Pulsed-field profile diversities of *Salmonella* Enteritidis, S. Infantis, and *S.* Corvallis in Japan

Koichi Murakami,[1,2] Tamie Noda,[2] Daisuke Onozuka,[3] Hirokazu Kimura,[1] Shuji Fujimoto[4]

[1]Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo; [2]Fukuoka Institute of Health and Environmental Sciences, Fukuoka; [3]Department of Health Care Administration and Management, Kyushu University Graduate School of Medical Sciences, Fukuoka; [4]Department of Health Sciences, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan

Abstract

The diversity of pulsed-field profiles (PFPs) within non-typhoidal *Salmonella* subtypes influences epidemiological analyses of *Salmonella* outbreaks. Therefore, determining the PFP diversity of each *Salmonella* serovar is important when evaluating current circulating strains. This study examined the PFP diversity of three important public health *Salmonella enterica* subspecies *enterica* serovars, *S.* Enteritidis (n=177), *S.* Infantis (n=205), and *S.* Corvallis (n=90), using pulsed-field gel electrophoresis. Isolates were collected from several sources, primarily from chicken-derived samples, in the Kyushu-Okinawa region of Japan between 1989 and 2005. *S.* Enteritidis isolates displayed 51 distinct PFPs (E-PFPs), with 92 (52.0%) and 32 (18.1%) isolates displaying types E-PFP1 and E-PFP10, respectively. The 205 *S.* Infantis isolates showed 54 distinct PFPs (I-PFPs), with 87 (42.4%) and 36 (17.6%) isolates being I-PFP4 and I-PFP2, respectively. I-PFP18 was the dominant I-PFP of layer chicken isolates across a 5-year period. Fourteen distinct *S.* Corvallis PFPs were detected. Simpson's index results for the genetic diversities of *S.* Enteritidis, *S.* Infantis, and *S.* Corvallis isolates were 0.70, 0.79, and 0.78, respectively. None of the E-PFPs or I-PFPs of layer chicken isolates overlapped with those of broiler chicken isolates, and the dominant clonal lines existed for >10 years. In conclusion, limited PFP diversities were detected amongst *S.* Enteritidis, *S.* Infantis, and *S.* Corvallis isolates of primarily chicken-derived origins in the Kyushu-Okinawa region of Japan. Therefore, it is important to take into account these limitations in PFP diversities in epidemiological analyses of *Salmonella* outbreaks.

Introduction

Pulsed-field gel electrophoresis (PFGE) subtype diversity within non-typhoidal *Salmonella* serovars can influence epidemiological analyses using pulsed-field profiling of *Salmonella* isolates, especially those using isolates from disease outbreaks. Pulsed-field profiling of non-typhoidal *Salmonella* strains is useful for public health as it enables detection of geographically dispersed outbreaks of this important diarrheal pathogen (Mishu Allos *et al.,* 2004). However, this type of analysis is complicated by the fact that some pulsed-field profiles (PFPs) may be common and widely distributed (Lindqvist and Pelkonen, 2007; Swaminathan *et al.,* 2001). For example, Pang *et al.* (2007) found limited genetic diversity amongst *Salmonella enterica* subspecies *enterica* serovar Enteritidis isolates in Taiwan and Germany. They observed that a single major worldwide clone of *S.* Enteritidis was present in several sources, including deer, pigs, fish, chickens, horses, other birds, rodents, eggs, corn, mushrooms, soil, and water, when PFPs were determined using three restriction enzymes.

Three serovars were chosen for analysis in the current study because these three serovars are relatively important for food hygiene. *S.* Infantis is the most common food-associated serovar in Japan, particularly in chicken meat (Murakami *et al.,* 2001), and is also one of the most important serovars for public health (Murakami *et al.,* 2007). *S.* Enteritidis is also commonly associated with human disease (NIID, 2006), and is related to layer chickens (Humphrey 2006). *S.* Corvallis is not a dominant serovar but is routinely isolated from chickens and poultry products (Murakami *et al.* 2001). While analysis of current PFP data is very important for public health, recording old PFP data is also important from a historical viewpoint. In addition, comparing historical PFP data with that from current isolates can provide important information, such as identification of recurring PFPs. PFGE data for the three chosen serovars is available from several previous papers; however, no long-term analysis has been carried out (Murakami *et al.,* 1999a, 1999b, 2001, 2007; Noda *et al.,* 2010, 2011). Therefore, we carried out a relatively long-term (1989–2005) analysis of historical isolates using PFGE. The aim of the current study was to determine the PFP diversity of *S.* Enteritidis, *S.* Infantis, and *S.* Corvallis isolates collected from several sources, mainly chicken-derived samples, in the Kyushu-Okinawa region of Japan over a 17-year period.

