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Epidemiological analysis of *Salmonella enterica* Enteritidis isolates in Japan by phage-typing and pulsed-field gel electrophoresis

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SUMMARY

Salmonella enterica serotype Enteritidis isolates of phage types (PTs) PT1, PT4, PT13a and PT22 derived from sporadic cases and outbreaks of food poisoning in Japan during 1994 and 1995 were analysed by pulsed-field gel electrophoresis (PFGE). While PT1 strains from 5 different outbreaks showed 14 PFGE patterns, 5 PFGE patterns were observed among PT4 isolates from 5 different outbreaks and 6 independent isolates from imported chicken. Interestingly, 8 out of 9 PT4 strains associated with foreign travel to Southeast Asia were indistinguishable in PFGE pattern from 5 independent isolates of imported chicken from England. Although both PT13a and PT22 were first reported in Japan in 1994, PT22 showed various PFGE patterns compared to PT13a which had the same pattern within an outbreak, unlike PT1. These results could indicate that multiple clonal lines of PT1 and PT22 had already spread while relatively fewer clonal lines of PT4 and PT13a might exist in Japan.

INTRODUCTION

Salmonella Enteritidis is a common cause of food-borne gastroenteritis and the incidence of human salmonellosis caused by strains of this serotype has increased dramatically in many European countries [1]. In Japan, the frequency with which Enteritidis infection have been reported has continued to increase since 1989 [2]. The rate of Enteritidis to all the *Salmonella* isolates in this country was 5% in 1988, but it suddenly increased to 24% in 1989. Thereafter, the rate became as high as 37% and 48% in 1992 and 1993, respectively [2]. Enteritidis can be subdivided into 44 different types by phage typing and this technique has been used for epidemiological investigation of Enteritidis infections [3, 4]. PT8 used to be the most prevalent phage type until 1988 but in 1989, PT34 accounted for 69% of Enteritidis associated outbreaks, which explained the sudden increase in isolation of Enteritidis in that year and it has been gradually replaced by PT1 and PT4 which became predominant during the period from 1992 to 1995 in Japan [2].

In recent years, molecular based techniques, such as plasmid profile analysis [5, 6], restriction fragment-length polymorphisms (RFLP) [7], ribotyping [8–10] and pulsed-field gel electrophoresis (PFGE) [11–13] have proven to be useful in discriminating isolates of *Salmonella* serotypes. In fact, PFGE has been successfully applied to determination of the molecular epidemiology of numerous organisms [14–17] including Enteritidis [18–20]. We employed phage-typing in order to epidemiologically investigate Enteritidis isolated from sporadic cases, epidemic outbreaks, and also from broilers and imported chickens in Japan, and further applied PFGE to analyse detailed molecular relationships between strains of the same phage type, especially strains of PT1 and PT4 which are currently predominant phage types in Japan. Other phage types, PT22 and PT13a which have emerged recently in Japan were also investigated to compare genotypic variations among the same phage type.

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Table 1. Salmonella *Enteritidis associated with outbreaks, 1994–5, Japan*

Year	Number of outbreaks due to Enteritidis phage type												Total outbreaks examined
	PT1	PT3	PT4	PT5	PT5a	PT8	PT9	PT13a	PT22	PT34	UT*	Mix†	
1994	41	—‡	31	1	1	2	4	2	3	3	4	3	95
1995	30	4	27	13	—	2	—	—	—	1	3	5	85

* Untypable.
† Multiple phage types.
‡ Not reported.

MATERIALS AND METHODS

Bacterial strains

A total of 74 strains of Enteritidis were isolated in different parts of Japan in 1994 and 1995. Isolates from 12 different outbreaks comprised 36 strains from humans and 10 strains from foods. Isolates from sporadic cases contain 12 strains associated with foreign travel and 3 strains from human sporadic cases. Some of the isolates were from broilers and imported chicks with no known association to any of the outbreaks.

Phage typing

Phage typing was done according to the method of Ward and colleagues [3].

