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Environmental survey of salmonella and comparison of genotypic character with human isolates in Western Japan

K. MURAKAMI[1]*, K. HORIKAWA[1], T. ITO[2] AND K. OTSUKI[3]

[1] *Pathology and Bacteriology Division, Department of Health Science, Fukuoka Institute of Health and Environmental Sciences, Mukaizano 39, Dazaifu, Fukuoka 818–0135, Japan*
[2] *Department of Veterinary Public Health and* [3] *Department of Veterinary Microbiology, Faculty of Agriculture, Tottori University, Tottori, Tottori 680–8553, Japan*

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SUMMARY

To estimate the prevalence and distribution of salmonellae, especially *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis), in Western Japan, an investigation was conducted of the chicken industry and environmental sources between 1995 and 1998. Salmonellae were isolated from 34 of 90 samples (37·8%) of raw chicken parts, 34 of 98 faecal samples (34·7%) at 35 broiler farms, 11 of 59 samples (18·6%) of liquid eggs, and from 71 of 272 samples (26·1%) of swab specimens from equipment and cracked or faecally soiled shell eggs at the processing facilities. Salmonellae, including *S.* Enteritidis, were also isolated from swab samples of henhouses associated with one of the shell-egg processing facilities (11 samples out of 55, 20%). In the broiler meat production environment, *S.* Infantis was dominant. Twenty-two of 36 sewage samples (61·1%) and 16 of 72 samples (22·2%) taken from 5 rivers contained salmonellae including *S.* Enteritidis. *S.* Enteritidis isolates were analysed with pulsed-field gel electrophoresis using enzyme *Bln* I. Thirty-four isolates from shell-egg processing facilities and henhouses, obtained over several years, had the same pulsed-field profile as isolates obtained from four individual outbreaks that occurred in this location in 1997. One of the clonal lines of *S.* Enteritidis, among multiple serovars of salmonellae in the environment, was thought to have distributed in reservoirs, laying hens, for several years, and continued to cause outbreaks in this area.

INTRODUCTION

The importance of nontyphoid salmonellosis has been increasing in many countries [1]. In Japan, cases of bacterial foodborne disease with salmonellae as the aetiological agents accounted for 36% of a total of 19089 in 1993, 49% of 29513 in 1994, and 36% of 22329 in 1995 [2]. Of the many serovars of salmonellae, *S. enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis) is commonly associated with foodborne disease in Japan as in other industrial countries [1–3]. Of outbreaks involving 10 or more cases, 41 were due to *S.* Enteritidis in 1993 (55%), 75 in 1994 (70%), 69 in 1995 (71%) and 84 in 1996 (76%) [2, 4]. This pathogen has the ability to contaminate eggs [5–7], so poultry products, eggs and egg-containing foods are important vehicles for the outbreaks caused by *S.* Enteritidis in industrial countries [3, 5, 8–10].

With *S.* Enteritidis, Terajima et al. speculated that multiple clonal lines of phage type 1 (PT1) and PT22 had already spread while relatively fewer clonal lines of PT4 and PT13a might exist in Japan based on

* Author for correspondence.

pulsed-field gel electrophoresis (PFGE) analysis [11]. They examined 74 strains of *S.* Enteritidis isolated in different parts of Japan in 1994 and 1995.

Compared with other industrial countries [12–14], Japan has had few reports on the prevalence of salmonellae (not only *S.* Enteritidis, but also other serovars) in food, commercial chicken egg production environments, and broiler meat production environments. There have been few investigations of the distribution of clonal lines of *S.* Enteritidis and relation between salmonellae isolates of humans and the environment in other countries as well. Thus it was important to evaluate the persistence and distribution of *S.* Enteritidis and other salmonellae serovars in such environments in Japan. We have reported that limited clonal lines of *S.* Enteritidis were causing outbreaks in a small area in Western Japan, and laying hens were suspected to be reservoirs of this pathogen [15]. We also pointed out the need to examine more isolates not only from human but also from environmental sources to elucidate the reservoirs and transmission chains of *S.* Enteritidis infection using an epidemiological method.

It is clear that the molecular typing of human and environmental strains of *S.* Enteritidis is an invaluable epidemiological tool. PFGE analysis provides precise information that can be used to evaluate epidemiological associations with a high degree of confidence. PFGE analysis is believed to offer a discriminating capacity greater than serotyping, ribotyping and other restriction fragment length polymorphism methods [16–18]. So, PFGE analysis was undertaken to determine whether the isolates were identical or not, whether the environmental isolates were similar or identical to human isolates, and the extent of genetic diversity among the isolates.

A survey of retail products and livestock products, poultry farms, shell-egg processing facilities and water environments including riverwater and sewage was conducted in Western Japan in 1995–8, to estimate the prevalence and distribution of salmonellae, especially *S.* Enteritidis. Environmental strains of *S.* Enteritidis were compared with human isolates with PFGE analysis, epidemiologically.