Correspondence: Koichi Murakami, Infectious Disease Surveillance Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-murayama, Tokyo 208-0011, Japan.
Tel: +81-42-848-7132; Fax: +81-42-565-3315.
E-mail: kmuraka@nih.go.jp

Key words: *Salmonella* Enteritidis; *Salmonella* Infantis; *Salmonella* Corvallis; Pulsed-field gel electrophoresis; Pulsed-field profile.

Conflict of interest: the authors declare no potential conflict of interest.

Funding: this work was supported in part by a grant from the Japanese Society for the Promotion of Science (KAKENHI; no. 15K08794), and by the Research Program on Emerging and Re-emerging Infectious Diseases from the Japan Agency for Medical Research and Development, AMED (17fk0108106j0101).

Acknowledgments: we are grateful to the late Mr. Toshihiro Mako, Fukuoka City Institute for Hygiene and the Environment, for his invaluable advice. We thank Dr. Maeda for providing selected S. Corvallis isolates, and Dr. Takenaka and Dr. Sera, Fukuoka Institute of Health and Environmental Sciences (FIHES), for their invaluable advice. We also thank Dr. Saeki and Ms Maruta of FIHES, and Ms. Doi and Ms. Yamada of National Institute of Infectious Diseases, for their technical assistance. We thank Tamsin Sheen, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

Received for publication: 22 May 2017.
Revision received: 13 July 2017.
Accepted for publication: 5 September 2017.

Italian Journal of Food Safety 2017; 6:6808
doi:10.4081/ijfs.2017.6808

Materials and Methods

Isolates

All isolates (n=472) belonging to the three serovars are listed in Table 1. Isolates were selected for long-term assessment of clonal lines, and included nearly all samples

from a collection of the three serovars from the Fukuoka Institute of Health and Environmental Sciences (FIHES), Dazaifu, Fukuoka Prefecture, in the Kyushu-Okinawa region of Japan. The Kyushu-Okinawa region consists of eight prefectures, including Japan's third largest island, and is located southwest of the main island of Honshu. Although the majority of isolates and their original samples were obtained from the Kyushu-Okinawa region, nine isolates were obtained from other regions. Sample details are documented in Table 1.

S. Enteritidis isolates (n=177) from 173 samples were collected between 1989 and 2005 (Tables 2 and 3). *S.* Infantis isolates (n=205) from 184 samples, and *S.* Corvallis isolates (n=90) from 88 samples, were collected between 1995 and 2005 (Tables 2 and 3). *S.* Enteritidis PFP (E-PFP) data for 64 isolates collected between 1989 and 2004, *S.* Infantis PFP (I-PFP) data for 134 isolates collected between 1995 and 2005, and *S.* Corvallis PFP (C-PFP) data for 16 isolates collected from food handlers in 1999 and 2000 were previously reported in a different context (Murakami *et al.,* 1999a, 1999b, 2001, 2007; Noda *et al.,* 2010, 2011), and were re-analyzed in the current study by PFGE for comparison with the newly tested isolates.

Isolates belonged to the following five categories: human, food, slaughterhouse, farm and shell egg production environment, and environmental inspection. Human samples included outbreak isolates from symptomatic patients, food handler isolates, and sporadic case isolates from symptomatic patients (Table 1). The outbreak isolates were obtained from outbreaks in Fukuoka Prefecture, and were isolated at the FIHES. The food handler isolates were obtained from a clinical laboratory and serotyped at the FIHES (Murakami *et al.,* 2007), except for one *S.* Infantis isolate, which was isolated at the FIHES. The sporadic case isolates were also obtained from a clinical laborato-

Table 1. *Salmonella* isolates examined in the present study.

