groups and the negative control group showed significantly higher frequency of moderate to severe liver lesions (80%, 60%, and 60%, respectively) than the Dtx-9RM-vaccinated groups (Fig 4.7). The grading of liver lesions is shown in Figure 4.6.

The humoral and mucosal antibody levels were compared, and Dtx-9RM-, Safe-9R-, and SG9R- vaccinated groups showed significantly higher levels than those of the negative control, but Dtx-9RL-vaccinated groups did not exhibit such levels (Fig 4.8). Dtx-9RL- and Dtx-9RM-vaccinated chickens showed a significantly higher percentage of $CD8^+$ T cells in PBMCs collected at 1 wpv compared to those observed in the negative control. However, Dtx-9RL had a lower increase even after the challenge than other vaccinated groups. Although not significant, Safe-9R-vaccinated groups and SG9R-vaccinated groups showed higher percentages of $CD8^+$ T cells than Dtx-9RL-vaccinated groups and negative control groups. After the challenge, Dtx-9RL responded rapidly than

the negative control showing an increased percentage of both T cells in 1wpv but did not rise as high as other vaccine groups. Safe-9R and Dtx-9RM showed a significantly increased percentage of both $CD4^+$ and $CD8^+$ T cells after the challenge (Fig 4.9).

## 4.4. Discussion

SG9R and SR2-N6 have been used to provide protection in chickens against FT, and paratyphoid caused by SE (Cho et al., 2015; Feberwee et al., 2001). The underlying reason of SG vaccines showing cross-protective efficacy to SE may be attributed to the intimate genetic relationship between SG and SE, and the competitive exclusion of SE by SG (Matthews et al., 2015; Rabsch et al., 2000). Since SG9R was permitted for use in commercial layer farms in 2001, food poisoning cases caused by SE have decreased gradually in the Republic of Korea. Although frequent FT outbreaks in broiler chickens have been reported, they have usually remained unvaccinated. Additionally, SE is frequently isolated in broiler chickens in the Republic of Korea (Cheong et al., 2007; Kim, 2010). In the EU, SG9R was used to reduce food poisoning cases caused by SE, until FT outbreaks were reported in SG9R-vaccinated layer farms (Van Immerseel et al., 2013). Although field pathogenic isolates originating from SG9R due to a single point mutation in *rfaJ* has never been reported, SG9R has been shown to cause FT in immunocompromised flocks (Kwon and Cho, 2011; Wigley et al., 2005). Therefore, removal of this reversion risk and maintenance of protective efficacy without residual pathogenicity are crucial for successful SG9R vaccination.

In this study, the reversion risk of SG9R was eliminated by knocking out *rfaJ* and demonstrated that the live Safe-9R vaccine was as efficacious as SG9R. OE Safe-9R induced production of significantly high titers of specific antibodies to linear epitopes of OmpA and OmpX, and OMPs at 2 wpv, as previously reported. However, these specific antibodies disappeared at 7 wpv (Fig 4.2) (Kim et al., 2020b). The correlation of higher

anti-OmpA and anti-OmpX antibodies with protective efficacy may highlight their immunoprotective roles. Even though significantly high anti-OMP antibody levels were apparent at 7 wpv, this observation did not highlight efficacy or showed even worse outcomes than the control group. This observation may be reminiscent of antibody-dependent enhancement (ADE), which is defined as the suppression of host defense by the immune complex composed of specific IgG and antigen, and the possibility of ADE in *Salmonella* has been reported (Halstead et al., 2010). Therefore, further studies on the protective efficacy of subunit vaccines composed of OmpA and OmpX epitopes and the relation of OMP antibodies against ADE should be conducted. The negative effect of OE Safe-9R on the body weight of young chicks necessitates the development of increased detoxified vaccine strains.

The detoxification of Safe-9R in terms of reduced induction of pro-inflammatory cytokines was apparent when *lpxL* or *lpxM* was knocked out compared to *pagP* and *phoP/Q* in an *in vitro* model (Fig 4.3). The detoxification levels of Dtx-9RL and Dtx-9RM and body weight of 2-week-old chicks were indistinguishable in the *in vitro* model, but they were clearly differentiated by the body weight in 1-week-old chicks (Fig 4.4). To date, endotoxin is known to be the cause of reduced body weight in chickens, and this study demonstrated that knockout of *lpxL* was the best strategy to eliminate these side effects (Lee et al., 2005).

In the PEM model, Dtx-9RL was also the least pathogenic, showing no lesions or re-isolation in the liver of inoculated chicks. Dtx-9RM showed mild lesions without re-isolation of bacteria, but Safe-9R and SG9R showed moderate to severe lesions with re-

isolation of the challenged strain from the liver. Therefore, different pathogenicity among detoxified strains was also clearly differentiated by the PEM model. Additionally, the significantly higher antibodies in the group administered with OE Dtx-9RL compared to the control group may support the use of OE vaccines for growing young chicks and laying hens that are sensitive to endotoxin.

The protective efficacies of live vaccines of Dtx-9RL and Dtx-9RM were verified in our radical protection model. Safe-9R- and SG9R-vaccinated chickens showed severe lesions after field strain challenge, and re-isolated bacteria were identified as the challenged field strains. This was an unexpected result and might be attributed to early infection by the field strain after the vaccination. Our previous study demonstrated that competition between field strains with different pathogenicity might result in the dominant isolation of more pathogenic strains over time (Kim et al., 2019). Therefore, pathogenicity of Safe-9R and SG9R may not be overcome by the host immunity within 1 wpv and subsequent challenge may result in the development of more severe lesions and predominant persistent infection by more pathogenic challenge strains. However, the protective efficacy of live Dtx-9RM was demonstrated in terms of bacterial re-isolation rate and severity of lesions. Particularly, the higher $CD8^+$ T-cell stimulation of Dtx-9RL and Dtx-9RM in 1 wpv suggested that the attenuation might lead to exhibition of rapid overcoming by the host, resulting in establishment of earlier protection (Fig 4.9). The priming of mucosal immunity by Dtx-9RM, similar to that observed with Safe-9R and SG9R, was also demonstrated (Kim et al., 2020b). The protective efficacy of Dtx-9RL was not immaculate. Although it showed a lower bacterial re-isolation rate and presence of

lesions, it could not induce production of serum-specific antibodies and establishment of mucosal immunity. However, although the percentage of $CD4^+$ and $CD8^+$ T cells did not surge after the challenge, Dtx-9RL showed a significantly higher value than the negative control in 1wpi, which might interpret the lesser lesions.