Pulsed-field gel electrophoresis

Strains of Enteritidis were cultured overnight at 37 °C in 3 ml of L-broth and 200 μl of the suspension were harvested in an Eppendorf microcentrifuge tube. After washing twice with 1·5 ml of TE (10 mM Tris. Cl, 1 mM EDTA pH 8·0), cells were resuspended in 500 μl of 0·8% low melting agarose (InCert agarose; FMC Bioproducts, Rockland, Maine) and poured into gel mould block to solidify. Solidified agarose gel blocks were first treated with 1 mg/ml of lysozyme solution at 37 °C overnight and then with lysis buffer containing 1 mg/ml of proteinase K, 1% Sarkosyl, 1 mM EDTA, pH 9·0 at 50 °C overnight. Gel blocks were then transferred into a tube containing 1 mM PMSF to inactivate proteinase K at 50 °C for 1 h twice. Gels were equilibrated in TE at 37 °C for 1 h twice, gel blocks were cut to an appropriate size and digested with 5–10 units of restriction endonuclease *Bln*I (Takara, Japan) at 37 °C overnight. Among several enzymes tested, *Bln*I provided the best discrimination with the most easily interpreted patterns (data not shown) and unless specifically indicated PFGE patterns were obtained by *Bln*I digestion. Samples were electrophoresed with lambda ladder (48·5 kb concatemers) and chromosomal DNA of *Saccharomyces cerevisiae* as standards through 1% agarose gel at 6 V/cm for 22 h. PFGE was performed using a CHEF DR II system (Biorad) and pulse times were ramped from 5–50 s during the run. The gels were stained with ethidium bromide and were photographed with a UV transilluminator. In the designation of sub-types, PFGE type was defined as the same sub-type if the number of band differences was less than three.

RESULTS

Phage types of Enteritidis

The distribution of phage types of Enteritidis isolates from human sources during the years 1994 and 1995 are shown in Table 1. PT1 and PT4 predominated (43·2 and 32·6% for 1994; 35·3 and 31·8% for 1995, respectively). Isolates of PT13a and PT22 from outbreaks were first reported in 1994. Phage types and other epidemiological information of the isolates used in this study are shown in Table 2. To determine whether it was possible to discriminate within the same phage type, the isolates were analysed by pulsed-field gel electrophoresis (PFGE).

PFGE analyses of PT1 and PT4 isolates

Even within a defined outbreak such as one in Hiroshima or Nagasaki, isolates of PT1 showed different PFGE patterns (Fig. 1, lanes a–c; lanes k–o). Among distinct outbreaks that occurred in the Fukuoka prefecture, PFGE patterns were different although their geographic locations were close (Fig. 1, lanes d–f). One of the outbreaks in Fukuoka showed the same PFGE pattern as an isolate from a poultry farm in Okayama prefecture (Fig. 1, lanes f, h).

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Table 2. *Enteritidis isolates*: *geographic origin*, *source*, *phage types*, *PFGE types*