S. enterica subsp. *enterica* serovar	Origin of isolates	Origin details	Isolates (n)	Samples (n)	Origin	Location	Year isolated
Enteritidis	Layer chicken-related	Pooled broken shell-eggs	14	14	2 facilities	Fukuoka Prefecture*	1996–1997
		Liquid egg	11	8	5 makers	Kyushu-Okinawa region	1995–1997
	Broiler chicken-related	Pooled feces of broiler chickens	6	5	4 farms	Kyushu-Okinawa region	1995–1996
	Human	Outbreaks (feces of symptomatic patients)	37	37	37 outbreaks	Fukuoka Prefecture	1989–1994, 1996–2001, 2003–2005
		Sporadic cases (feces)	93	93	93 cases	Kyushu-Okinawa region and Kinki region (3 isolates)°	1999–2000
		Food handlers (feces)	14	14	14 handlers	Kyushu-Okinawa region	1999–2000
	Other	River water	1	1	1 river	Fukuoka Prefecture	1996
		Sewage	1	1	1 facility	Fukuoka Prefecture	1996
		Subtotal	177	173	-	-	-
Infantis	Layer chicken-related	Pooled broken shell-eggs	2	2	1 farm	Fukuoka Prefecture	1996
		Swabs from shell-eggs and egg production environment	5	5	2 facilities	Kyushu-Okinawa region	1995, 1999, 2000
	Broiler chicken-related	Broiler chicken meat	88	83	71 shops	Fukuoka Prefecture	1995–1997, 1999–2005
		Pooled feces of broiler chickens	29	19	15 farms	Kyushu-Okinawa region	1995–1996
		Autopsy materials of broiler chickens	6	4	4 farms	Kyushu-Okinawa region	1995
		Sporadic cases and outbreaks (symptomatic) (feces)	10	10	1 outbreak and nine sporadic cases	Kyushu-Okinawa region and Kinki region (1 isolate)°	2000, 2005
		Food handlers (feces)	56	56	56 handlers	Kyushu-Okinawa region	1996, 1999–2000
	Other	Chicken cases in a slaughterhouse (not identified as broiler- or layer-related)	2	1	1 facility	Kyushu-Okinawa region	1995
		Pork	1	1	1 shop	Fukuoka Prefecture	2002
		River water	2	1	1 river	Fukuoka Prefecture	1995
		Sewage	4	2	1 facility	Fukuoka Prefecture	1995
		Subtotal	205	184			
Corvallis	Layer chicken-related	Pooled broken shell-eggs, swabs of shell-eggs, and egg production environment	33	33	11 facilities	Kyushu-Okinawa region and Chugoku regions (5 isolates)°	1996, 1998–2000, 2002, 2003–2005
		Layer chicken slaughterhouse samples (chilling water and carcass)	3	3	1 facility	Fukuoka Prefecture	1998–1999
	Broiler chicken-related	Broiler chicken meat	9	8	8 shops	Kyushu-Okinawa region	1996, 1998, 2000, 2005
		Broiler chicken slaughterhouse samples (chilling water and carcass)	10	10	3 facilities	Fukuoka Prefecture	1997–1999
	Human	Food handlers (feces)	25	25	25 handlers	Kyushu-Okinawa region	1998–2000
	Other	River water	7	7	4 rivers	Fukuoka Prefecture	1995, 1998–1999
		Sewage	1	1	1 facility	Fukuoka Prefecture	1995
		Beef	2	1	1 shop	Fukuoka Prefecture	1998
		Subtotal	90	88			

*Fukuoka Prefecture is located in the Kyushu-Okinawa region, which consists of eight prefectures, including Japan's third largest island, and is located southwest of the main island of Honshu; °Kinki and Chugoku regions are outside of Kyushu.

Table 2. Pulsed-field profiles of *Salmonella* isolates and their origins.