Both *lpxL* and *lpxM* are responsible for late secondary acylation, which provide lauroyl and myristoyl groups, respectively, to the different sites of the tetra-acylated intermediate, lipid $IV_A$ (Clementz et al., 1996; Clementz et al., 1997). Therefore, different chemical structures may be variably sensed by the innate and acquired immune systems of chickens. According to previous reports, in contrast to *lpxM,* defects in *lpxL* resulted in a reduced phagocyte resistance and detoxified reaction in the *Limulus* amebocyte lysate test (Clements et al., 2007; Jones et al., 1997; Lee et al., 2009; Mills et al., 2017). Therefore, *in vivo* tests performed in this study may be useful strategies to differentiate *lpxL*- and *lpxM*-knockout mutants. The attenuation of Dtx-9RL and Dtx-9RM can also be explained by the reduced growth rate at the normal body temperature of chickens at 42°C. The vulnerability of the *lpxL* mutant to high growth temperature has already been reported in *E. coli* and *Salmonella* serovar Typhimurium, but this study observed a similar effect of the *lpx*M-knockout mutation in SG (Karow and Georgopoulos, 1992; Sunshine et al., 1997).

In conclusion, optimal vaccine strains were developed that were safe and highly protective even in young chicks, and they might be useful for providing protection against FT as well as paratyphoid caused by SE in poultry.

**Table 4.1. Bacterial strains used in this study**

| Strain | Description |
|---|---|
| SG-SNU5161 | An isolate similar to SG9R |
| SG0197 | A virulent field isolate of *Salmonella* Gallinarum |
| Safe-9R | *ΔrfaJ* mutant of SG-SNU5161 |
| *ΔphoP/phoQ* | *ΔrfaJ, ΔphoP/phoQ* mutant ofSG-SNU5161 |
| *ΔlpxL* | *ΔrfaJ, ΔlpxL* mutant of SG-SNU5161; Dtx-9RL |
| *ΔlpxM* | *ΔrfaJ, ΔlpxM* mutant of SG-SNU5161; Dtx-9RM |
| *ΔpagP* | *ΔrfaJ, ΔpagP* mutant of SG-SNU5161 |

**Table 4.2. Primers used in this study.**

| Primers | 5' to 3' | Purpose | Reference |
|---|---|---|---|
| *rfaJ* deletion F | CTTTAAACGTAAACTTCTTGAATAAA ACCCATAGGTGATGTAATGGATTAAA TTAACCCTCACTAAAGGGCG | *rfaJ* deletion | This study |
| *rfaJ* deletion R | AGTTTTTAATCTTTTTTTCAATAATCA TAATAGAGATTTAGGCAGGGGAATA ATACGACTCACTATAGGGCTC | | |
| *phoP/phoQ* deletion F | CAACGCTAGACTGTTCTTATTGTTAA CACAAGGGAGAAGAGATGATGCGC AATTAACCCTCACTAAAGGGCG | *phoP/phoQ* deletion | |
| *phoP/phoQ* deletion R | ATAACGGATGCTTAACGAGATGCGT GGAAGAACGCACAGAAATGTTTATT TAATACGACTCACTATAGGGCTC | | |
| *lpxL* deletion F | CAAAAAGATGCGAGAATACGGGGA ATTGTTCGTTGAAAGACAGGATAGA AAATTAACCCTCACTAAAGGGCG | *lpxL* deletion | |
| *lpxL* deletion R | AAAGCTAAAAGAGGGGAAAAATTG CAGCCTGACGGCTGCAATCCTGTCA ATAATACGACTCACTATAGGGCTC | | |
| *lpxM* deletion F | GACGTCGCTACACTATTCACAATTCC TTTTCGCGTCAGCAGACCCTGGAAA ATTAACCCTCACTAAAGGGCG | *lpxM* deletion | |
| *lpxM* deletion R | CATCAGGTAGTACAGGGTTTGTCAG CATAAAGCCTCTCTTACGAGAGGCTT AATACGACTCACTATAGGGCTC | | |
| *pagP* deletion F | TATTCAGGTTAATGTTGTTATTATCAC AGTCGAATTTTTGAACGGTATGTAAT TAACCCTCACTAAAGGGCG | *pagP* deletion | |
| *pagP* deletion R | GGCTTTTTAATTCACAACAGAACAAT GCCCTTCTCCGTCAAAACTGGAAAT AATACGACTCACTATAGGGCTC | | |
| *phoP/phoQ* F | CTGTTTATCCCCAAAGCACC | Deletion confirmation | |
| *phoP/phoQ* R | GCGAGAGCGGATCAATAAAG | | |
| *lpxL* F | GCTCAACGCAAAAAGATGCG | | |
| *lpxL* R | AGGGTGACATAGCGTTCCAC | | |
| *lpxM* F | CGATTAACAAATGCGCTGAC | | |
| *lpxM* R | GTTCAACCAATACCACGCGT | | |
| *pagP* F | CGCCGTTAACCCGATACTCT | | |
| *pagP* R | GCTGTGTCGGATACCAGTAC | | |

| | | | |
|---|---|---|---|
| *rfaJ* F | TCCAGTCGATGCTGATACTG | | |
| *rfaJ* R | GTAAACCCTTCTCGCCGAAC | | |
| *TNF-α* F | CCCCTACCCTGTCCCACAA | qPCR | Han et al. (2017) |
| *TNF-α* R | TGAGTACTGCGGAGGGTTCAT | | |
| *GAPDH* F | CCCCAATGTCTCTGTTGTTGAC | | |
| *GAPDH* R | CAGCCTTCACTACCCTCTTGAT | | |
| IL-1β F | GCTCTACATGTCGTGTGTGATGAG | | |
| *IL-1β* R | TGTCGATGTCCCGCATGA | | |
| *iNOS* F | GCATTCTTATTGGCCCAGGA | | |
| *iNOS* R | CATAGAGACGCTGCTGCCAG | | |
| *IL-18* F | ACGTGGCAGCTTTTGAAGAT | | Rychlik et al. (2009) |
| *IL-18* R | GCGGTGGTTTTGTAACAGTG | | |
| *TLR-4* F | GGCAAAAAATGGAATCACGA | | Rajput et al. (2013) |
| *TLR-4* R | CTGGAGGAAGGCAATCATCA | | |

**Abbreviations:** TNF-α, tumor necrosis factor-α; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; iNOS, inducible nitric oxide synthase; TLR-4, Toll-like receptor-4.

