FULL PAPER *Public Health*

Lysine Decarboxylase-Negative *Salmonella enterica* Serovar Enteritidis: Antibiotic Susceptibility, Phage and PFGE Typing

Shogo YAMASAKI[1,2)]*, Kenshi HARA[1)], Hidemasa IZUMIYA[3)], Haruo WATANABE[3)], Naoaki MISAWA[2,4)], Karoku OKAMOTO[2,5)] and Kozo TAKASE[2,5)]

[1)]Nagasaki Prefectural Institute for Environmental Research and Public Health, 2–1306–11 Ikeda, Omura, Nagasaki 856–0026, [2)]United Graduate School of Veterinary Sciences, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753–8515, [3)]Department of Bacteriology, National Institute of Infectious Diseases, 1–23–1 Toyama, Shinjuku, Tokyo 162–8640, [4)]Faculty of Agriculture, Miyazaki University, 1–1 Gakuenkibanadai-Nishi, Miyazaki 889–2192 and [5)]Faculty of Agriculture, Kagoshima University, 1–21–24 Korimoto, Kagoshima 890–0065, Japan

(Received 15 January 2007/Accepted 19 April 2007)

ABSTRACT. One hundred twenty *Salmonella* Enteritidis isolates collected from1992 to 2005 in Nagasaki prefecture (65 isolates from 40 outbreak cases, 44 from sporadic diarrhea patients, and 11 from chicken-related products) were investigated by their antibiotic susceptibility profiles, phage typing, and pulsed-field gel electrophoresis (PFGE) typing. Out of them, 18 were identified as lysine decarboxylase (LDC)-negative isolates, and 15 showed resistance toward streptomycin. Based on the PFGE typing, the isolates were classified into five clusters by UPGMA clustering method. Three LDC-negative isolates belonged to cluster A and were of phage type (PT) 4 and isolated between 2000 and 2004. Other 15 LDC-negative isolates belonged to cluster E. They were PT1, reacted but did not conform (RDNC), or untypable and were isolated between 2001 and 2004. LDC-negative isolates of the cluster A differed from LDC-negative isolates of the cluster E in antibiotic susceptibility profiles, phage typing, and PFGE typing. LDC-negative isolates of the cluster E were isolated after 2001 in Nagasaki prefecture.

KEY WORDS: cluster analysis, epidemiology, lysine decarboxylase, PFGE, *Salmonella* Enteritidis.

J. Vet. Med. Sci. 69(8): 813–818, 2007

Salmonella enterica serovar Enteritidis (*S.* Enteritidis) is the most common serovar that causes salmonellosis in humans. Since 1980s, there has been dramatic increase of salmonellosis in Europe and worldwide [2, 5, 12]. In Japan, *S.* Enteritidis has been the predominant serotype since 1989 [7, 10]. The most common reservoir for *S.* Enteritidis has been chickens [12].

Lysine decarboxylase (LDC) activity is one of the most important criteria to identify *Salmonella* species. In recent years, however, isolates that lack this biochemical activity have been observed in *S.* Enteritidis isolates from foodborne outbreaks and cases of sporadic diarrhea in Japan [8–10]. Morita *et al.* [4] clarified the mechanism for this loss of LDC activity and suggested that these isolates have spread already throughout Japan. However, no nationwide epidemiological survey comparing LDC-negative and positive isolates has been reported yet.

In the present study, we performed an epidemiological survey of LDC-negative *S.* Enteritidis isolates in Nagasaki prefecture, Japan.