PFPs		No. of isolates by origin				
		Layer chicken-related	Broiler chicken-related	Humans	Others*	Total
Salmonella enterica subspecies *enterica* serovar *(S.)* Enteritidis-PFPs	E-PFPs found in several sources	E-PFP 1 (13)°		E-PFP 1 (79)		124
		E-PFP 10 (3)		E-PFP 10 (29)		
	E-PFPs found in a single source	E-PFP 2 (1)				53
		E-PFP 9 (1)				
		E-PFP 11 - E-PFP 14 (1 each)				
		E-PFP 15 (3)				
			E-PFP 3 - E-PFP 8 (1 each)			
				E-PFP 18 - E-PFP 29 (1 each)		
				E-PFP 30 - E-PFP 38 (1 each)		
				E-PFP 39 (3)		
				E-PFP 40 - E-PFP 41 (1 each)		
				E-PFP 42 (1)		
				E-PFP 43 - E-PFP 46 (1 each)		
				E-PFP 47 (1)		
				E-PFP 48 - E-PFP 49 (1 each)		
				E-PFP 50 - E-PFP 51 (1 each)		
				E-PFP 16 - E-PFP 17 (1 each)		
	Subtotal	25	6	144	2	177
S. Infantis-PFPs	I-PFPs identified in several sources		I-PFP 2 (21)	I-PFP 2 (15)		145
			I-PFP 4 (59)	I-PFP 4 (26)	I-PFP 4 (3)	
			I-PFP 9 (1)		I-PFP 9 (1)	
		I-PFP 20 (1)			I-PFP 20 (1)	
			I-PFP 25 (7)	I-PFP 25 (2)		
			I-PFP 37 (2)	I-PFP 37 (1)		
				I-PFP 38 (4)		
			I-PFP 40 (1)	I-PFP 40 (1)		
	I-PFPs identified in a single source	I-PFP 10 (1)				60
		I-PFP 17 (1)				
		I-PFP 18 (4)				
			I-PFP 3 (1)			
			I-PFP 5 - I-PFP 8 (1 each)			
			I-PFP 11 (2)			
			I-PFP 12 - I-PFP 14 (1 each)			
			I-PFP 15 (3)			
			I-PFP 16 (1)			
			I-PFP 21 (1)			
			I-PFP 23 - I-PFP 24 (1 each)			
			I-PFP 26 - I-PFP 32 (1 each)			
			I-PFP 34 - I-PFP 36 (1 each)			
			I-PFP 41 - I-PFP 42 (1 each)			
			I-PFP 47 (1)			
			I-PFP 51 (3)			
				I-PFP 1 (1)		
				I-PFP 39 (1)		
				I-PFP 43 (1)		
				I-PFP 44 (1)		
				I-PFP 46 (1)		
				I-PFP 48 (1)		
				I-PFP 50 (1)		
				I-PFP 52 (5)		
				I-PFP 53 - I-PFP 55 (1 each)		
				I-PFP 56 (2)		
					I-PFP 19 (1)	
					I-PFP 21 - I-PFP 22 (1 each)	
					I-PFP 33 (1)	
	Subtotal	7	123	66	9	205
S. Corvallis-PFPs	C-PFPs identified in several sources		C-PFP 1 (2)	C-PFP 1 (12)		78
			C-PFP 2 (1)	C-PFP 2 (1)		
		C-PFP 4 (23)	C-PFP 4 (7)	C-PFP 4 (6)	C-PFP 4 (3)	
		C-PFP 5 (1)	C-PFP 5 (1)			
		C-PFP 6 (1)		C-PFP 6 (1)	C-PFP 6 (1)	
		C-PFP 7 (1)	C-PFP 7 (4)		C-PFP 7 (3)	
			C-PFP 8 (2)		C-PFP 8 (2)	
		C-PFP 9 (1)	C-PFP 9 (2)			
		C-PFP 11 (2)			C-PFP 11 (1)	
	C-PFPs identified in a single source			C-PFP 3 (5)		12
		C-PFP 10 (3)				
		C-PFP 12 (2)				
		C-PFP 13 (1)				
		C-PFP 14 (1)				
	Subtotal	36	19	25	10	90

PFPs, pulsed-field profiles. *Including *S.* Infantis PFPs from broiler chicken slaughterhouse samples (chilling water and carcass); °no. of isolates.

ry and serotyped at the FIHES (Murakami *et al.,* 2007). The sporadic case samples were obtained from periodic inspections of food handlers in the Kinki (three *S.* Enteritidis isolates and one *S.* Infantis isolate) and Kyushu-Okinawa regions. In the food category, isolates were collected from beef, broiler meat, liquid-egg, and pork (Table 1). These food isolates were obtained during a food hygiene inspection survey for foodborne pathogens in Fukuoka Prefecture, and were isolated at the FIHES. In the slaughterhouse category, isolates were collected from broiler slaughterhouse samples (chilling water and carcass), chicken samples from a slaughterhouse (not identified as broiler- or layer-derived), and layer slaughterhouse samples (chilling water and carcass) (Table 1). These isolates were obtained from five slaughterhouses in the Kyushu-Okinawa region and were isolated at the Fukuoka Prefectural Meat Safety Inspection Center, Chikushino, Fukuoka Prefecture, and at another laboratory in Fukuoka Prefecture.

In the farm and shell egg production environment category, isolates were grouped into those from swabs from egg shells and the egg production environment, pooled broken shell eggs, pooled feces of broiler chickens, and necropsy materials from broilers (Table 1). The swabs from egg shells and egg production environment isolates and the pooled broken shell egg isolates were obtained from 32 chicken farms and three egg-packing facilities. Thirty-one of the 32 farms that donated samples were located in the Kyushu-Okinawa regions, while one farm that donated four samples of *S.* Corvallis was outside of the region. Some of these isolates were provided by a livestock hygiene service center. Two of the three egg-packing facilities (facilities A and B) provided isolates from swab samples obtained from eggshells and the egg production environment. Facility A packs 360,000 eggs per day, and these eggs are supplied from nine farms. Facility B has an integrated operation with 240,000 eggs supplied daily from their own farm. Retail data were not available from farms, or for the third egg-packing facility.

Finally, in the environment inspection category, isolates were obtained from river water and sewage samples (Table1). The samples were obtained from Fukuoka Prefecture, and bacteria were isolated at the FIHES (Murakami *et al.,* 2001). These isolates were re-categorized into the following four groups: layer chicken-related, broiler chicken-related, human, and other isolates (Table 2).

One isolate from each sample was analyzed, except in the case of four *S.* Enteritidis-containing samples, 21 *S.* Infantis-containing samples, and two *S.* Corvallis-containing samples for which two isolates from each sample were analyzed. In these samples, two representative isolates showed different PFPs, indicating the presence of more than one strain. Therefore, both isolates were analyzed from these 27 samples (totaling 54 isolates).