**Table 4.3. Protective efficacy test[a] of OE Safe-9R**

| Group | Vaccination | Survival rate [b] (%) (No. of survived/No. of vaccinated) | | | Total |
|---|---|---|---|---|---|
| OE Safe-9R – 2wpv[c] | Vaccinated | 60 (6/10) * | 50 (5/10) | 80 (8/10) | 63 (19/30) |
| | Non-vaccinated | 0 (0/10) | 11.1 (1/9) | 50 (5/10) | 21 (6/29) |
| OE Safe-9R[a] – 7wpv[c] | Vaccinated | 87.5 (7/8) | 50 (5/10) | 70 (7/10) | 68 (19/28) |
| | Non-vaccinated | 80 (8/10) | 90 (9/10) | 90 (9/10) | 87 (26/30) |

[a]Oil emulsion (OE) vaccine inoculated in 1 week-old and challenged in 3 week-old (2wpv) and 8 week-old (7wpv), respectively
[b]Survival rate observed for 17 days, including the last 3 days in fasting condition
[c]wpv refers to week-post vaccination
*indicates a significant difference from the negative control ($P$<0.05)

**Table 4.4. High temperature sensitivity test[a]**

| Group | Agar | | | Broth | | |
|---|---|---|---|---|---|---|
| **Sample** | **Dtx-9RL** | **Dtx-9RM** | **Safe-9R** | **Dtx-9RL** | **Dtx-9RM** | **Safe-9R** |
| **Proliferation** | -[b] | -[b] | + | + | + | + |

[a]Single colony of each strain was inoculated into Mac Conkey agar and LB broth and incubated at 42℃ overnight
[b]No visible colony until 2 days after inoculation

**Table 4.5. Recovery of detoxified strains of SG in protein-energy malnutrition model***

| Groups | Dtx-9RL | Dtx-9RM | Safe-9R | SG9R | Neg |
|---|---|---|---|---|---|
| **Lesion**[a] | 0/5 | 5/5 | 5/5 | 5/5 | 0/5 |
| **Recovery**[b] | 0/5 | 0/5 | 4/5 | 3/5 | 0/5 |

*fasted for three days after 2 weeks post-vaccination

[a]Presence of the liver lesions (No. of positive/No. of inoculated)

[b]Recovery of the inoculated strains (No. of recovered/No. of inoculated)

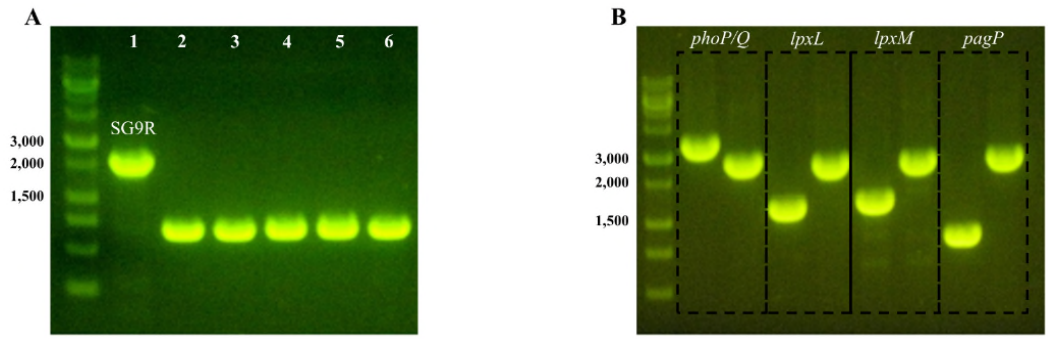


**Figure 4.1. Deletion of *rfaJ* in Safe-9R and detoxified strains.** (A) Deleted mutants of *rfaJ* in Safe-9R and its detoxified strains. Lanes: 1, SG9R; 2, Safe-9R; 3, *ΔphoP/Q*; 4, *ΔlpxL;* 5*, ΔlpxM;* 6*, ΔpagP.* (B) Deleted mutants of lipid A biosynthesis related genes. The dotted rectangles indicate each knocked-out gene confirmed by PCR. Each lane within the rectangles was in the order of Safe-9R and detoxified strains.

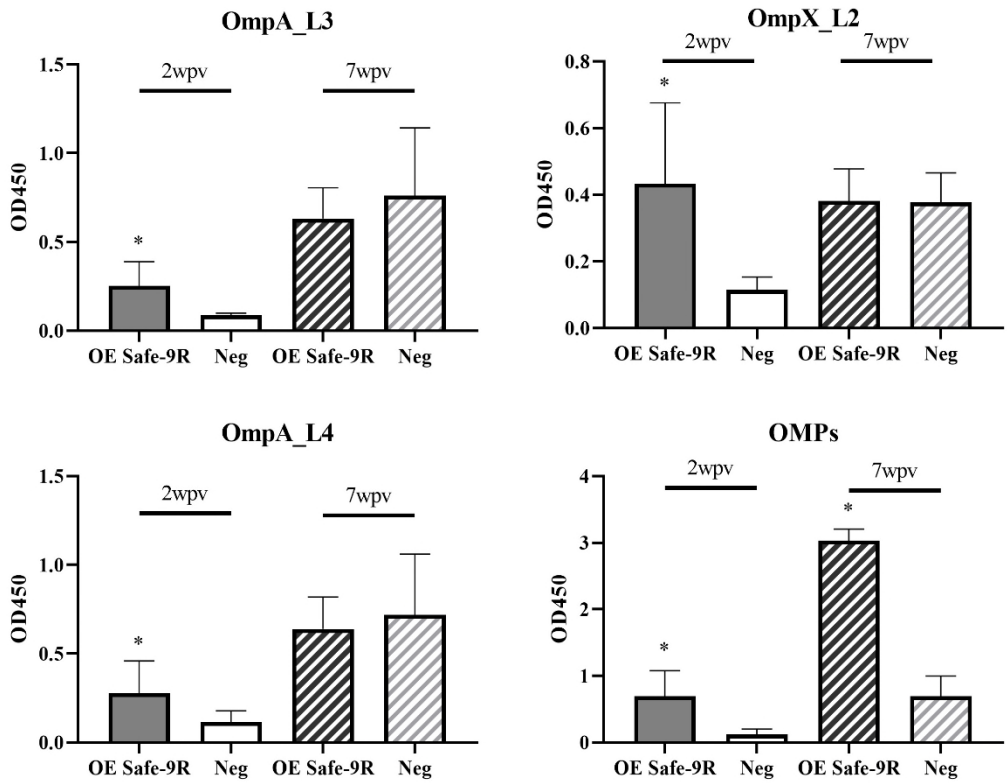


**Figure 4.2. Humoral immune responses of the OE Safe-9R vaccines**. Each group received OE Safe-9R at 1 week of age, and serum samples were collected at (A) 2 weeks post-vaccination (wpv) and (B) 7 wpv, respectively. The immune response was analyzed by ELISA made by SG immunogenic outermembrane proteins (OMP), OmpA and OmpX, and total OMP extracts. *Indicates a significant difference compared with Neg ($P$<0.05).