Sample no.	Year of isolation	Geographic origin	Source	PT*	PFGE type *Bln*I (*Xba*I)
1	1994	Hiroshima	Human stool, OB† 1	1	1
2	1994	Hiroshima	Human stool, OB 1	1	2
3	1994	Hiroshima	Human stool, OB 1	1	3
4	1995	Fukuoka	Human stool, OB 2	1	4
5	1995	Fukuoka	Human stool, OB 3	1	5
6	1995	Fukuoka	Human stool, OB 4	1	6
7	1995	Okayama	Disposed water used for washing eggs	1	7
8	1995	Okayama	Poultry	1	6
9	1995	Okayama	Poultry	1	8
10	1995	Okayama	Poultry	1	9
11	1995	Nagasaki	Suspected food, OB 5	1	10
12	1995	Nagasaki	Human stool, OB 5	1	11
13	1995	Nagasaki	Human stool, OB 5	1	12
14	1995	Nagasaki	Human stool, OB 5	1	13
15	1995	Nagasaki	Human stool, OB 5	1	14
16, 17, 18, 19,20	1989	Hyogo	Imported chicks from England	4	15 (X1)
21	1989	Tochigi	An imported chick from England	4	15 (X1)
22, 23, 24, 25	1994	Tokushima	Human stool, OB 6	4	15 (X1)
26, 27	1994	Tokushima	Suspected food, OB 6	4	15 (X1)
28, 30, 31, 33	1994	Akita	Human stool, OB 7	4	16 (X2)
29	1994	Akita	Human stool, OB 7	4	17 (X2)
32	1994	Akita	Human stool, OB 7	4	18 (X2)
34, 35, 36	1994	Akita	Suspected food, OB 7	4	16 (X2)
37, 38, 39	1994	Toyama	Human stool, OB 8	4	19 (X2)
40, 41, 42, 43	1994	Niigata	Human stool, OB 9	4	15 (X1)
44, 45, 46	1994	Niigata	Suspected food, OB 9	4	15 (X1)
47	1994	Chiba	Human stool, OB 10	4	15 (X1)
48, 49	1991, 1993	Thailand	Human stool, tourist	4	15 (X1)
50	1991	Hong Kong	Human stool, tourist	4	15 (X1)
51	1992	Hong Kong	Human stool, tourist	4	20 (X1)
52, 53	1993, 1994	Singapore	Human stool, tourist	4	15 (X1)
54	1991	Indonesia	Human stool, tourist	4	15 (X1)
55	1993	Philippine	Human stool, tourist	4	15 (X1)
56	1994	Malaysia	Human stool, tourist	4	15 (X1)
57	1993	Shizuoka	Human stool, sporadic case	22	21
58	1993	Shizuoka	Human stool, sporadic case	22	22
59	1994	Tokyo	Broiler	22	21
60	1994	Tokyo	Broiler	22	23
61, 62, 63, 64	1994	Yamanashi	Human stool, OB 11	22	24
65	1994	Hiroshima	Broiler	22	25
66	1994	Tokyo	Human stool, sporadic case	22	26
67, 68, 69	1993	Overseas	Human stool, tourist	13a	27
70	1994	Aomori	Suspected food, OB 12	13a	28
71, 72, 73, 74	1994	Aomori	Human stool, OB 12	13a	28

* Phage type.
† Outbreak.

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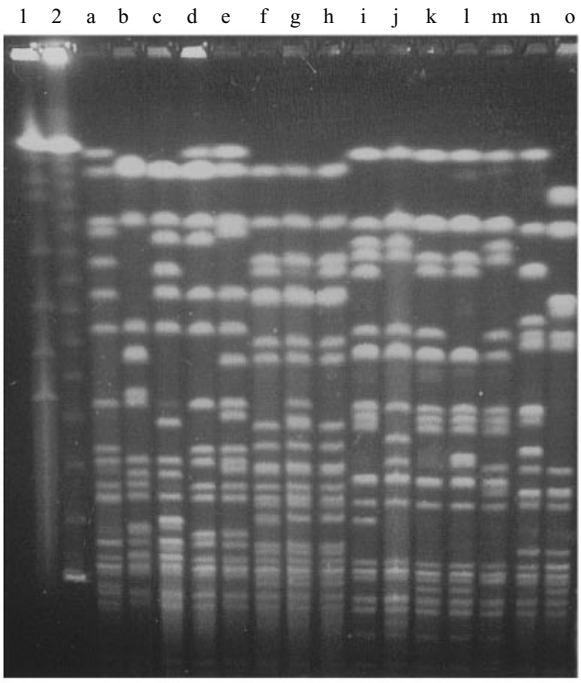

Fig. 1. PFGE patterns of PT1 isolates in 1994 and 1995. Lane 1, *S. cerevisiae*; lane 2, Lambda ladder as molecular size markers; lanes a–o correspond to sample numbers from 1 to 15 respectively shown in Table 2.