Our results show that *S.* Enteritidis is commonly associated with the chicken egg production environment and *S.* Infantis with the broiler meat production environment. The molecular genotypes of human *S.* Enteritidis isolates were similar to those encountered in eggs and at the egg layer farm. These findings reveal an epidemiological link between chicken eggs and the chicken egg production environment, and human infection with *S.* Enteritidis.

MATERIALS AND METHODS

Poultry and live stock products

Fifty-nine retail samples of unpasteurized liquid eggs (31 brands), 90 raw chicken parts (74 brands), 53 cuts of beef (from 38 retail shops), and 15 cuts of pork (from 13 retail shops), in Western Japan were randomly sampled and tested for the presence of salmonellae during the period from January 1995 to March 1998 (Table 1). Ten samples of unpasteurized liquid eggs in January 1995, 28 samples (consisting of 10 unpasteurized liquid eggs, 10 raw chicken parts and 8 cuts of beef) in June 1995, 15 samples (5 raw chicken parts, 7 cuts of beef and 3 cuts of pork) in September 1995, 10 samples of unpasteurized liquid eggs in January 1996, 38 samples (10 unpasteurized liquid eggs, 13 raw chicken parts, 14 cuts of beef and 1 cut of pork) in June 1996, 41 raw chicken parts in September 1996, 69 samples (19 unpasteurized liquid eggs, 20 raw chicken parts, 19 cuts of beef and 11 cuts of pork) in June 1997, and 6 samples (1 raw chicken part and 5 cuts of beef) in September 1997, were tested, respectively.

Samples (25 g) were homogenized with 225 ml of enterobacteria enrichment mannitol (EEM) broth (Eiken Chemical Co., Tokyo) for 1 min in a stomacher, and incubated for 18 h at 35 °C. After incubation, 1 ml aliquots of the pre-enriched test portions were subcultured in three tubes with 10 ml of selenite brilliant green broth (Eiken Chemical Co.) and three tubes with 10 ml of selenite broth (Nissui Pharmaceutical Co., Tokyo) in parallel. These cultures of 6 tubes were selectively enriched for 18 h at 42 °C, and then streaked for isolation on differential plating media, using 6 *Salmonella-Shigella* agar (Eiken Chemical Co.), 6 DHL agar (Eiken Chemical Co.) and 6 XLD agar (Difco Laboratories, Detroit, MI, USA) plates, and incubated for 24 h at 37 °C. Potential salmonellae colonies (1–10 colonies/plate) were then Gram-stained and transferred into triple sugar iron agar (Eiken Chemical Co.), lysine decarboxylase broth (Eiken Chemical Co.) and sulphide indole motility medium agar (Eiken Chemical Co.) for biochemical profiling. In addition, methylred-Voges-Proskauer (MR-VP) medium (Nissui Pharmaceutical Co.) Simmons citrate agar (Eiken Chemical Co.), malonate broth (Eiken Chemical Co.) and cytochrome-oxidase test strip (Nissui Pharmaceutical Co.)

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Table 1. *Contamination with salmonella in poultry and livestock products*

Samples	No. of samples	No. of positive samples (%)	Isolates: Serovars: O-group	Isolates: Serovars: Name	Isolates: Number
Unpasteurized liquid eggs	59	11 (18·6)	D_1	*S.* Enteritidis	9
			A	*S.* Nitra	1
			C_3	Untypeable strains in O-group C_3*	1
Raw chicken parts†	90	34 (37·8)	C_1	*S.* Infantis	23
			B	*S.* Typhimurium	4
			B	*S.* Haifa	2
			B	*S.* Agona	1
			B	*S.* Untypeable strains in O-group B*	2
			C_1	*S.* Thompson	1
			C_3	*S.* Corvallis	1
			C_3	Untypeable strains in O-group C_3*	2
			E_1	*S.* Uganda	1
Beef	53	1 (1·9)	C_2	*S.* Hadar	1
Pork	15	0 (0·0)			
Total	217	46 (21·2)			

* Not identified as a serovar.
† Three samples yielded two serovars each.

were used as necessary. Isolates with a biochemical profile consistent with salmonellae were serotyped using somatic (O) antisera and flagella (H) antisera (Denka Seiken Co., Tokyo). Phage-typing was completed on *S.* Enteritidis isolates recovered from samples in this study according to the method of Ward et al. [19] at the National Institute of Infectious Disease.

Poultry farms

From March 1995 to February 1996, 98 and 15 samples (about 100 g each) of chicken faeces were collected from each of 35 broiler farms (Table 2) and 5 layer farms, respectively. Thereafter, one gram aliquots (a total of 6 g) of replicated portions from each 100 g sample were selectively enriched in parallel in three tubes with 10 ml of selenite brilliant green broth and three tubes with 10 ml of selenite broth for 18 h at 42 °C, using the bacteriological test procedures described above.