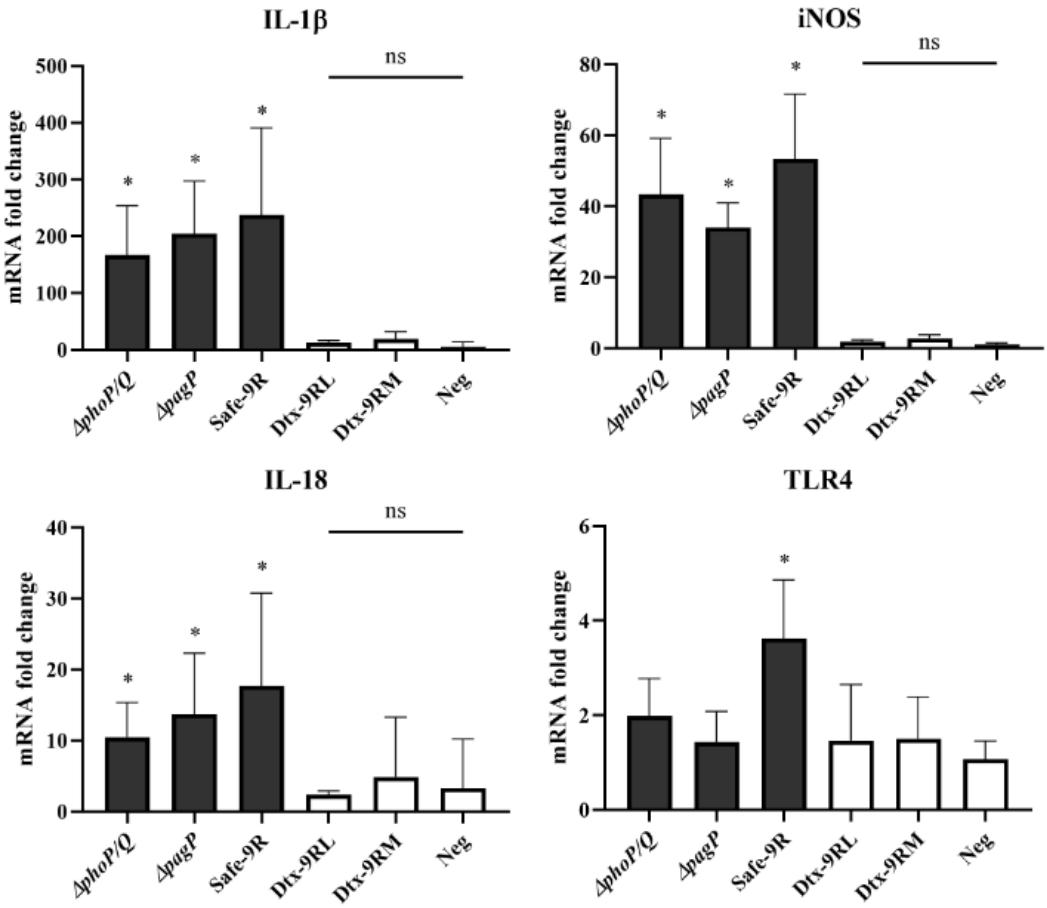


**Figure 4.3. Comparison of *Salmonella enterica* serovar Gallinarum biovar Gallinarum (SG) strains with regard to induction of pro-inflammatory cytokines and related genes in HD11 cells.** Relative comparison of mRNA expression in HD11 was performed using the $2^{-\Delta\Delta Ct}$ method. The significance was compared to the negative control. Statistical significance indicated as follows: *ns* not significant, *significantly different compared with Neg ($P$<0.05).

