



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

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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, we 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. We 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.

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 LD₅₀ of SG by approximately 10⁶-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.,

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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, we investigated the PPL lengths of Korean field isolates, virulent reference strains, a vaccine strain (SG 9R) of SG and other *Salmonella* serovars. 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, we aimed to unravel the molecular evolution of SG in the field and the molecular epidemiology of FT in Korea.

2. Materials and methods

2.1. 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).

2.2. 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_NQWN00000000.1), 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). 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).

2.3. 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 Supplementary Table 1. 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 s, 55 °C for 30 s, 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).

2.4. 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, we sorted the genes whose putative impact was high and analyzed them.

2.5. 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, 2005).

2.6. 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 (OD₆₀₀) 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 1000 rpm for 10 min. The supernatants (5 ml) were transferred to 15 ml conical tubes and centrifuged at 3000 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.

3. Results

3.1. Distribution and characterization of *spvB* in *Salmonella Enterica* subspecies *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

Table 1
Salmonella strains used in this study and the length of their PPL.

Strain	Length of PPL	Serotype	Year of isolation	Strain	Length of PPL	Serotype	Year of isolation
SG 9R	9	SG	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 ^a	–
BP-SG67	15	SG	2001	SD_CT02021853	11	SD ^a	–
BP-SG86	15	SG	2001	SD_pOU1115	11	SD ^a	–
BP-SG88	15	SG	2001	SNU1093	7	SE	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	–
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 ^b	–
BP-SG183	15	SG	–	ST_DT104	7	ST ^b	–
BP-SG187	15	SG	–	–	–	–	–

^a *Salmonella* serotype Dublin.

^b *Salmonella* serotype Typhimurium.

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).

3.2. Variation in the PPL length of *SpvB* among field isolates of SG

We determined the PPL length of 61 field isolates of SG and SG 9R by PCR and sequencing (Table 2). 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.

3.3. Identification of specific pseudogenes in Korean SG isolates

Of 61 SG field isolates, we selected five isolates with different PPL lengths (BP-SG002, SNU16035, BP-SG177, BP-SG221, and BP-SG52) and SG 9R 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 2. 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 2). Therefore, the Korean isolates 287/91 and SG 9R had 28–31, 22 and 24 pseudogenes, respectively (Table 2 and 3), and the strains could be differentiated by their pseudogene profiles.

Table 2
Identified pseudogenes, variable or common, among the SG isolates and strains.

Gene	Function	Description	
<i>araA</i>	L-arabinose isomerase	Pseudogenes only common in Korean isolates (15)	
<i>aphA</i>	acid phosphatase		
<i>fliC</i>	flagellin		
<i>fliK</i>	flagellar hook length control protein		
<i>lamB</i>	porin involved in the transport of maltose and maltodextrins		
<i>lpfC</i>	outer membrane usher protein		
<i>murP</i>	PTS sugar transporter subunit IIC		
<i>phsA</i>	thiosulfate reductase		
<i>sfbA</i>	metal ABC transporter substrate-binding protein		
<i>sirB1</i>	putative regulator		
<i>tsr</i>	methyl-accepting chemotaxis protein		
<i>ytcJ</i>	amidohydrolase		
<i>yfiP</i>	DTW domain-containing protein		
<i>yhbW</i>	LLM class flavin-dependent oxidoreductase		
<i>yhhJ</i>	ABC transporter permease	Isolate-specific pseudogenes in Korean isolates (6)	
<i>gspE</i>	type II secretion system protein		
<i>rfbP</i>	undecaprenyl-phosphate galactose phosphotransferase		
<i>SG_RS06090</i>	putative phage protein		
<i>terC</i>	integral membrane protein		
<i>ybdH</i>	oxidoreductase		
<i>ygiD</i>	4,5-DOPA dioxygenase extradiol		287/91-specific pseudogenes (9)
<i>aidB</i>	probable acyl Co-A dehydrogenase		
<i>asnA</i>	aspartate-ammonia ligase		
<i>dcuA</i>	anaerobic C4-dicarboxylate transporter		
<i>fadI</i>	acetyl-CoA C-acyltransferase		
<i>foxA</i>	TonB-dependent siderophore receptor		
<i>mdIA</i>	multidrug ABC transporter permease/ATP-binding protein		
<i>SG_RS20965</i>	fumarate hydratase		
<i>ybgE</i>	cyd operon protein		
<i>yjcC</i>	environmental sensor c-di-GMP phosphodiesterase	SG 9R-specific pseudogenes (7)	
<i>bgIA</i>	6-phospho-beta-glucosidase		
<i>btuB</i>	vitamin B12 transporter		
<i>cueO</i>	multicopper oxidase		
<i>brkB</i>	virulence factor BrkB protein		
<i>rfaJ</i>	lipopolysaccharide glucosyltransferase		
<i>ybiR</i>	anion transporter		
<i>ydfI</i>	putative D-mannonate oxidoreductase		
<i>dinG</i>	probable ATP-dependent helicase DinG		Pseudogenes common in Korean isolates, 287/91, SG 9R and SP (13)
<i>emrB</i>	multidrug resistance protein B		
<i>gabT</i>	4-aminobutyrate aminotransferase		
<i>kefB</i>	glutathione-regulated potassium-efflux system protein (K(+)/H(+)) antiporter		
<i>phoE</i>	outer membrane pore protein E precursor		
<i>SPUL_1682</i>	transposase		
<i>SPUL_2451</i>	outer membrane protein		
<i>SPUL_2756</i>	large repetitive protein		
<i>SPUL_3734</i>	putative membrane transport protein		
<i>sspH2</i>	secreted effector protein		
<i>ydiQ</i>	putative electron transfer flavoprotein subunit		
<i>yjeH</i>	putative permease		
<i>ynhG</i>	LysM peptidoglycan-binding domain-containing protein		