Shell-egg processing facilities and related henhouses

From April 1995 to March 1998, 2 shell-egg processing facilities in Western Japan were monitored for salmonellae. Processing facility A packs 360000 eggs per day, and these eggs are supplied from 9 farms. Facility B has an integrated operation with 240000 eggs supplied daily from their own farm in line. One pooled sample of about 100 cracked or faecally soiled shell eggs (shell and contents), 2 swab samples of egg handling equipment and two litres of wastewater from the egg washer were sampled once every month in facility A during the 3-year-period (in total, 108 samples of swab and waste water, and 34 samples of shell eggs). In shell-egg processing facility B, one pooled sample of about 100 cracked or faecally soiled shell eggs, one swab sample of egg handling equipment and two litres of wastewater from the egg washer were sampled once a month in the first 12 months, and two pooled cracked or faecally soiled shell egg samples and two swab samples in the last 24 months during the 3-year-period (in total, 72 samples of swab and waste water, and 58 samples of shell eggs).

In addition, a survey of the hens supplying the eggs to facility B was done. Thirty-three samples (about 100 g each) of chicken faeces and 22 swab samples of henhouse equipment were tested for salmonellae during the test period.

Shell egg samples (200 g) of replicated portions from pooled samples were placed in two litres of EEM broth and tested as described above. The two litres of wastewater was filtered through 0·45 μm membranes (Toyo Roshi Kaisha, Tokyo). The membranes were then placed in 250 ml of EEM broth and subjected to

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Table 2. Salmonella *serovars isolated from broiler faeces of 35 farms*

	No. of samples			No. of isolates of each serovar of salmonellae from samples				
Farm no.	Tested	Positive (%)		*S.* Enteritidis	*S.* Infantis	*S.* Typhimurium	Others (no. of isolates)	Total
1	4	2	(50·0)		1	1		2
2	3	0	(0·0)					0
3	1	0	(0·0)					0
4	2	1	(50·0)		1			1
5	2	1	(50·0)	1				1
6	6	3	(50·0)	2	1	1		4*
7	4	2	(50·0)		2			2
8	15	5	(33·3)	1	1	2	*S.* Hadar (3)	7†
9	4	4	(100·0)	1	3	3	Untypeable strain in O-group B (1)	8§
10	4	0	(0·0)					0
11	5	0	(0·0)					0
12	8	2	(25·0)				*S.* Bredeney (1), *S.* Hadar (1)	2
13	1	0	(0·0)					0
14	4	0	(0·0)					0
15	7	0	(0·0)					0
16	4	0	(0·0)					0
17	2	0	(0·0)					0
18	1	0	(0·0)					0
19	2	2	(100·0)		2			2
20	3	2	(66·7)			1	*S.* Liverpool (1)	2
21	1	1	(100·0)		1			1
22	1	0	(0·0)					0
23	1	0	(0·0)					0
24	1	0	(0·0)					0
25	1	1	(100·0)		1			1
26	1	1	(100·0)		1			1
27	1	1	(100·0)		1			1
28	1	1	(100·0)		1			1
29	1	1	(100·0)		1			1
30	1	0	(0·0)					0
31	2	1	(50·0)				*S.* Hadar (1)	1
32	1	1	(100·0)		1			1
33	1	1	(100·0)		1			1
34	1	1	(100·0)	1			*S.* Hadar (1)	2*
35	1	0	(0·0)					0
Total	98	34	(34·7)	6	19	8	9	42

* One sample yielded two serovars.
† Two samples yielded two serovars each.
§ Two samples yielded two serovars each, and one sample yielded three serovars.

bacteriological tests as described above. Faecal samples were directly enriched in selenite brilliant green broth and selenite broth as described above.

Water survey

A total of 36 waste water samples of influent sewage, secondary treated sewage and final effluent from a sewage treatment plant were examined for the presence of salmonellae. The selected sewage plant which is typical for Western Japan was monitored once a month between April 1995 and March 1996. The plant processes daily 180000 m^3 of wastewater from 7500 hectares of residential area using an activated sludge (diffused air) treatment with no tertiary treatment. Each one litre wastewater sample was pre-enriched in one litre of EEM broth (twofold

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concentration), selectively enriched and plated as described above.

A total of 72 samples of riverwater (each sample contained one litre of riverwater) were collected in sterile bottles at six sampling points from five rivers in urban areas in Western Japan once a month from April 1995 to March 1996 and examined for the presence of salmonellae. The water samples were pre-enriched in EEM broth (twofold concentration), and analysed as previously described.

Genotyping using PFGE analysis

Sixty-five isolates of *S*. Enteritidis in this survey were characterized with PFGE analysis. These 63 isolates were compared with 71 isolates of *S*. Enteritidis from 6 outbreaks that occurred in 1996 and 1997 in Fukuoka Prefecture in this locality. These outbreaks occurred in May 1997 (385 cases), June 1997 (43 cases), June 1997 (47 cases), September 1997 (31 cases), July 1996 (33 cases), and October 1996 (644 cases). DNA for PFGE analysis was prepared as described previously [20]. After appropriate preparations for restriction endonuclease digestion were made, the DNA in each plug was digested with 20 U *Bln* I (Takara Shuzo, CO., Kyoto, Japan) at 37 °C for 15 h. We found *Bln* I digestion better for distinguishing *S*. Enteritidis from isolates with different genotypes than PFGE analysis with other restriction enzymes [15]. So, we induced *Bln* I digestion in this study.