Table 3. Chronological appearance of each pulsed-field profile of *Salmonella enterica* subspecies *enterica* serovars Enteritidis (*S.* Enteritidis), *S.* Infantis, and *S.* Corvallis over a 17-year period.

			Isolation year																	Total	(%)
			1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005		
S. Enteritidis (177 isolates)	E-PFPs observed for 2 or more years	E-PFP 1		1	1			1		7	10	4	13	50	1		1	2	1	92	(52.0)
		E-PFP 10								2	2		5	22	1					32	(18.1)
	The remaining 49 E-PFPs observed in a single year only		1			2	3	4	4	9	5		10	13	1				1	53	(29.9)
S. Infantis (205 isolates)	I-PFPs observed for 2 or more years	I-PFP 2							4	4			2	15		2	6	1	2	36	(17.6)
		I-PFP 4							16	10	4		4	32	1	6	2	5	7	87	(42.4)
		I-PFP 9							1					1						2	(1.0)
		I-PFP 15							1						2					3	(1.5)
		I-PFP 18								1			2	1						4	(2.0)
		I-PFP 20							1				1							2	(1.0)
		I-PFP 25								2	1		1	2	1				2	9	(4.4)
		I-PFP 37												2				1		3	(1.5)
		I-PFP 51												1			2			3	(1.5)
		I-PFP 52											4	1						5	(2.4)
	The remaining 44 I-PFPs observed in a single year only								21	6	3		1	18	1		1			51	(24.9)
S. Corvallis (90 isolates)	C-PFPs observed for two or more years	C-PFP 1										1	10	3						14	(15.6)
		C-PFP 3											2	3						5	(5.6)
		C-PFP 4								9	1	16	3			2		7	1	39	(43.3)
		C-PFP 5									1	1								2	(2.2)
		C-PFP 6											2				1			3	(3.3)
		C-PFP 7										7	1							8	(8.9)
		C-PFP 8							2	2										4	(4.4)
		C-PFP 9												2				1		3	(3.3)
		C-PFP 10											1					1	1	3	(3.3)
		C-PFP 11										1						2		3	(3.3)
	The remaining 4 C-PFPs observed in a single year only											2		2			1	1		6	(6.7)

PFPs, pulsed-field profiles.

Pulsed-field gel electrophoresis

Clonal lineages of the *S.* Enteritidis, *S.* Infantis, and *S.* Corvallis serovars were determined by PFGE analysis. PFGE was performed as described previously (Murakami *et al.* 1999b; Murakami *et al.* 2007), with the following modifications. After preparation for restriction endonuclease digestion, the DNA in each *S.* Corvallis plug was digested with 20 units of *Xba*I (Takara Bio, Otsu, Japan) at 37°C for 15 h, and then electrophoresed at 200 V for 22 h, with a switched pulse time of 5–50 s at 14°C. Plugs of *S.* Enteritidis and *S.* Infantis were then digested with 20 units of *Bln*I (Takara Bio) at 37°C for 15 h, and electrophoresis was performed at 200 V for 24 h with a switched pulse time of 2–43.1 s at 14°C. DNA fragment patterns were assessed visually, and different PFPs were assigned based on the presence or absence of bands. Similarity and cluster analyses were performed using the Dice coefficients of similarity and an unweighted pair group method with average linkage, respectively, using FPQuest Software (Bio-Rad Laboratories, Hercules, CA, USA). *S.* Enteritidis strain ATCC 13076, *S.* Infantis strain ATCC 51741, and *S.* Corvallis strain K54-1 were used as respective reference strains in all analyses.

Simpson's index analysis

Simpson's index of diversity (Hunter and Gaston 1988) was used to evaluate genetic diversity within each serovar. This index is given by the following equation:

$$= 1 - (\Sigma n(n-1))/(N(N-1))$$

where n is number of isolates belonging to the nth type and N is total number of isolates in the same population. A value of 1 indicates infinite diversity, and a value of 0 indicates no diversity.

Statistical analysis

PFP associations between layer and broiler chicken isolates of each of the three serovars were evaluated using the Wilcoxon rank-sum test (Hollander and Wolfe, 1973) using SAS Software version 9.1.3 (SAS Institute, Cary, NC, USA) to assess population differences between the two host types.