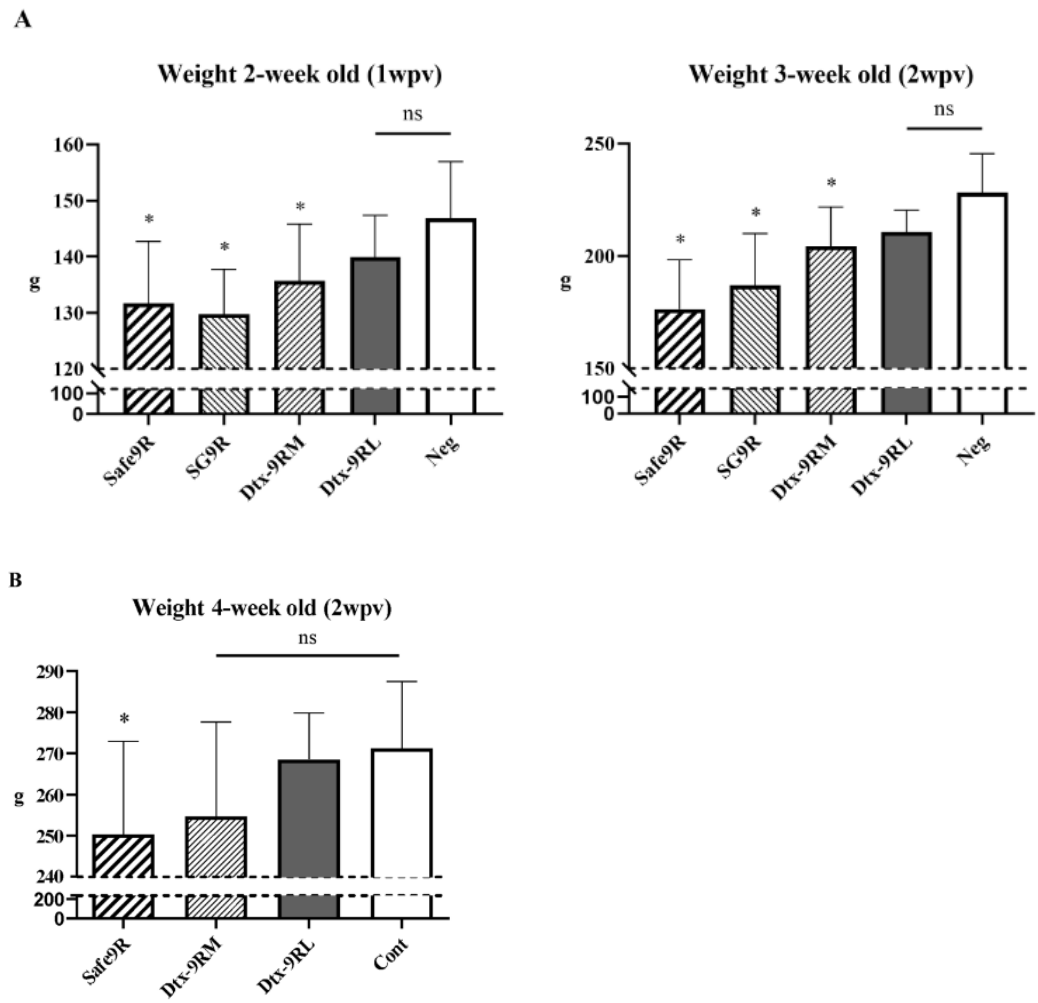


**Figure 4.4.** ***In vivo*** **verification of detoxification by using 1-week-old chick body weight model.** Inactivated vaccines were inoculated in (A) 1-week-old and (B) 2-week-old chicks and the differences in body weight were examined. The significance was compared to the negative control. Statistical significance indicated as follows: *ns* not significant, *significantly different compared with Neg ($P$<0.05).

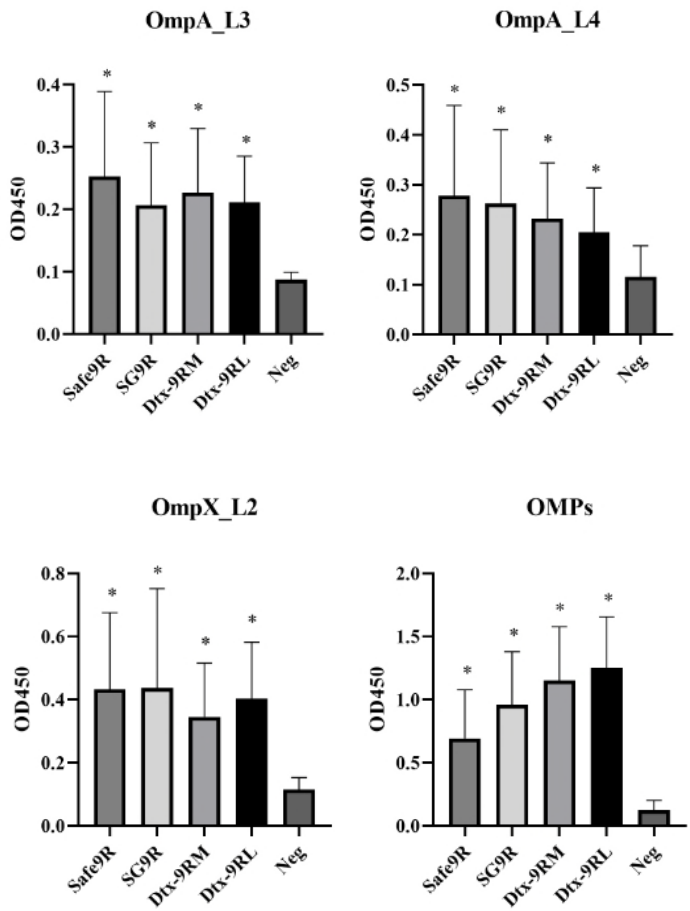


**Figure 4.5. Humoral immunogenicity of oil emulsion (OE) vaccines with the detoxified strains.** Vaccines were inoculated at 1 week of age, and antibody titers were determined after 2 weeks. All groups showed a significant immune response compared to the negative control and there was no difference from SG9R. The significance was compared to the negative control. *Indicates a significant difference compared with Neg ($P$<0.05).

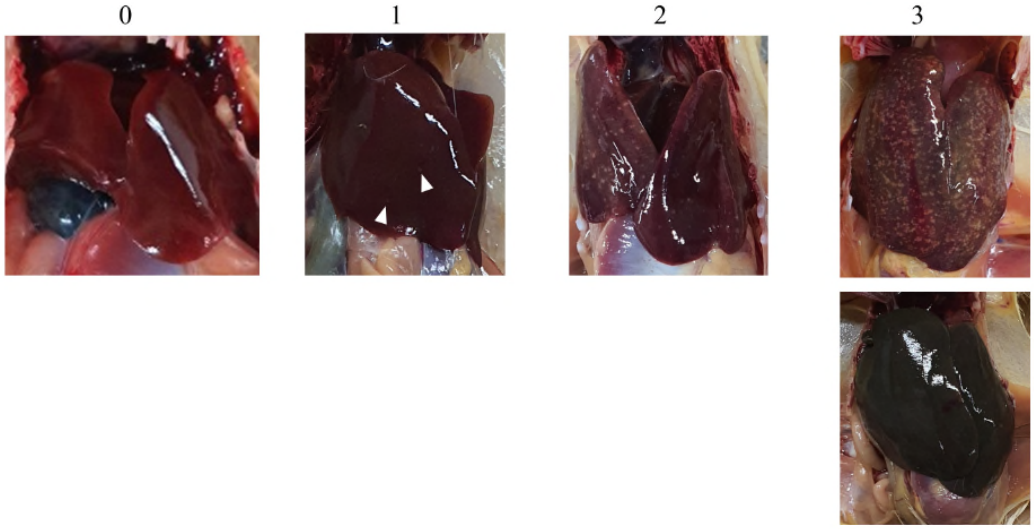


**Figure 4.6. Grading of liver lesions from 0 to 4.** 0: normal, 1: single to several necrotic foci (<5), 2: large and multiple necrotic foci (<100) and hepatomegaly, 3: highly multiple necrotic foci (countless) and severe hepatomegaly, 4: death. The white arrows are indicating mild necrotic foci.

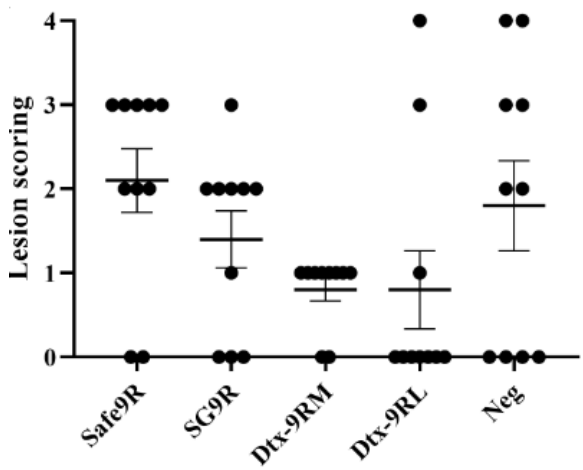


**Figure 4.7. Lesion scoring of live detoxified strains.** Detoxified vaccines were inoculated in 1-week-old and challenged at 2-week-old. Liver lesion scoring was assessed at 2 weeks after the challenge. 0: No lesion; 1: single to several necrotic foci (<5); 2: large and multiple necrotic foci (<100) and hepatomegaly; 3: highly multiple necrotic foci (countless) and severe hepatomegaly (enlarged 2 times or more); 4: death. A lesion score of 2 or higher was regarded as a severe liver lesion. Liver lesion scoring is demonstrated in Figure 4.6. The data are presented as the mean ± SEM.