DNA fragment patterns were assessed visually. The presence, absence and intensity of a band was scored and strains that differed by one band were assigned different pulsed-field profiles (PFPs). The similarities of PFPs were scored by the Dice coefficient of similarity [21]. This coefficient, F, expresses the proportion of shared DNA fragments in two isolates and was calculated by the following formula: $F = 2n_{xy}/(n_x + n_y)$, where n_x is the total number of DNA fragments from isolate X, n_y is the total number of DNA fragments from isolate Y, and n_{xy} is the total number of DNA fragments that were identical in the two isolates. An F value of 1·0 indicates that the two isolates have an identical PFP. *S*. Enteritidis ATCC 13076 and IFO 3313 were used as controls in PFGE of this serovar.

RESULTS

Poultry and live stock products

The results of the analysis of the 217 products are shown in Table 1. Eleven samples from 59 (18·6%) unpasteurized liquid eggs contained salmonellae, including *S*. Enteritidis in 9 samples, *S*. Nitra in 1 sample and an untypeable strain in 1 sample. However, among 31 brands of unpasteurized liquid eggs, 2 brand samples were highly contaminated with salmonellae, relatively (2 out of 4 samples and 4 out of 5 samples were salmonella-positive, respectively). The raw chicken parts were highly contaminated (37·8%), in comparison with the beef and pork, with *S*. Infantis which was the dominant serovar of salmonellae. However, *S*. Enteritidis, the major serovar of salmonellae isolated from liquid eggs, was isolated neither from raw chicken parts, beef or pork. One unpasteurized liquid egg in January 1995, 4 samples (consisting of 1 unpasteurized liquid egg, 2 raw chicken parts and 1 cut of beef) in June 1995, 3 raw chicken parts in September 1995, 7 samples (3 unpasteurized liquid eggs and 4 raw chicken parts) in June 1996, 16 raw chicken parts in September 1996, and 15 samples (6 unpasteurized liquid eggs and 9 raw chicken parts) in June 1997 were salmonella-positive.

Poultry farms

Forty poultry farms, consisting of 35 broiler farms and 5 layer farms, were analysed for salmonellae. From 20 of 35 broiler farms (57·1%) and 1 of 5 layer farms (20%), salmonellae were isolated. In broiler farms, 8 out of 12 samples in March 1995, 2 of 6 samples in April 1995, 1 of 14 samples in May 1995, none of 5 samples in June 1995, 2 of 11 samples in July 1995, none of 8 samples in August 1995, none of 6 samples in September 1995, 3 of 9 samples in October 1995, 13 of 19 samples in November 1995, 1 of 2 samples in December 1995, 1 of 2 samples in January 1996 and 3 of 4 samples in February 1996, were salmonella-positive.

Table 2 shows the serovars of salmonellae isolated from the broiler farms. *S*. Infantis, which was isolated from 19 samples, accounted for 45.2% of the isolates among 6 serovars. In layer farms, *S*. Enteritidis was isolated from 1 sample (in May 1995) out of 9, which were collected from 1 farm. From the other 4 farms' samples (total of 8 samples), salmonellae were not isolated.

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Table 3. *Survey of* Salmonella *Enteritidis* (●) *and other serovars* (○) *in shell-egg processing facilities during April 1995 to March 1998**

Facilities	Samples	No. of samples in each month	Apr.	May	Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.
			1995†									**1996**		
A	Swab samples of equipment and wastewater of egg washer	3	—	—	—	—	○	○	—	○	○	—	—	—
	Pooled cracked or faecally soiled shell eggs	1	NT§	NT§	—	○	○	○	○	○	—	●	—	—
B	Swab samples of equipment and wastewater of egg washer	2	—	—	—	—	—	—	—	—	—	—	—	●
	Pooled cracked or faecally soiled shell eggs	1	NT§	NT§	—	—	—	—	—	—	—	●	—	—
			1996									**1997**		
A	Swab samples of equipment and wastewater of egg washer	3	—	●	—	—	○	○	—	○	—	—	—	—
	Pooled cracked or faecally soiled shell eggs	1	—	—	—	—	○	○	—	—	—	—	—	—
B	Swab samples of equipment	2	—	—	—	○	●	—	○	—	—	—	—	—
	Pooled cracked or faecally soiled shell eggs	2	—	●	○○	○⊙	⊙	○○	○	○	○○	○	○○	○●
			1997									**1998**		
A	Swab samples of equipment and wastewater of egg washer	3	—	○	—	●	○	○	○	—	—	—	—	—
	Pooled cracked or faecally soiled shell eggs	1	—	—	—	●	—	○	●	—	—	—	○	—
B	Swab samples of equipment	2	—	●	—	○	○○	○○	○	○	●	●	—	—
	Pooled cracked or faecally soiled shell eggs	2	●	●	○○	○○	○○	○○	—	○●	●	●	—	—

* ●, indicates a sample that was positive for *S.* Enteritidis; ○, indicates a sample that was positive for at least one strain of other salmonella serovars; and ⊙, indicates one sample that was positive for *S.* Enteritidis and at least one strain of other salmonella serovars at the same time.