Results

Pulsed-field gel electrophoresis

The three serovars showed limited PFP diversities. Although the *S.* Enteritidis isolates displayed 51 distinct PFPs (Tables 1 and 2), 92 (52.0%) and 32 (18.1%) isolates displayed E-PFP1 and E-PFP10, respectively. In particular, E-PFP1 was detected every year except one between 1990 and 2005 (Table 3), and no E-PFPs other than E-PFP1 and E-PFP10 were detected in more than one year (Table 3). No E-PFPs were shared between layer chicken isolates (pooled broken shell eggs and liquid-egg isolates) and broiler chicken isolates (pooled feces) (Tables 1 and 2). In addition, many E-PFPs associated with broiler-derived isolates grouped together in clades that were distinct from the layer chicken isolates (Appendix Figure 1).

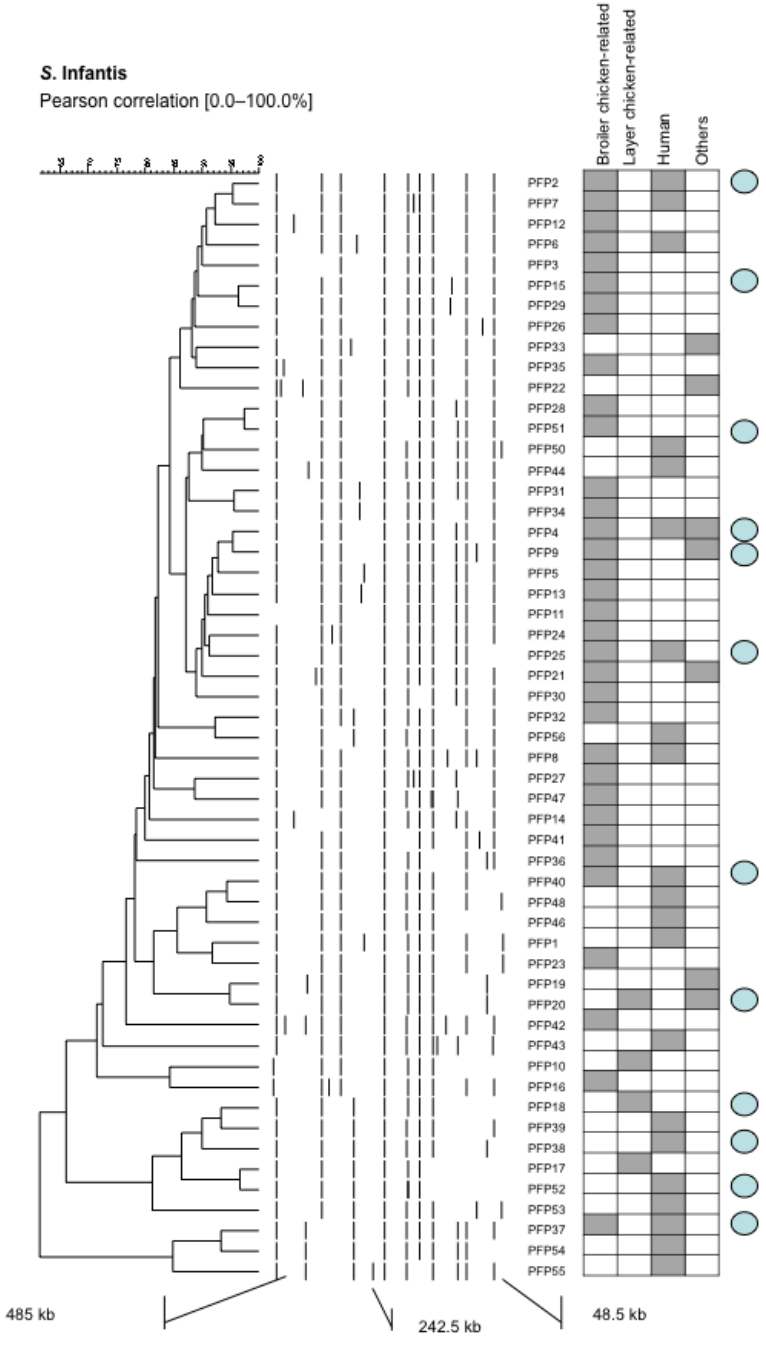

Figure 1. Dendrogram of pulsed-field profiles for *Salmonella enterica* subspecies *enterica* serovar Infantis (I-PFPs) following *BlnI* digestion. Fifty-four different I-PFPs were obtained from 205 isolates. Numbers indicate fragment sizes. Origins of each I-PFP are indicated. I-PFPs with closed circles were observed in two or more years. Some I-PFPs were assigned numbers in our previous study, and thus are not numbered consecutively. The scale indicates the percentage similarity, as determined using Dice coefficients.