MATERIALS AND METHODS

Bacterial strains: *S.* Enteritidis isolates used in the present study were obtained from four sources: 65 isolates from 44 outbreaks, 44 from sporadic diarrhea patients, 10 from chicken eggs, and 1 from commercial chicken meat. One hundred twenty isolates examined were collected for the past 14 years from 1992 to 2005 in Nagasaki prefecture of Kyushu Island. The isolates were preserved in our laboratory and in municipal hospitals until use. The reference strains used for PFGE typing were *S.* Enteritidis strains GTC131 (Gifu University, Gifu, Japan) and NBRC 3313 (National Institute of Technology and Evaluation, Biological Resource Center, Kisarazu, Japan). The LDC-negative strains of Honshu Island associated isolates were Fukuoka 101 (PT4) isolated in Fukuoka prefecture (Fukuoka Institute of Health and Environmental Sciences, Dazaihu, Japan) from a traveler to Honshu Island and Okayama S-HC359 (PT14b) isolated from a food poisoning case in Okayama prefecture of Honshu Island (Okayama Institute for Environmental Sciences and Public Health, Okayama, Japan).

LDC activity test: LDC activity of the isolates was examined with LIM medium (Nissui Pharmaceutical, Tokyo, Japan) and Moeller's decarboxylase medium (Becton, Dickinson and Company, Sparks, MD, U.S.A.) by adding 1%(+)-lysine hydrochloride.

Antibiotic susceptibility test: Antibiotic susceptibility was tested for all isolates using BD Sensi-Disc antimicrobial discs (Becton, Dickinson and Company, Sparks, MD, U.S.A.) in accordance with Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards [NCCLS]) guidelines [1]. Antimicrobial agents used in this study were as follows; streptomycin (SM), chloramphenicol (CP), tetracycline (TC), kanamycin (KM), ampicillin (ABPC), nalidixic acid

* CORRESPONDENCE TO: YAMASAKI, S., Nagasaki Prefectural Institute for Environmental Research and Public Health, 2–1306–11 Ikeda, Omura, Nagasaki 856–0026, Japan.
e-mail: syamasaki@pref.nagasaki.lg.jp

(NA), fosfomycin (FOM) and sulfamethoxazole-trimethoprim (ST).

Phage typing: Phage typing was performed by using the *S.* Enteritidis phage-typing scheme provided by Health Protection Agency (HPA) of the United Kingdom according to the method of Ward *et al.* [15].

Clustering analysis based on PFGE: A total of 120 isolates were characterized by clustering analysis with PFGE, which was performed as previously described using the *Salmonella* Braenderup H9812 as DNA size standard strain [3, 11], with the following modifications. Cells grown overnight at 35°C in 10 m*l* of brain heart infusion broth (Becton, Dickinson and Company, Sparks, MD, U.S.A.) were centrifuged and washed with 1 m*l* of sterilized phosphate-buffered saline (pH 7.2). The washed cell pellets were resuspended in 500 μl of sterilized and deionized distilled water. Plugs were prepared by adding the sample to 500 μl of 1% SeaKem Gold agarose gel (Cambrex Bio Science, Rockland, ME, U.S.A.). The mixtures were poured into 0.7 mm sample-plug casters and kept at 4°C. The plugs were then transferred to tubes containing 2 m*l* of 0.5M-EDTA solution (pH 8.0) containing 1 mg/m*l* of proteinase-K (Wako Pharmacia, Osaka, Japan) and 1% *N*-lauroylsarcosin (Sigma Aldrich, St. Louis, MO, U.S.A.). The plugs were incubated overnight at 50°C and washed three times in 1 m*l* of 1 × TE buffer for 30 min each. The plugs were incubated in 200 μl of 1 × restriction buffer (H buffer; Roche Diagnostics GmbH, Mannheim, Germany) for 30 min at 4°C to allow equilibration, followed by addition of 100 μl of restriction buffer containing 20 U of restriction enzyme *Bln* I (Roche Diagnostics GmbH, Mannheim, Germany), and digestion was performed overnight at 37°C. The plugs were placed in 1% SeaKem Gold agarose gel (dissolved in 0.5 × tris-borate-EDTA buffer). Digested DNA was separated with the following electrophoresis conditions: voltage, 6 V/cm at 14°C for 19 hr; initial pulse, 2.2 s; final pulse, 63.8 s; angle, 120°. The run was developed in a CHEF DR-III (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with 0.5 × tris-borate-EDTA buffer to which 50 μM thiourea was added. The gels were stained with ethidium bromide, and visualized with UV transilluminator.