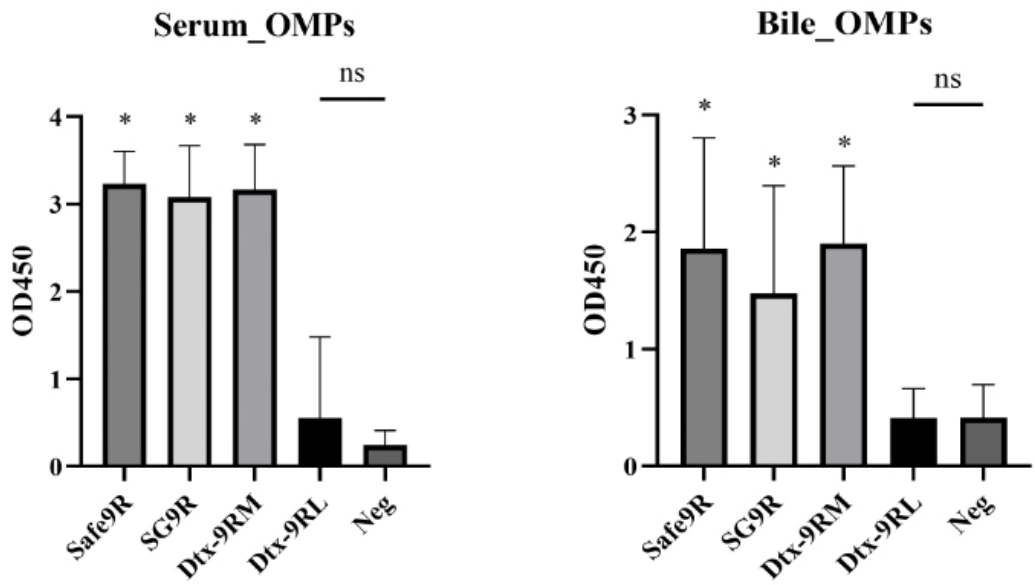


**Figure 4.8. Humoral and mucosal immunogenicity of live vaccines with the detoxified strains after challenge.** Detoxified vaccines were inoculated in 1-week-old chicks and the virulent field strain was challenged after 1 week of vaccination. Blood and bile samples were collected after 2 weeks of the challenge and analyzed with the ELISA. Statistical significance indicated as follows: *ns* not significant, *significantly different compared with Neg ($P$<0.05).

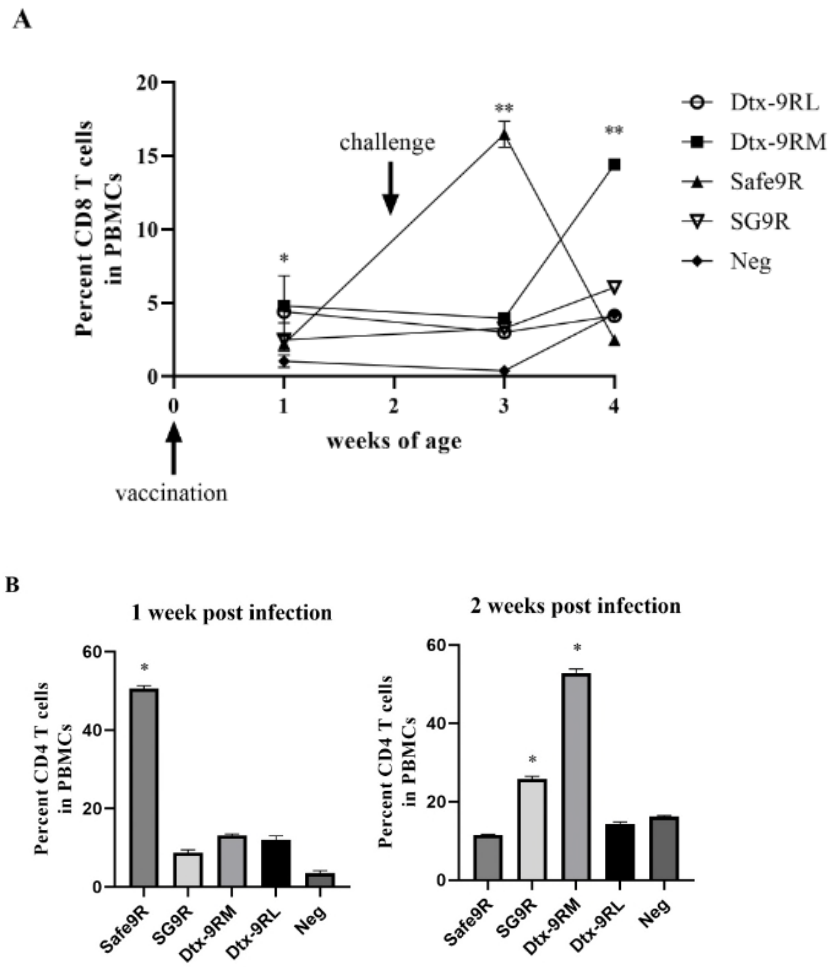


**Figure 4.9. Proportion of CD8$^+$ T cells in peripheral blood mononuclear cells (PBMCs) determined by fluorescence activated cell sorting.** Detoxified vaccines were inoculated in 1-day-old chicks and the whole blood samples were collected after 1 week in heparin-containing tubes. The samples were pooled by group and PBMCs were isolated. The percentage of (A) CD8$^+$ T cells and (B) CD4$^+$ T cells were analyzed. The significance was compared to the negative control. Statistical significance indicated as follows: $^*P$ <0.05, $^{**}P$<0.001.

# General Conclusion

Since the first outbreak of the fowl typhoid in Korea, SG has spread across the country and having difficulty in eradicating. Considering the extensive use of the fowl typhoid vaccine, the revertant of vaccine or evolution into more pathogenic SG have been suspected. Therefore, it is necessary to develop a new vaccine based on the detailed epidemiological investigation and the study of immune response to SG.