† Salmonella-negative.

§ Not tested.

Table 4. *Number of salmonella isolates obtained from shell-egg processing facilities (A and B) and from henhouses associated with shell-egg processing facility B during April 1995 to March 1998*

Site	Sample	No. of samples		Serovars of isolates	
		Tested	Positive (%)	O-group	Name (no.)
Shell-egg processing facility A					
	Swabs and wash water*	108	13 (12·0)	D_1	*S.* Enteritidis (2)
				B	*S.* Hato (1), *S.* Typhimurium (1)
				C_1	*S.* Mbandaka (2), *S.* Oranienburg (1)
				C_3	Untypeable (2)
				K	Untypeable (4)
	Shell eggs†	34	12 (35·3)	D_1	*S.* Enteritidis (3)
				B	*S.* Agona (1)
				C_1	*S.* Mbandaka (3), *S.* Oranienburg (2)
				C_3	*S.* Corvallis (1)
				G	Untypeable (1)
				K	Untypeable (1)
Shell-egg processing facility B					
	Swabs and wash water*	72	14 (19·4)	D_1	*S.* Enteritidis (5)
				B	*S.* Agona (2)
				C_1	*S.* Braenderup (3), *S.* Montevideo (3), *S.* Virchow (3)
				K	Untypeable (1)
	Shell eggs†	58	32 (55·2)	D_1	*S.* Enteritidis (10)
				B	*S.* Agona (3)
				C_1	*S.* Livingstone (1), *S.* Braenderup (4), *S.* Montevideo (13), *S.* Thompson (1), *S.* Virchow (2), *S.* Infantis (2)
Henhouses associated with shell-egg processing facility B					
	Swabs	22	11 (50·0)	D_1	*S.* Enteritidis (2)
				B	*S.* Agona (1)
				C_1	*S.* Braenderup (7), *S.* Montevideo (2)
	Faeces	33	0 (0·0)		
Total		327	82 (25·1)		

* Swab samples of equipment and wastewater of egg washer.
† Pooled cracked or faecally soiled shell eggs.

Shell-egg processing facilities and related henhouses

In shell-egg processing facility A, 25 of 142 samples (17·6%) were salmonella-positive including swab samples of equipment and shell eggs, and 7 serovars and 8 untypeable strains of salmonellae were isolated (Tables 3, 4). *S.* Mubandaka was isolated from 5 of 142 samples of both environments. *S.* Enteritidis contaminated 3 of 34 (8·8%) cracked or faecally soiled shell egg samples and 2 of 108 (1·9%) swab samples of equipment and waste water of egg washer. In shell-egg processing facility B, 8 serovars and an untypeable strain of salmonellae were isolated from swab samples of equipment and shell eggs. *S.* Montevideo and *S.* Enteritidis were the dominant serovars, being isolated from 16 and 15 samples, respectively.

Only *S.* Agona and *S.* Enteritidis were isolated from both facilities. No salmonellae was isolated from faecal samples of the layer henhouses supplying processing facility B. However, *S.* Enteritidis, *S.* Agona, *S.* Braenderup, and *S.* Montevideo, which were the same serovars isolated in processing facility B, were isolated from swab samples of the handling equipment in these henhouses.

Water survey

All 12 samples of influent sewage contained at least 1 strain of salmonellae (Table 5). The secondary treated sewage contained salmonellae in all samples except for 2 taken in April and August, 1995. The final

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Table 5. *Serovars of salmonella isolated from sewage samples in a one-year study*

	Sampling point			
	Influent	Secondary treated	Final effluent	Total
No. of tested samples	12	12	12	36
No. of positive samples	12	10	0	22
(%)	(100·0)	(83·3)	(0·0)	(61·1)
No. of isolates in each serovar				
O-group D_1 *S.* Enteritidis	1			1
O-group B *S.* Agona		2		2
O-group B *S.* Kiambu		2		2
O-group B *S.* Saintpaul		1		1
O-group B *S.* Typhimurium	1	1		2
O-group B Untypeable	1			1
O-group C_1 *S.* Infantis	1			1
O-group C_1 *S.* Larochelle	1			1
O-group C_1 *S.* Thompson	4			4
O-group C_1 *S.* Virchow		1		1
O-group C_2 *S.* Hadar		3		3
O-group C_3 *S.* Corvallis	1			1
O-group C_3 Untypeable		1		1
O-group E_1 *S.* Amsterdam	1	1		2
O-group E_1 *S.* Anatum	6	4		10
O-group E_1 *S.* Give		1		1
O-group E_1 *S.* Zanzibar	1			1
O-group E_4 *S.* Senftenberg	3	2		5
O-group K Untypeable		1		1
Total				41*

* 22 of 36 samples collected from sewage were positive for at least one strain of salmonella. Eleven samples yielded 1 serovar, 4 samples yielded 2 serovars, 6 samples yielded 3 serovars and 1 sample yielded 4 serovars.