Banding patterns were analyzed by the unweighted pair-group method with arithmetic averages (UPGMA) using Lane Multi Screener for Windows version 3 (ATTO, Tokyo, Japan). Clusters were designated by genetic dissimilarity of 0.236 in this study.

RESULTS

Longitudinal study of LDC-negative S. Enteritidis isolates: One hundred twenty strains of *S.* Enteritidis were collected from 1992 to 2005. Eighteen isolates (13 from outbreak cases and 5 from sporadic diarrhea patients) were LDC-negative. These LDC-negative isolates were found between 2000 and 2004 (Table 1).

Antibiotic susceptibility profiles: Of the 120 isolates, 56 (46.7%) were sensitive to all the drugs tested, 51 (42.5%) were SM-resistant, 11 (9.2%) were resistant to ABPC and SM, 1 showed resistance to NA, and 1 was resistant to SM and TC. Of the 18 LDC-negative isolates, 15 showed SM-resistance, and 3 were sensitive to all the drugs (Table 1). In addition, Fukuoka 101 and Okayama S-HC359 strains were sensitive to all the drugs.

Phage typing: The predominant phage type was PT4 (29.2%) and PT1 (27.4%) among the 113 isolates examined. The remaining 49 isolates were classified into 11 different phage types. LDC-negative isolates belonged to PT1, PT4, reacted but did not conform (RDNC), or untypable group (Table 2).

Clustering analysis by PFGE: Based on the *Bln* I-PFGE

Table 1. Number of *S.* Enteritidis isolates used in this study and Antibiotic suceptibility profiles

Year	No. of isolates	Source			Antimicrobial resistance pattern				
		Outbreak cases	Sporadic patients	Food	Sensitive	SM	NA	ABPC•SM	SM•TC
1992	4	2	2		2	2			
1993	7	1	6			2		5	
1994	4	1	3		1			3	
1995	2		2					2	
1996	3		3		2			1	
1997	10	9	1		4	6			
1998	10	7	3		7	2			1
1999	14		4	10	1	13			
2000	24 (2)	20 (2)	4		18 (2)	6			
2001	4 (2)	4 (2)				4 (2)			
2002	3 (3)		3 (3)			3 (3)			
2003	7 (2)	4 (2)	2	1	3 (1)	3 (1)	1		
2004	25 (9)	17 (7)	8 (2)		15	10 (9)			
2005	3		3		3				
Total	120 (18)	65 (13)	44 (5)	11	56 (3)	51 (15)	1	11	1

( ): No. of LDC-negative isolates. SM: Streptmycin, NA: Nalidixic acid, ABPC: Ampicillin, TC: Tetracycline. All isolates were sensitive to chloramphenicol, kanamycin, fosfomycin, and sulfamethoxazole-trimethprim.

Table 2. Phage type distribution of *S.* Enteritidis isolates

Year	No. of isolates	Phage type																ND
		1	1c	3	4	4b	5a	6	6a	21	30	36	RDNC	UT				
1992	4	1									1		2					
1993	7	3	3															1
1994	4	1	2		1													
1995	2		2															
1996	3	1				2												
1997	10	1		1		3												5
1998	10	5			2		2											1
1999	14	3			1				9	1								
2000	24 (2)	9			8 (2)				1	2		1	1		2			
2001	4 (2)												4 (2)					
2002	3 (3)	2 (2)													1 (1)			
2003	7 (2)	3 (1)			3 (1)			1										
2004	25 (9)	2 (2)			15								5 (5)		3 (2)			
2005	3				3													
Total	120 (18)	31 (5)	7	1	33 (3)	5	2	1	10	3	1	1	12 (7)		6 (3)			7

( ): No. of LDC-negative isolates, RDNC: Reacted but did not conform, UT: Untypable, ND: Not done.

typing, the 120 isolates were divided into five clusters, A, B, C, D, and E, which contained 57, 13, 5, 27 and 18 isolates, respectively (Fig. 1). Of the 18 LDC-negative isolates, 3 belonged to the cluster A and 15 to the cluster E.