The 205 *S.* Infantis isolates showed 54 I-PFPs, with 87 (42.4%) and 36 (17.6%) isolates being I-PFP4 and I-PFP2, respectively (Table 1, Figure 1). I-PFP2 was found in isolates from both broiler chicken and human samples, while I-PFP4 appeared in isolates from pork, broiler chickens, and humans (Table 2). I-PFP18 was the dominant I-PFP of layer chicken isolates across a 5-year period (Table 3). Among the 54 I-PFPs, 10 were detected over multi-year periods, and 44 (51 isolates) were each found in a single year. Some I-PFPs were assigned numbers in our previous study, and thus are not numbered consecutively in this study. Layer and broiler chicken isolates shared no common I-PFPs (Tables 1 and 2). Many layer chicken isolate-associated I-PFPs grouped together in clades that were distinct from those of the broiler chicken isolates (Figure 1).

Fourteen distinct *S.* Corvallis C-PFPs were detected (Tables 1 and 2, and Appendix Figure 2). C-PFP4 was the dominant C-PFP for over 10 years, and was found in 39 (43.4%) of 90 isolates (Table 3). Among the 14 C-PFPs, nine were present over multi-year periods (Table 3).

Analyses using Simpson's index

Simpson's index results for the genetic diversities of *S.* Enteritidis, *S.* Infantis, and *S.* Corvallis were 0.70, 0.79, and 0.78, respectively.

Statistical analysis

PFP patterns of the *S.* Infantis isolates were significantly different ($P<0.001$) between isolates associated with layer and broiler chickens. No statistical differences between these groups were found for *S.* Enteritidis ($P=0.147$) and *S.* Corvallis ($P=0.597$).

Discussion

Our study had three major findings. First, Simpson's index analysis indicated that there was little PFP diversity within the serovars. Second, dominant PFPs of *S.* Enteritidis, *S.* Infantis, and *S.* Corvallis were observed in both chicken- and human-derived strains. The dominant PFPs of *S.* Enteritidis and *S.* Infantis persisted for a relatively long time (>10 years). Finally, of the population structures of the PFPs for the three serovars, only the I-PFPs of broiler chicken isolates differed significantly from those of layer chicken isolates, based on the Wilcoxon rank-sum test. Moreover, none of the PFPs detected for *S.* Enteritidis and *S.* Infantis from layer chicken isolates overlapped with those from broiler chicken isolates. Based on these findings, we concluded that one E-PFP was dominant in the Kyushu-Okinawa region of Japan (Table 3), and that *S.* Infantis from several sources showed limited PFP diversity.

Comparing Simpson's index results determined in the current analysis to those from other countries, the diversity of the *S.* Enteritidis isolates (0.70) was lower than that determined in previous studies: 0.76 for a general US survey (using *Bln*I) (Zheng *et al.,* 2007), 0.79 in Minnesota, USA (using *Xba*I) (Rounds *et al.,* 2010), and 0.79 in France (using *Xba*I) (Kérouanton *et al.,* 2007). The value for *S.* Infantis (0.79) was also lower than those reported in other studies: 0.97 in Minnesota (using *Xba*I) (Rounds *et al.,* 2010) and 0.88 in France (using *Xba*I) (Kérouanton *et al.,* 2007). The differences between the current and previous studies may stem from physiographical differences or clonalities of the serovars. We collected samples mainly from the Kyushu-Okinawa region, which may be limited in comparison with other studies. However, the values determined in all non-typhoidal *Salmonella* studies are lower than those for other salmonellae or foodborne pathogens such as *S.* Typhi (0.952) (Kubota *et al.,* 2005) or *Escherichia coli* O157 (0.98) (Avery *et al.,* 2002), illustrating the limited clonal populations of these three *Salmonella* serovars in the Kyushu-Okinawa region of Japan.

There are several explanations for the observed lower genetic diversity of the *Salmonella* serovars examined in the current study. Hauser *et al.* suggested genetic stability or broad dissemination of a recent ancestor as possible reasons for the high clonality of *S.* Infantis (Hauser *et al.,* 2012). However, another reason might be the limited genealogical diversity of industrial poultry chickens. Almost all commercial layer and broiler chicken flocks in many developed countries, including Japan, are derived from a few great-grandparental flocks that are imported from limited countries (Leeson and Summers, 2000), likely limiting the impact of genealogical factors. Therefore, *S.* Enteritidis and *S.* Infantis serovars might have evolved to adapt to the limited chicken genetic population, as is described by the theory of co-evolution (Pfennig, 2001). If all members of a population adapt to an evolutionarily stable state, no mutations can evolve under the influence of natural selection according to Maynard-Smith (1982).

Conclusions

Limited PFP diversities were detected in *S.* Enteritidis, *S.* Infantis, and *S.* Corvallis isolates collected between 1989 and 2005 from primarily chicken-derived origins in the Kyushu-Okinawa region of Japan. It is important to account for these limited PFP diversities in epidemiological analyses of outbreaks caused by these *Salmonella* serovars.