Table 6. *Serovars of salmonella isolated from riverwater samples in a one-year study*

	Sampling point						
	A	B	C	D	E	F	Total
No. of tested samples	12	12	12	12	12	12	72
No. of positive samples	3	1	4	4	2	2	16
(%)	(25·0)	(8·3)	(33·3)	(33·3)	(16·7)	(16·7)	(22·2)
No. of isolates in each serovar							
O-group D_1 *S.* Enteritidis	1						1
O-group B *S.* Typhimurium						1	1
O-group C_1 *S.* Infantis				1			1
O-group C_1 *S.* Mikawasima			1		1		2
O-group C_1 *S.* Thompson		1	1				2
O-group C_3 *S.* Corvallis			1			1	2
O-group E_1 *S.* Anatum				1			1
O-group E_1 *S.* Bolton				1			1
O-group E_1 *S.* Give	1						1
Untypeable	1		1	1	1		4

effluent samples did not contain any salmonellae. *S.* Anatum, which was isolated from 10 samples, was dominant among the 16 different serovars and other untypeable strains of salmonellae isolated from 36 sewage samples. *S.* Enteritidis and *S.* Infantis were isolated from only 1 sample.

Sixteen out of 72 samples from 6 sampling points in 5 rivers contained salmonellae (Table 6). At sampling

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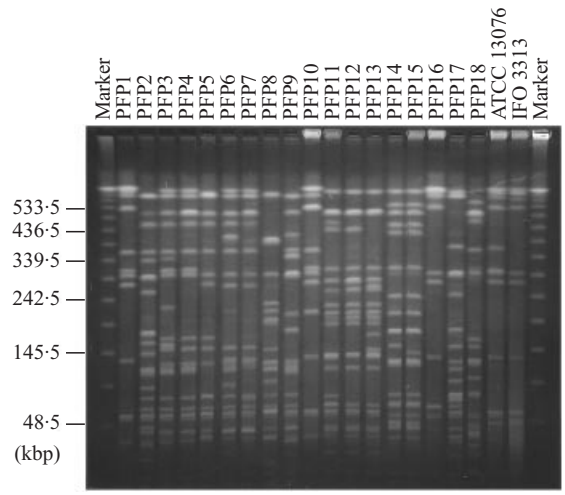

Fig. 1. Comparison of pulsed-field profiles (PFPs) of *Salmonella enterica* subsp. *enterica* serovar Enteritidis isolates digested with *Bln* I. PFP1-PFP18 were isolated from various sources. *S.* Enteritidis ATCC 13076 and IFO 3313 were used as control in pulsed-field gel electrophoresis analysis of this serovar. Marker shows the DNA size standard used was a bacteriophage lambda consisting of concatemers starting at 48·5 kilobase pairs.

point A, 3 samples contained salmonellae in April 1995, July 1995 and January 1996. At sampling point B, 1 sample contained salmonellae in August 1995. At sampling point C, 4 samples contained salmonellae in July, September, and November 1995 and January 1996. At sampling point D, 4 samples contained salmonellae in May, July, November and December 1995. At sampling point E, 2 samples contained salmonellae in August and October 1995. At sampling point F, 2 samples contained salmonellae in December 1995 and January 1996. No salmonellae was isolated from any riverwater sample in February, March or June. Nine serovars and other untypeable strains of salmonellae were isolated and there was no dominant serovar. *S.* Enteritidis and *S.* Infantis were each isolated from only one sample.

PFGE analysis and phage types of *S.* Enteritidis Isolates

A PFGE of the fragments obtained on *Bln* I digestion of genomic DNA from 134 isolates of *S.* Enteritidis showed 18 distinct PFPs with 6–14 resolvable chromosomal fragments, ranging approximately above 100 kbp in size (Fig. 1, Table 7). The evaluation in this range may be helpful for the interpretation of PFP excluding plasmid DNA [11].

Six *S.* Enteritidis isolates from shell-egg processing facility A, 16 isolates from shell-egg processing facility B and 48 human isolates from 4 outbreaks exhibited the same PFP (PFP1) (Fig. 2), and PT-typed isolates showed a PT1, PT4 or RDNC pattern (react with the typing phages but do not conform to any of the current recognized patterns). Isolates with the character of both PFP1 and PT4 (PFP1-PT4) were found in 32 isolates of 2 outbreaks and 4 isolates of shell-egg processing facilities. Four isolates obtained from 3 liquid egg samples of the same brand in June 1996 and June 1997, and human isolates from an outbreak in July 1996 showed the same PFP (PFP10) (Fig. 2). PFP1 isolates, PFP10 isolates and PFP16 isolates (obtained from riverwater), showing relatively high similarity ($F = 0{\cdot}80$–$0{\cdot}94$), were placed in Group A (Table 8). According to the Dice coefficient of similarity, there were 3 other PFP groups, B ($F = 0{\cdot}85$–$0{\cdot}96$), C ($F = 0{\cdot}91$) and D ($F = 0{\cdot}56$–$0{\cdot}90$), and 5 distinct PFPs. Groups B, C and D consisted of liquid egg isolates, liquid egg isolates and broiler faeces isolates, respectively. Isolates obtained from the outbreak in October 1996, which occurred among schoolchildren, showed quite a unique PFP (PFP18), the F value against other PFPs being 0·00–0·44.