3.4. 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, Supplementary Table 2) (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).

3.5. Subgrouping of Korean SG isolates by variable pseudogenes

To verify the pseudogene profiles of the Korean field isolates, we synthesized primers for 37 pseudogenes, excluding the 13 common SG

pseudogenes in Table 2, and performed PCR and sequencing with an additional twenty-three SG field isolates (Table 2). 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. 2, Table 2). 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).

Table 3
Subgrouping of Korean field isolates of SG (n = 28) based on pseudogenes and PPL length.

Strain	PPL length	Year of isolation	Subgroup	<i>gspE</i>	<i>rfbP</i>	<i>SG_RS06090</i>	<i>terC</i>	<i>ybdH</i>	<i>ygiD</i>	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%)
16,035	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%)

3.6. 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 4).

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

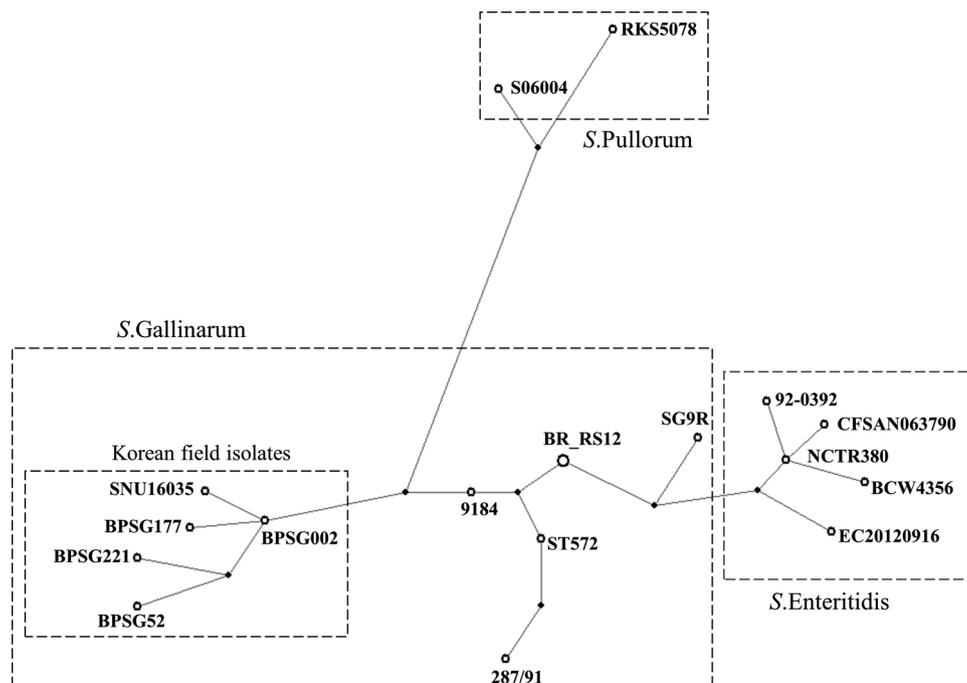


Fig. 1. Evolutionary relationship of SE, SG, and SP. Seventeen strains and isolates of SE, SG, and SP were analyzed with Network software. A total of 122 genes were entered as 0 (intact gene) and 1 (pseudogene), and these binary data were used for median joining network analysis.