Figure 2 showed major banding patterns of the electrophoresis in each clusters. Especially, cluster E showed every different pattern of the LDC-negative isolates. Banding pattern of the electrophoresis of the LDC-positive isolates in the cluster A (Spo11/4/94 strain) was similar to that of the reference strain GTC131. In contrast, the band pattern of the LDC-negative isolates (Out21a/4/00 and Out29/4/03 strains) in the cluster A differed from those of the LDC-positive strains. It was also largely different from those of the LDC-negative isolates in cluster E. In addition, the band pattern of these isolates in the cluster E varied from each other. Furthermore, the patterns of the clusters B, C and D differed from those of other LDC-negative isolates.

DISCUSSION

The numbers of *S.* Enteritidis food poisoning cases and sporadic diarrhea patients due to *S.* Enteritidis have decreased in Japan since 2000 [10]. It is thought that this decrease was due to the result of biosecurities taken in chicken farms and related facilities to prevent egg contamination against *Salmonella* [13]. In contrast, cases due to LDC-negative *S.* Enteritidis have been reported recently in western Japan, such as Kyoto City and Yamaguchi prefecture [8–10]. According to the report from the Yamaguchi prefecture, percentage of LDC-negative isolates from diarrhea patients have risen from 16.4% in 2002 to 71.3% in 2003, and finally to 100% in 2004 [9]. Much attention should be paid to epidemiology of the LDC-negative *S.* Enteritidis.

In our study, 18 strains out of the 120 isolates of *S.* Enteritidis were found to be LDC-negative. In phage typing, these LDC-negative isolates were divided into four groups: PT1, PT4, RDNC and untypable. The surveillance data on phage typing of *S.* Enteritidis outbreaks showed that PT4 was the most common phage type after 1990, PT1 was secondary after 1992, and RDNC have increased since 1997 in Japan [10]. In Nagasaki prefecture, 64 isolates (56.6%) were of the types PT4 and PT1. Moreover, 12 (10.0%) RDNC isolates were found in this research, with a tendency. Although the type PT14b has increased in western Japan, Kobe city [6], Kyoto city [8], and Yamaguchi prefecture [10], this type was not detected in Nagasaki prefecture.

The analysis of LDC-negative isolates for drug resistance, phage type, and cluster analysis by PFGE showed that they did not belong to a single group. Therefore, it is suggested that the LDC-negative strains isolated in Nagasaki prefecture may have different origins.

Although three LDC-negative (PT4) strains isolated in Nagasaki between 2000 and 2003 belonged to the cluster A, the epidemiological survey demonstrated that these were isolated from travelers who visited to Honshu Island. Additionally, the drug resistance patterns and PFGE electrophoresis were the same as those of the Honshu Island associated strains Fukuoka 101 (PT4) and Okayama S-HC359 (PT14b) (see Fig. 2). Morita *et al.* showed the PFGE profiles of LDC-negative 10 strains, of which isolation areas in Japan were not reported [4]. The four PT4 and four PT14b strains were isolated from Honshu Island, the one PT4 strain was isolated in Fukuoka prefecture, and the remaining one PT1 strain was isolated in Nagasaki prefecture, which was identical to the Out36/RDNC/04 strain of cluster E in this study (personal communication from Dr. H. Izumiya of National Institute of Infectious Diseases). The nine among 10 strains reported by Morita *et al.* [4] showed similar PFGE patterns to our three LDC-negative PT4 strains which belong to the cluster A in this study. These three LDC-negative isolates from two cases belonging to the cluster A are likely to have originated from Honshu Island. Moreover, of the LDC-negative isolates belonging to the