DISCUSSION

We reported here a survey of salmonellae in the environment, and the molecular characterization of strains of *S.* Enteritidis isolated from environmental sources and their relationship with human isolates with PFGE analysis.

There was no direct relation between the suspected vehicle of four individual outbreaks that occurred in this location in 1997 and the shell-egg processing facilities that were surveyed in this study, according to the inspector's report. Based on PFGE analysis, similarity of *S.* Enteritidis isolates with PFP1 between four individual outbreaks, shell-egg processing facilities and hen houses was indicated in a few clonal lines which were related to each other. A clonal line with the character of both PFP1 and PT4 (PFP1-PT4) which was found in isolates of human outbreaks and shell-egg processing facilities, accumulated in some chicken production environments and caused outbreaks in the area during the 3-year period 1995–7, surprisingly. Comparative analysis of isolates obtained 7 years apart outbreak (1990) [15] also indicates these genotypes of *S.* Enteritidis (PFP1-PT4) to be stable and to persist over a considerable period.

Many molecular analyses have led to the prevailing view that most epidemics of infectious disease in-

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Table 7. *Distribution of* Bln *I pulsed-field gel electrophoresis profiles in isolates of Salmonella Enteritidis from shell-egg processing facilities and other sources*

Source	PT	No. of isolates with each PT	PFP	
			Found in several sources	Found in each source
Shell-egg processing facility A	4	2	PFP1§	
	NT*	4	PFP1	
	NT	2		PFP2
Shell-egg processing facility B	4	2	PFP1	
	NT	14	PFP1	
Henhouses associated with shell-egg processing facility B	NT	12	PFP1	
Broiler faeces	1	1		PFP4
	1	1		PFP5
	1	1		PFP6
	1	1		PFP7
	UT	1		PFP3
	NT	2		PFP8
Liquid eggs	1	2		PFP9
	NT	4	PFP10	
	NT	1		PFP11
	NT	1		PFP12
	NT	2		PFP13
	NT	1		PFP14
	NT	5		PFP15
Riverwater	4a	2		PFP16
Sewage	1	2		PFP17
Human and vehicular food in outbreaks of *S.* Enteritidis in Fukuoka, Western Japan				
July 1996	7	11	PFP10	
October 1996	1	12		PFP18
May 1997	RDNC†	11	PFP1	
June 1997	1	5	PFP1	
June 1997	4	15	PFP1	
September 1997	4	17	PFP1	
Total		134		

* NT, not tested.
† RDNC, reacts with the typing phages but does not conform to any of the currently recognized patterns.
§ PFP, pulsed-field profile.

cluding salmonellosis are caused by a pathogen genotype with special fitness properties [22–25]. *S.* Enteritidis occupies an ancestral and pivotal position among some serovars of salmonellae and has a high degree of genotypic homogeneity [26, 27]. A limited background of genetic diversity of PT4 strains, one of the dominant phage types in *S.* Enteritidis outbreaks worldwide, was reported in several studies [11, 28, 29]. In the present study, the PT4 *S.* Enteritidis isolates had PFP1, showing non-genetic diversity. Thus, our results further support the hypothesis that a few clonal lines of *S.* Enteritidis, highly clonal nature (often PT4 in character) and carried by laying hens, caused human outbreaks over a long period among multiple clonal lines of *S.* Enteritidis coexisting independently simultaneously in the region, showing local adaptation. This clonal line would escape natural selection in the host, laying hens, and remain mono- or pauciclonal line among the heterogeneous array of *S.* Enteritidis. The mechanism of survival of the PFP1–PT4 clonal line without selection remains unclear. The parasite's local adaptation results from that parasites infected locally common host genotypes significantly more often than rare host genotypes in each geographical area [30]. This finding was made with snail and digentic trematode. So, we do not know

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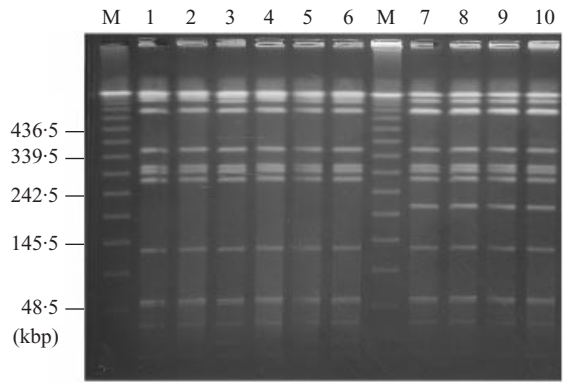