In chapter I, molecular epidemiology of SG in Korea was investigated. The prevalence and its genetic diversity by pulse field gel electrophoresis have previously investigated. However, in recent studies on *Salmonella* epidemiology, pseudogene analysis has become mainstream with the development of NGS. The early isolates from 1992-1994 were grouped into S1-1, the most major subgroup. It might have evolved into S1-2, S1-3, S2, S3, S4-1, and S4-2, acquiring pseudogenes and/or changing PPL length during vertical and horizontal transmission. The S6 subgroup is clearly different from S1-1 as it possesses the *ygiD* pseudogene and shortened PPL. Therefore, it was speculated that the early outbreaks of FT in Korea may have been caused by at least two different but closely related subgroups (S1 and S6). The origin of the first SG strains was unclear. Still, considering that the same nonsense mutation at codon 495 of the *fliC* gene was also shared by 3 strains isolated in the Middle East, it might provide a clue to the transmission of fowl typhoid in Korea.

In chapter II, the humoral immunity of various forms of SG antigens were evaluated via the immunogenic OMPs, OmpA and OmpX. The rough strain showed higher

immune response than the smooth strain, which was speculated that the LPS might compete or conceal the immune response of OMP. Live SG vaccine was predicted to have a low humoral immune response as the live SG hide in the reticuloendothelial system. However, it demonstrated similar response to oil adjuvanted killed vaccine. The PBS suspended killed vaccine hardly stimulated the immunity, but it showed a synergistic effect that sustained the antibody titer much longer when co-injected with the live vaccine. When the pathogenic field strain was infected, humoral immunity did not respond for about two weeks, suggesting that the B cell immunity couldn't react to the acute death of fowl typhoid.

In chapter III, the commercialized fowl typhoid vaccine SR2-N6 was characterized with comparative genomics, and proteomics analysis. The deletion of the *rfaL* gene made the strain rough mutant, and the antibiotic resistance to quinolones was increased by the missense mutations of the *gyrA* gene. The proteomics showed reduced expression of DNA starvation/stationary phase protection protein (dps), which was confirmed in cold susceptibility test with the parent strain, SG002. The *Salmonella* D group antibody ELISA was performed that the revertant or its inner core reactivity of SG9R might distort the test result. The positive result was obtained from SG9R and *ΔrfaJ* SG9R inoculated group suggesting that regardless of the revertant, the inner core of SG9R could show positive ELISA results.

In chapter IV, endotoxin of the Safe-9R (*ΔrfaJ* SG9R) was detoxified via mutating the lipid A biosynthesis-related genes. The detoxified strains were evaluated for mRNA expression level in chicken macrophage cells, HD11. The *lpxL* (Dtx-9RL) and *lpxM* (Dtx-9RM) mutants showed substantial reduction in IL-1β, IL-18, iNOS and TLR4. The two

promising candidates were inoculated as an oil adjuvanted killed vaccine with Safe-9R and SG9R to 1 week old chickens. The Dtx-9RL showed almost no reduction in the bodyweight compared to negative control, and the Dtx-9RM also had a huge difference from un-detoxified strains. Although live Dtx-9RL was unable to stimulate the humoral and mucosal immunity, its killed vaccine showed significant antibody rising. The live Dtx-9RM vaccine was protective against the challenge within 1 week post-vaccine while the SG9R inoculated chicken showed severe liver lesion and re-isolation of challenged strain. Therefore, the more detoxified strain Dtx-9RL might suitable for killed vaccine and Dtx-9RM mutant could be the superior substitute of SG9R.

The result of this study not only provides insight into the evolution of SG in Korea but also presents the humoral immunity of SG, which could be the base of future vaccine development. Furthermore, it offers a novel effective vaccine candidates for both killed and live form, which might contribute the eradication of fowl typhoid in Korea.

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국 문 초 록

# *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum의 진화 및 면역학적 특성 분석

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*Salmonella* Gallinarum (SG)은 가금티푸스를 일으키는 원인체로 높은 폐사율과 산란율 저하로 가금 산업의 심대한 경제적 피해를 일으키는 병원균이다. SG는 수평전염뿐 아니라 난계대 전염으로 확산되고, 세포내 잠복감염이 가능하므로 박멸하기 어려운 것으로 알려져 있다. 현재 여러 백신들이 상용화 되었으나 여전히 농가에서는 가금티푸스가 박멸되지 않고 있다. 현재 국내에서는 약독화 생균백신주인 SG9R과 SR2-N6가 상용화

되었고, SG9R이 접종된 후 큰 효과를 보았으나 최근 정기적인 백신 접종에도 불구하고, 상당수의 농가에서 가금티푸스 피해가 보고되고 있다. 이에 따라, 백신주의 병원성 회복 또는 야외 변이주의 출현이 의심되고 있고, 기존 백신주와 백신 프로그램에 대한 개선 요구가 증대되고 있다.

SG의 경우 닭의 세포 내에서 잠복 감염하며 진화한 결과 닭에 대한 숙주특이성을 갖게 되었고, 다양한 기능을 잃어버린 유전자(pseudogene)가 축적되었으며 이러한 pseudogene profiles을 이용하는 경우 야외주 간의 진화적인 근연관계를 규명할 수 있을 것으로 판단하였다. 또한, *Salmonella* 병원성 기전에서 중요한 역할을 하는 *spvB* 유전자의 polyproline linker (PPL)의 길이를 비교하였으며 국내 분리주들은 11개에서 21개까지의 prolines으로 구성된 서로 다른 PPL을 가지고 있어 해외의 PPL에 비해 더 긴 것을 확인하였다. PPL 길이에 따른 병원성 차이를 검증하기 위해 15개와 17개 PPL을 갖는 야외주를 동일 역가로 혼합하여 닭에 접종한 결과 17개 PPL을 갖는 야외주가 우점하는 것을 확인하였다. 국내 분리주들의 pseudogene profiles은 국외 분리주들과는 다른 고유의 cluster를 형성하였다. Pseudogene profiles과 PPL 길이에 따라 국내 분리주들은 7개의 subgroups으로 분류되었고, 유전적으로 유사하지만 서로 다른 두개의 초기 국내 분리주들이 분화한 결과로 추정되었다. 초기 국내 분리주들의 기원과 유입 경로에 대해 밝혀지지 않았지만 국내 분리주들에서 발견되는 편모 합성 유전자(*fliC*)가 pseudogene이 되는 과정에서 획득한 돌연변이를 중동 분리주가 보유하는 것을 볼 때 국내

분리주들과 중동 분리주들의 기원이 같을 것으로 추정되었다.