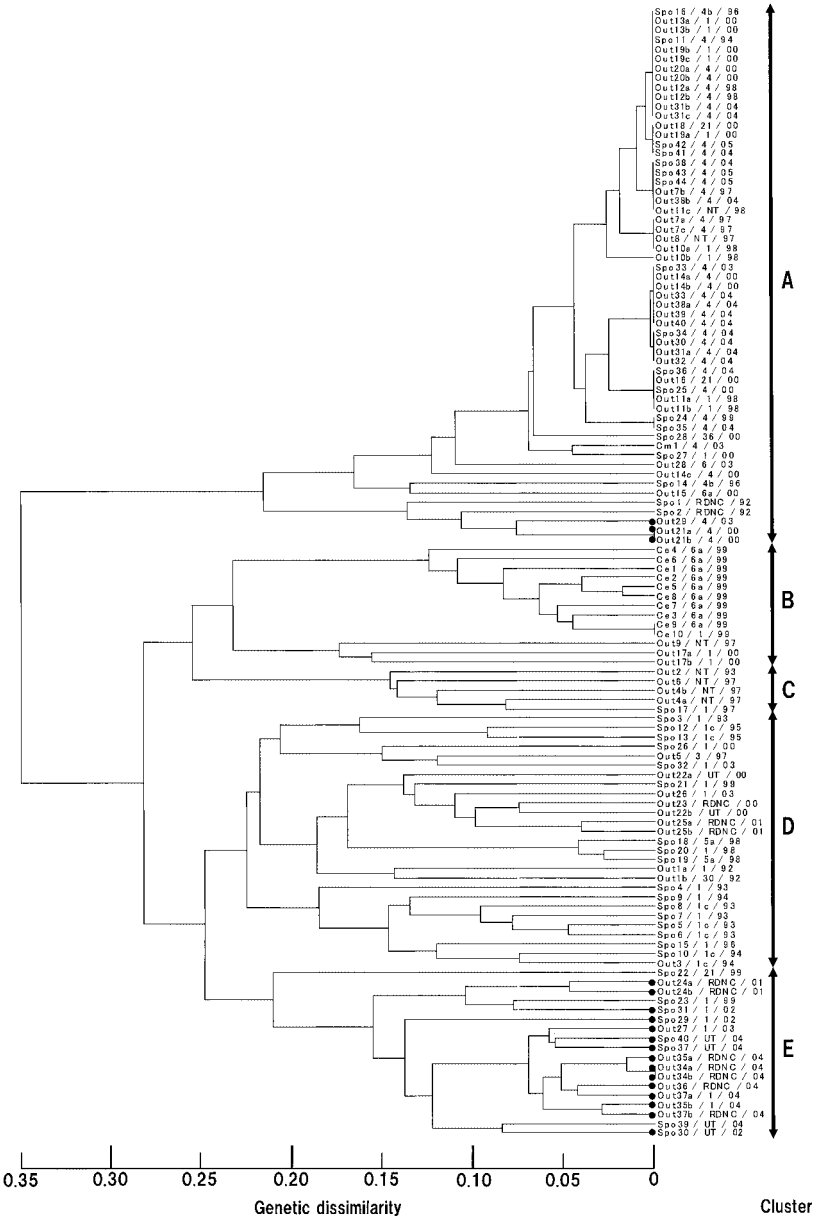

Fig. 1. UPGMA clustering dendrogram using *Bln*I PFGE patterns of *S.* Enteritidis isolates. ●: negative to lysine decarboxylase, Out: outbreak case, Spo: sporadic patient, Ce: chicken egg, Cm: chicken meat, RDNC: reacted but did not conform, UT: untypable. Strain no.: case no. in each sources/phage type/isolation year.

cluster E, five isolates were PT1, seven were RDNC, and three were untypable; these were isolated from 2001 to 2004. These 15 LDC-negative isolates were SM-resistant. Their PFGE electrophoresis patterns differed largely from the LDC-negative isolates that preserved to be in Honshu Island. It was speculated that these isolates belonged to different groups. LDC-negative isolates of the cluster E appeared to be "category of closely related" in accordance with criteria of Tenovar *et al.* [14]; however, these could be different group since they showed three different phage typing patterns.