Fig. 2. Comparison of pulsed-field gel electrophoresis profiles of *Salmonella enterica* subsp. *enterica* serovar Enteritidis isolates obtained from environmental sources and humans. Lanes: 1, isolate from shell-egg processing facility A sample (PFP1); 2, isolate from shell-egg processing facility B sample (PFP1); 3, isolate from a human outbreak that occurred in May 1997 (PFP1); lane 4, isolate from an outbreak in June 1997 (PFP1); lane 5, isolate from an outbreak in June 1997 (PFP1); lane 6, isolate from an outbreak in September 1997 (PFP1); lane 7, isolate from a liquid egg sample in June 1996 (PFP10); lane 8, isolate from a liquid egg sample in June 1997 (PFP10); lane 9, isolate from a liquid egg sample in June 1997 (PFP10); lane 10, outbreak in July 1996 (PFP10); M, marker which shows that the DNA size standard used was a bacteriophage lambda concatemer starting at 48·5 kilobase pairs.

whether this theory applies to the relation between bacterium (salmonella) and host (hen). However, with regard to future work, it would be of interest, for example, to determine the genotype of the laying hen in the region and correlation between host and parasite (salmonella).

Our results show that *S*. Enteritidis is commonly associated with the chicken egg production environment, and by contrast, *S*. Infantis is commonly associated with the broiler meat production environment, among not only *S*. Enteritidis and *S*. Infantis but also multiple serovars of salmonellae isolated in this area. Broiler meat is an important vehicle of infection by zoonotic salmonellae of human [31, 32]. The high rate of salmonellae contamination in broiler meat environments and raw chicken parts reported here is similar to that of studies done in other countries [14, 33]. In the USA, *S*. Derby, *S*. Hadar, and *S*. Kentucky were common serovars in 31 broiler farms [12]. *S*. Virchow was the main isolate from chickens and the common salmonellae serovar isolated from humans in England and Wales [34]. *S*. Hadar was dominant in Tochigi Prefecture in Eastern Japan [35]. However, in the present study, neither *S*. Virchow, *S*. Derby nor *S*. Kentucky was isolated from chicken parts or faecal samples of broiler farms in Western Japan. *S*. Infantis was the dominant serovar isolated from raw chicken parts and faecal samples of broiler farms, making up 62 and 45%, respectively, of those isolates. An association has been found between the high rate of *S*. Infantis contamination in broiler farms and raw chicken parts.

We previously reported that relatively few clonal lines of *S*. Infantis had spread widely whereas multiple clonal lines exist in Western Japan [20]. In addition, the epidemiological link between the chicken egg production environment and human infection with *S*. Enteritidis was revealed with molecular genotyping in this study. Thus, eradication of these clonal lines of *S*. Enteritidis and *S*. Infantis in poultry farms should lead to further reductions in the number of contaminated eggs and chicken meat products and hopefully also the incidence of human salmonellosis.

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Table 8. *Similarity matrix of* Salmonella *Enteritidis based on PFPs*

	Isolates		*F* value among PFPs																	
PFP	Origin	No.	1	10	16	2	9	11	12	13	14	15	3	4	5	6	7	8	17	18
1	Human Facility*, henhouse	82	Group A					Group B			Group C		Group D							
10	Human, liquid eggs	15	0·94																	
16	Riverwater	2	0·86	0·80																
2	Facility*	2	0·21	0·20	0·12															
9	Liquid eggs	2	0·22	0·21	0·13	0·38														
11	Liquid eggs	1	0·19	0·09	0·00	0·00	0·09													
12	Liquid eggs	1	0·20	0·10	0·00	0·09	0·09	0·96												
13	Liquid eggs	2	0·19	0·09	0·00	0·00	0·09	0·85	0·88											
14	Liquid eggs	1	0·11	0·20	0·00	0·09	0·29	0·25	0·26	0·25										
15	Liquid eggs	5	0·11	0·20	0·00	0·09	0·29	0·25	0·26	0·25	0·91									
3	Broiler faeces	1	0·45	0·43	0·20	0·32	0·42	0·22	0·15	0·15	0·16	0·16								
4	Broiler faeces	1	0·53	0·50	0·24	0·36	0·57	0·25	0·17	0·25	0·18	0·27	0·80							
5	Broiler faeces	1	0·47	0·33	0·40	0·40	0·42	0·18	0·10	0·09	0·10	0·10	0·61	0·80						
6	Broiler faeces	1	0·53	0·40	0·35	0·36	0·48	0·17	0·09	0·08	0·18	0·18	0·56	0·64	0·60					
7	Broiler faeces	1	0·59	0·44	0·40	0·40	0·42	0·09	0·10	0·09	0·10	0·10	0·61	0·70	0·67	0·90				
8	Broiler faeces	2	0·24	0·22	0·13	0·10	0·11	0·18	0·19	0·18	0·10	0·10	0·09	0·30	0·22	0·10	0·11			
17	Sewage	2	0·24	0·33	0·13	0·20	0·32	0·09	0·10	0·09	0·10	0·10	0·17	0·30	0·11	0·50	0·44	0·33		
18	Human	12	0·24	0·22	0·13	0·10	0·11	0·09	0·10	0·09	0·20	0·20	0·09	0·20	0·44	0·40	0·44	0·00	0·11	

*Facility, shell-egg processing facility.

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