다양한 형태의 SG 균체 항원에 대한 체액성 면역 반응의 차이를 규명하기 위한 연구를 수행하였다. 면역원성이 높은 외막단백질 (OMP)을 선정하기 위해 SR2-N6의 항혈청과 OMP 추출물로 2D-gel electrophoresis와 western blotting을 실행하였다. 강한 면역반응을 보이는 단백질들로 LC-MS/MS 분석을 한 결과 OmpA와 OmpX로 동정 되었다. 두 단백질의 3차구조와 B-세포 에피톱 예측 프로그램 결과를 비교 분석하여 OmpA 유래 2개 에피톱과 OmpX 유래 1개 에피톱을 최종 선발하였고, 에피톱 아미노산 서열로 구성된 peptides를 합성하여 3종의 peptide-ELISA를 확립하였다. Peptide-ELISA로 SG002와 SR2-N6 유중수 사균을 접종하여 수확한 혈청의 체액성 면역을 비교한 결과, SR2-N6가 SG002 대비 더 강한 체액성 면역반응을 유발하였다. SG9R 생균, 사균, 생균/사균혼합, 유중수 사균을 접종하여 동일한 방식으로 체액성 면역을 비교한 결과 유중수 사균의 경우 생균 접종 수준의 항체역가가 2주간 관찰되었고, 생균/사균혼합의 경우 OmpA peptide-ELISA에서 3주 면역혈청에서도 대조군 대비 유의적으로 높은 항체역가를 유지하였다. 그러나 사균의 경우 모든 면역혈청에서 대조군과 차이가 없었다. 야외주를 감염시켜 수집한 면역혈청의 경우 상기의 항원을 접종한 경우보다 2주정도 항체반응이 지체되는 것을 확인하였고, 담즙 내 IgA도 검출되지 않았다. 생균이나 사균만을 접종한 경우 모두 담즙 내 IgA가 검출되지 않았으나 SG9R 생균 접종 후 야외주를 감염시키는 경우 담즙내 IgA 역가가 유의적인

수준으로 증가하였다.

SR2-N6 백신주는 현재 농장에서 사용되고 있으나 약독화와 관련된 유전체학 및 단백질체학적 기전은 밝혀지지 않았다. 따라서, SR2-N6와 모균주인 SG002의 게놈과 프로테옴을 비교하였고, 그와 관련된 표현형을 비교하였다. SR2-N6는 O-항원을 지질다당체의 outer-core에 연결해주는 효소를 암호화하는 *rfaL* 유전자에서 13개 염기 결손이 확인되어 조면집락을 형성하였고, *gyrA* 유전자의 퀴놀론 저항 결정 영역의 변이를 통해 퀴놀론 계 항생제에 대한 저항성이 모균주 대비 8배 증가하였다. 또한, *Salmonella*의 large virulence plasmid에 존재하는 *spvB*와 *spvC* 유전자가 PCR에 의해 증폭되지 않아 SG9R 대비 병원성이 낮아진 것을 확인하였다. 저온 감수성 실험 결과 SR2-N6가 SG002 대비 증가하였는데 이는 DNA starvation/stationary phase protection gene의 발현량 감소에 의한 것으로 추정되었다.

SG9R은 *rfaJ* 유전자 중 단 한 개의 nonsense mutation으로 인해 조면집락을 형성하여 약독화 된 것으로 알려져 있다. 그러나 해당 염기에 돌연변이가 발생하는 경우 활면집락을 갖는 병원성주로 복귀할 수 있는 위험성이 있으며 국외에서 실제 사례가 보고된 바 있다. 따라서 *rfaJ* 유전자 돌연변이에 의한 병원성 회복 가능성을 원천적으로 제거하기 위해 *rfaJ* 유전자를 적중(knock-out)한 Safe-9R 주를 제작하였고, 생균 및 사균백신의 효능을 조사한 결과 기존 SG9R과 동등한 생균백신 효능을 확인하였다. 그러나 유중수 사균백신은 어린 병아리에서 유의적인 항체역가 상승을

유발하였으나 심한 체중 감소를 보였다. 이러한 체중 감소는 내독소인 lipid A에 의한 것으로 추정되었다. lipid A의 독성을 감소시키면 생균의 병원성도 감소되기 때문에 lipid A 합성 및 변형에 관련된 *lpxL*, *lpxM*, *pagP*, *phoP/phoQ* 단일 유전자 적중(*ΔlpxL*, *ΔlpxM*, *ΔpagP*, *ΔphoP/phoQ*) 돌연변이주들을 제작하여 그 효과를 평가하였다. 닭 대식세포 (HD11)에 감염시켜 염증성 사이토카인의 발현량을 비교한 결과 *lpxL*과 *lpxM* 적중 돌연변이주들(Dtx-9RL, Dtx-9RM)에서 유의적으로 감소하였으며 유중수 사독백신을 제조하여 1주령 병아리에 접종하였을 때 Dtx-9RL은 대조군과 유의적인 체중 차이를 보이지 않았다. Dtx-9RL과 Dtx-9RM 생균을 접종한 후 절식하여 면역을 억제한 결과 간병변이 전혀 없거나 미약하였고, 균의 재분리도 일어나지 않았다. Dtx-9RL과 Dtx-9RM 생균을 피하 접종한 후 병원성 야외주로 공격접종한 결과 Dtx-9RL의 폐사율은 대조군과 비슷한 수준이었으나 Dtx-9RM 접종군에서는 완전한 방어효과를 보였으며 간병변 역시 Safe-9R이나 SG9R 접종군 보다 경미 하였고, 세균도 재분리 되지 않았다. 따라서, Safe-9R은 SG9R과 동등한 백신 효능을 보이지만 역돌연변이가 생기지 않는 안전한 대체제가 될 수 있을 것으로 기대되며, Dtx-9RL은 체중저하 등 부작용이 거의 없어 어린 병아리나 산란중인 닭의 사독백신주로 활용성이 높을 것으로 판단된다. Dtx-9RM은 1일령 병아리에 접종 시 체중에 대한 영향이 크지 않은 경우 조기 백신으로써의 활용성이 높을 것으로 평가된다.

결론적으로 국내 야외주는 국내 유입된 후 게놈과 *spvB* 유전자에서

점진적인 돌연변이가 축적되고 있고, 병원성이 증가된 변이주가 출현하여 이에 대한 대비가 필요하며 기존 SG9R을 개선한 Safe-9R, Dtx-9RL, Dtx-9RM은 기존 백신주와 백신 프로그램을 개선하여 보다 효과적인 백신 전략을 수립하는데 유용할 것으로 사료된다.

주요어: 가금티푸스, *Salmonella* Gallinarum, 게놈 분석, 백신, 체액성면역

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