The LDC-negative *S.* Enteritidis were isolated after 2000 in Japan [8–10], although their routes of infection have not yet been clarified. However, our results showed that their some differences in epidemiological markers between LDC-negative isolates from Honshu Island and those from Nagasaki prefecture. It will be necessary to initiate a more extensive nationwide epidemiological investigation to clarify the distribution of the LDC-negative strains, and prevent their food poisoning as well as those by LDC-positive *S.*

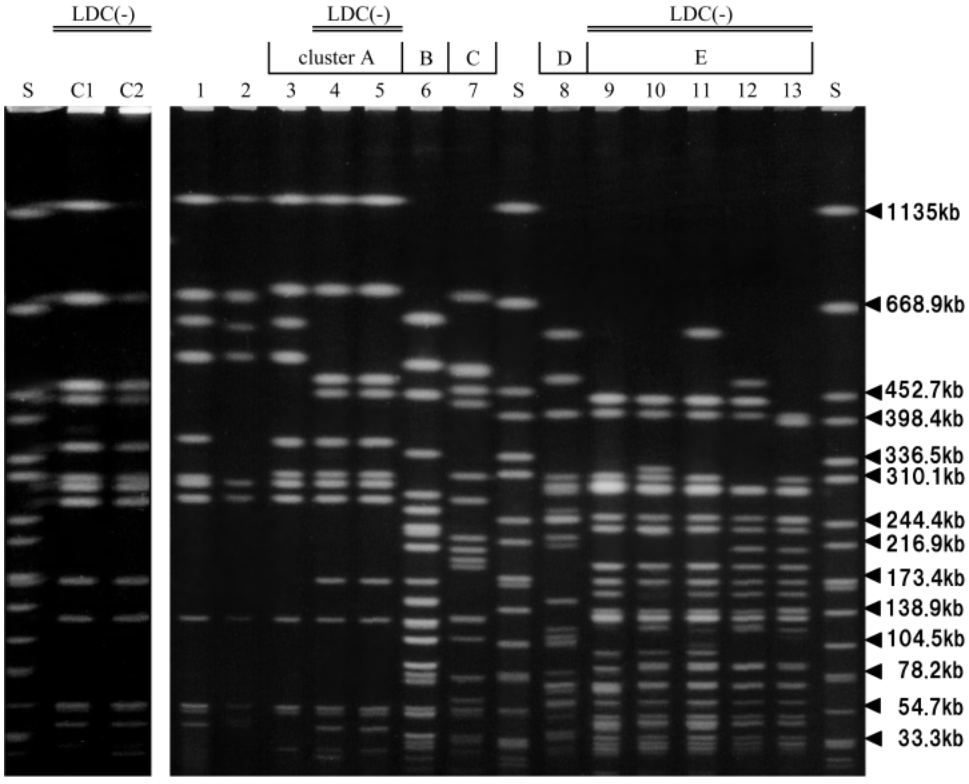

Fig. 2. Comparison of *Bln*I PFGE pattern. Photograph shows PFGE typing of representative strains from each cluster (A-E, see Fig. 1) and isolates in Honshu Island. C1: isolated in Fukuoka prefecture from a traveler to Honshu Island (strain no. Fukuoka 101, PT4), C2: isolated in Okayama prefecture of Honshu Island from a food poisoning case (strain no. Okayama S-HC359, PT14b). Lane 1: GTC131, 2: NBRC3313. Lane 3: Spo11/4/94, 4: Out21a/4/00, 5: Out29/4/03, 6: Ce9/6a/99, 7: Spo17/1/97, 8: Spo6/1c/93, 9: Out34a/RDNC/04, 10: Out27/1/03, 11: Out36/RDNC/04, 12: Spo31/1/02, 13: Spo29/1/02. Lane S: *Salmonella* Braenderup H9812 CDC-PulseNet Standard Strain; *Xba*I digested.