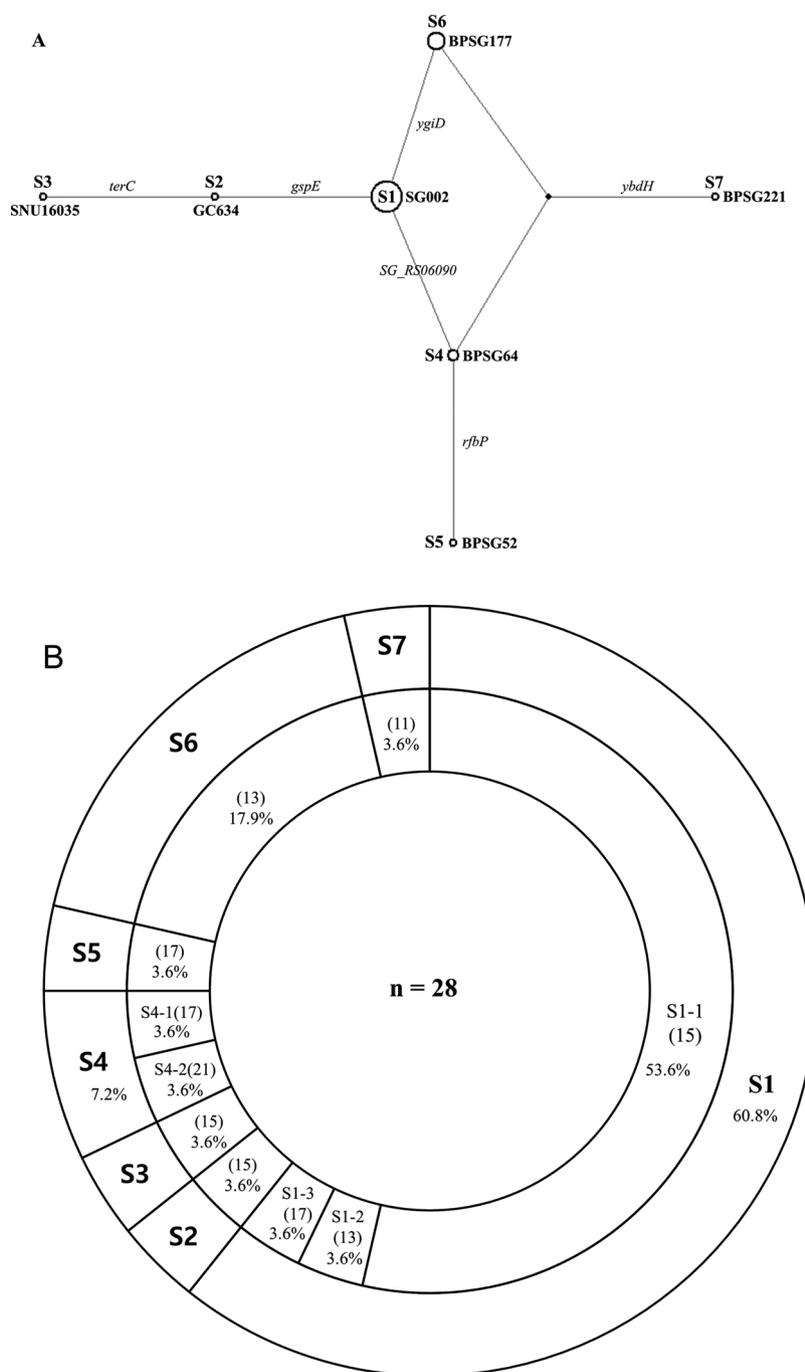


Fig. 2. Subgrouping of SG field isolates in Korea by pseudogene profiles and PPL lengths. Twenty-eight Korean field isolates were divided into 10 subgroups by six isolate-specific pseudogenes (*gspE*, *rfbP*, *SG_RS06090*, *terC*, *ybdH*, and *ygiD*) and PPL lengths. (A) Schematic view of pseudogene 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.

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 to 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 (16,035) 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

Table 4
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.

^d nt: not tested.

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. (Bäumler 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 *fljC* 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.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vetmic.2019.05.019>.

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