References

Avery SM, Liebana E, Reid CA, Woodward MJ, Buncic S, 2002. Combined use of two genetic fingerprinting methods, pulsed-field gel electrophoresis and ribotyping, for characterization of Escherichia coli O157 isolates from food animals, retail meats, and cases of human disease. J Clin Microbiol 40:2806-12.

Hauser E, Tietze E, Helmuth R, Junker E, Prager R, Schroeter A, Rabsch W, Fruth A, Toboldt A, Malorny B, 2012. Clonal dissemination of Salmonella enterica serovar Infantis in Germany. Foodborne Pathog Dis 9:352-60.

Hollander M, Wolfe DA, 1973. Nonparametric statistical methods. John Wiley & Sons Inc., New York, NY, USA.

Humphrey T, 2006. Public health aspects of Salmonella enterica in food production. In: P Mastroeni P, Maskell D eds. Salmonella infections, clinical, immunological and molecular aspects. Cambridge University Press, Cambridge, UK, pp 89-116.

Hunter PR, Gaston MA, 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol 26:2465-6.

Kérouanton A, Marault M, Lailler R, Weill FX, Feurer C, Espié E, Brisabois A, 2007. Pulsed-field gel electrophoresis subtyping database for foodborne Salmonella enterica serotype discrimination. Foodborne Pathog Dis 4:293-303.

Kubota K, Barrett TJ, Ackers ML, Brachman PS, Mintz ED, 2005. Analysis of Salmonella enterica serotype Typhi pulsed-field gel electrophoresis patterns associated with international travel. J Clin Microbiol 43:1205-9.

Leeson S, Summers JD, 2000. Broiler breeder production. University books, Guelph, Canada.

Lindqvist N, Pelkonen S, 2007. Genetic surveillance of endemic bovine Salmonella Infantis infection. Acta Vet Scand 49:15.

Maynard-Smith J, 1982. Evolution and the theory of games. Cambridge University Press, Cambridge, UK.

Mishu Allos B, Moore MR, Griffin PM, Tauxe RV, 2004. Surveillance for sporadic foodborne disease in the 21st century: the FoodNet perspective. Clin Infect Dis. 38(Suppl.3):115-20.

Murakami K, Horikawa K, Ito T, Otsuki K, 2001. Environmental survey of salmonella and comparison of genotypic character with human isolates in Western Japan. Epidemiol Infect 126:159-71.

Murakami K, Horikawa K, Otsuki K, 1999a. Epidemiological analysis of Salmonella enteritidis from human outbreaks by pulsed-field gel electrophoresis. J Vet Med Sci 61:439-42.

Murakami K, Horikawa K, Otsuki K, 1999b. Genotypic characterization of human and environmental isolates of Salmonella choleraesuis subspecies choleraesuis serovar Infantis by pulsed-field gel electrophoresis. Microbiol Immunol 43:293-6.

Murakami K, Ishihara T, Horikawa K, Oda T, 2007. Features of Salmonella serovars among food handlers in Kyushu, Japan. New Microbiol 30:155-9.

NIID, 2006. National Institute of Infectious Diseases and Tuberculosis and Infectious Diseases. Salmonellosis in Japan as of June 2006. Infect Agents Surv Rep 27:191-2.

Noda T, Murakami K, Asai T, Etoh Y, Ishihara T, Kuroki T, Horikawa K, Fujimoto S, 2011. Multi-locus sequence typing of Salmonella enterica subsp. enterica serovar Enteritidis strains in Japan between 1973 and 2004. Acta Vet Scand 53:38.

Noda T, Murakami K, Ishiguro Y, Asai T, 2010. Chicken meat is an infection source of Salmonella serovar Infantis for humans in Japan. Foodborne Pathog Dis 7:727-35.

Pang JC, Chiu TH, Helmuth R, Schroeter A, Guerra B, Tsen HY, 2007. A pulsed field gel electrophoresis (PFGE) study that suggests a major world-wide clone of Salmonella enterica serovar Enteritidis. Int J Food Microbiol 116:305-12.

Pfennig KS, 2001. Evolution of pathogen virulence: the role of variation in host phenotype. Proc Biol Sci 268:755-60.

Rounds JM, Hedberg CW, Meyer S, Boxrud DJ, Smith KE, 2010. Salmonella enterica pulsed-field gel electrophoresis clusters, Minnesota, USA, 2001-2007. Emerg Infect Dis 16:1678-85.

Swaminathan B, Barrett TJ, Hunter SB, Tauxe RV, CDC PulseNet Task Force, 2001. PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerg Infect Dis 7:382-9.

Zheng J, Keys CE, Zhao S, Meng J, Brown EW, 2007. Enhanced subtyping scheme for Salmonella enteritidis. Emerg Infect Dis 13:1932-5.