Enteritidis.

ACKNOWLEGEMENTS. We thank Dr. K. Murakami (Fukuoka Institute of Health and Environmental Sciences) and Mr. H. Kariya (Okayama Institute for Environmental Science and Public Health) for providing the reference strains. We also acknowledge the help provided by hospital staff and public health related agency in Nagasaki prefecture for collecting the isolates and epidemiological information.

REFERENCES

1. Kawakita, T. 1987. Guideline of NCCLS M2-A3. pp. 247–257. *In*: Drug Receptivity Examination, Kindai Syuppan, Tokyo (in Japanese).
2. Hickman-Brenner, F. W., Stubbs, A. D. and Farmer, J. J. III. 1991. Phage typing of *Salmonella enteritidis* in the United States. *J. Clin. Microbiol.* **29**: 2817–2823.
3. Hunter, S. B., Vauterin, P., Lambert-Fair M. A., Van Duyne, M. S., Kubota, K., Graves, L., Wrigley, D., Barrett, T. and Ribot, E. 2005. Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols: converting the national databases to the new size standard. *J. Clin. Microbiol.* **43**: 1045–1050.
4. Morita, M., Mori, K., Tominaga, K., Terajima, J., Hirose, K., Watanabe, H. and Izumiya, H. 2006. Characterization of lysine decarboxylase -negative strains of *Salmonella enterica* serovar Enteritidis disseminated in Japan. *FEMS Immunol. Med. Microbiol.* **46**: 381–385.
5. Murase, T., Okitsu, T., Nakamura, A., Matsushima, A. and Yamai, S. 1996. An epidemiological study of *Salmonella enteritidis* by pulsed-field gel electrophoresis (PFGE): several PFGE patterns observed in isolates from a food poisoning outbreak. *Microbiol. Immunol.* **40**: 873–875.
6. Murase, M., Kurokawa, M., Kurihara, K., Ikeda, R., Izumiya, H. and Watanabe, H. 2002. Epidemiological analysis by phagovar in food poisoning caused by *Salmonella* Enteritidis between 1989 and 2002 in Kobe City. *Jpn. J. of Food Microbiol.* **19**: 195–199.
7. National Institute of Infectious Diseases. 2000. Salmonellosis in Japan as of June 2000. *Infect. Agents Surveillance Rep.* **21**: 162–163.
8. National Institute of Infectious Diseases. 2004. An outbreak of *Salmonella* Enteritidis food poisoning caused by uncaked sushi mixed with various delicacies prepared by a caterer, August 2003 -Kyoto City. *Infect. Agents Surveillance Rep.* **25**: 154–155.
9. National Institute of Infectious Diseases. 2005. Change in sero-

type, biochemical nature and phage type of Salmonella isolates during April 2002-December 2004-Yamaguchi. *Infect. Agents Surveillance Rep.* **26**: 93–94.
10. National Institute of Infectious Diseases. 2006. Salmonellosis in Japan as of June. *Infect. Agents Surveillance Rep.* **27**: 191–192.
11. Ribot, E. M., Wierzba, R. K., Angulo, F. J. and Barrett, T. J. 2002. Salmonella enterica seroype Typhimurium DT104 isolated from humans, United States, 1985, 1990, and 1995. *Emrg. Infect. Dis.* **8**: 387–391.
12. Rodrigue, D. C., Tauxe, R. V. and Rowe, B. 1990. International increase in *Salmonella* enteritidis: a new pandemic? *Epidemiol. Infect.* **105**: 21–27.
13. Sato, S. 2004. A present state and measures of chicken's salmonellosis. *J. Jpn. Vet. Med. Assoc.* **57**: 671–677.
14. Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. and Swaminathan, B. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* **33**: 2233–2239.
15. Ward, L. R., de Sa J. D. H. and Rowe B. 1987. A phage-typing scheme for Salmonella enteritidis. *Epidemiol. Infect.* **99**: 291–294.