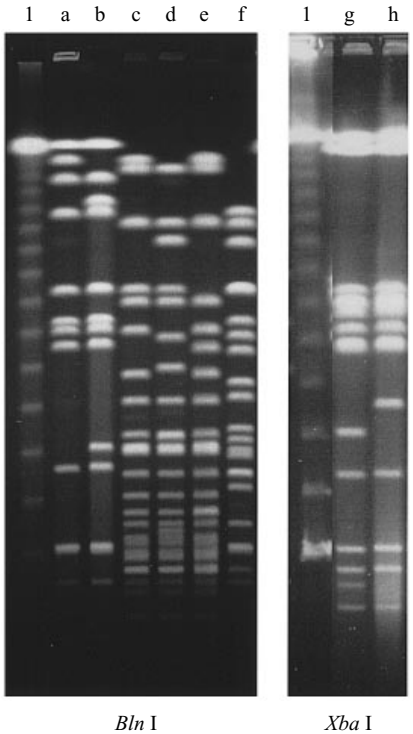

Fig. 2. Representatives of PFGE patterns of PT4 isolates in 1989 and 1994. Lane 1, lambda ladder: lane a, PFGE type 15 pattern; lane b, PFGE type 20; lanes c–f, PFGE types 16–19 respectively when digested with *Bln*I as shown in Table 2. Lanes g and h show PFGE pattern X2 and X1 when digested with *Xba*I respectively as shown in Table 2.

Isolates from a single poultry farm in Okayama showed three different PFGE patterns (Fig. 1, lanes h–j).

PFGE pattern was the same among PT4 isolates from imported chickens from England in 1989 and from outbreaks in Tokushima, Niigata and Chiba prefectures in 1994 (Table 2, PFGE type 15, Fig. 2, lane a). Among these outbreaks, isolates from contaminated foods which contained inadequately cooked eggs also showed the same PFGE pattern. There were four independent PFGE patterns observed in isolates from Akita and Toyama in 1994 (Fig. 2, lanes c–f). However, these isolates showed the same PFGE pattern when digested with a different restriction endonuclease *Xba*I (Table 2, PFGE type X2, Fig. 2, lane g).

PT4 isolated from imported chickens from England and from travellers to Southeast Asian countries showed identical (Table 2, PFGE type 15, Fig. 2, lane a) or similar PFGE patterns (Table 2, PFGE type 20, Fig. 2, lane b) although the year and country of isolation were different. They also showed the same PFGE pattern as those isolates from outbreaks in Tokushima, Niigata and Chiba prefectures in 1994 when digested with *Xba*I (Table 2, PFGE type X1, Fig. 2, lane h).

PFGE analyses of PT22 and PT13a isolates

Isolates of PT22 from sporadic cases and broilers showed six distinct patterns although isolates from an outbreak in Yamanashi prefecture showed the same pattern within the outbreak (Fig. 3). Within one outbreak of PT13a observed in Aomori prefecture in 1994, the PFGE pattern was identical among isolates from the suspected food source and the patients (Fig. 4, lane d, and lanes e–h, respectively). Isolates of PT13a from travelers to overseas showed a different pattern (Fig. 4, lanes a–c).

DISCUSSION

Increasing numbers of reports of outbreaks and sporadic cases caused by Enteritidis have been noted in Japan since 1989. Since most of the poultry is being imported from overseas to Japan, it can be speculated that some of the isolates derived from food-borne gastrointestinal infections could have originated from imported Enteritidis strains. A tendency to annual change in the predominant phage types of Enteritidis isolated in Japan since 1989 [2] may support the

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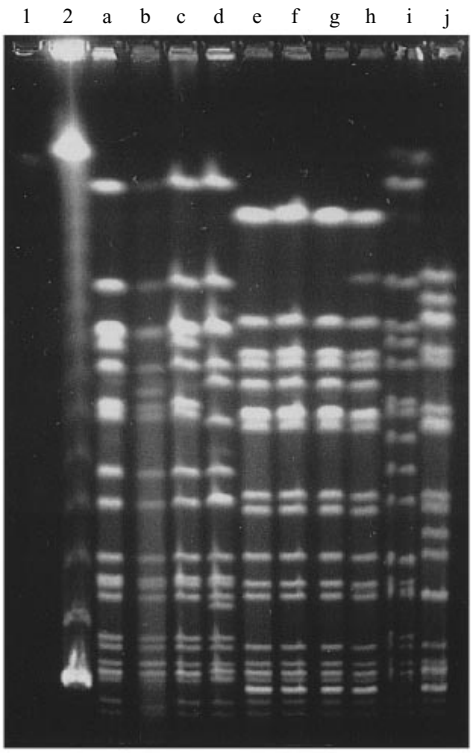

Fig. 3. PFGE patterns of PT22 isolates in 1994. Lane 1, *S. cerevisiae*: lane 2, lambda ladder: lanes a, b, isolates from a sporadic case in Shizuoka prefecture; lanes c, d, isolates from broilers in Tokyo; lanes e–h, isolates from patients of an outbreak in Yamanashi prefecture; lane i, an isolate from an sporadic case in Hiroshima prefecture; lane j, an isolate from sporadic case in Tokyo.

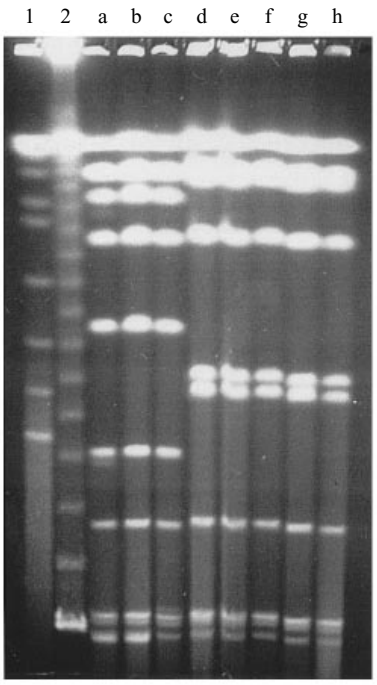

Fig. 4. PFGE patterns of PT13a in 1994. Lane 1, *S. cerevisiae*; lane 2, lambda ladder: lanes a–c, isolates from tourist; lane d, an isolate from the suspect food source in an outbreak in Aomori prefecture; lanes e–h, isolates from the patients of the same case.

speculation that although recent phage types found in Japan have drifted toward PT1 and PT4, several other phage types are being reported every year. In order to epidemiologically investigate Enteritidis that were derived from sporadic cases and outbreaks in Japan during 1994 and 1995, PFGE was applied. This technique has proved to be useful for further discrimination of Enteritidis after the application of conventional typing methods such as phage typing [11, 13, 18–20]. Among restriction enzymes tested, *Bln*I provided the best discrimination with the most easily interpreted patterns that separated fragments between approximately 100 and 700 kb very well. This range of separation may be helpful for interpretation of PFGE pattern because it may exclude restriction polymorphism due to the 57 kb virulence associated plasmid observed in all strains of PT1 and PT22 in this study (data not shown). It has been suggested by Liebisch and colleagues [21] that large plasmid bands did not account for the restriction fragment polymorphism detected in their PFGE experiments.

Isolates of PT1 from 5 different outbreaks and 4 individual chickens at a poultry farm could be subdivided into 14 genomic groups. There appeared to be multiple PFGE patterns observed even within a defined outbreak. These results were in good agreement with the data of Olsen and colleagues [11] who showed the usefulness of PFGE for discrimination among PT1 strains. There were more distinguishable PFGE patterns of isolates of PT1 from sporadic cases that occurred in geographically distinct areas in Japan from 1990 to 1995 (data not shown). Therefore it may be concluded that a diverse population of PT1 strains exists in Japan. However, some of the diversity observed in PT1 isolates in this study may be attributed to minor differences of DNA sequence in genomes, which can be considered as variation of a specific genotype. Since we defined the sub-type according to the number of band differences of more than two, this may have put genotypically close strains into different sub-types. In fact, there are strains of PT1 whose PFGE patterns are similar to each other, although the number of band differences was more than two. Since it is extremely difficult to determine the frequency of mutation in chromosomes of Enteritidis, in a defined period of time and we have no information of true origin of the isolates it was difficult to determine whether variations observed in

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PFGE patterns of PT1 in Japan were due to introduction of new strains from overseas or genomic change by mutations that occurred in indigenous isolates.

Most of the PT4 isolates shared identical PFGE patterns whether from imported chickens or distinct outbreaks. These isolates appeared to be homogeneous since they shared the same pattern when digested with *Bln*I which has been shown to be more discriminative than *Xba*I [13]. Another important observation was that PFGE patterns of these PT4 isolates were the same as PT4 isolates from Japanese tourists who traveled to Southeast Asian countries. Since the Enteritidis PT4 isolate obtained from chicken from England, where strains of PT4 predominated and comprised 85% of Enteritidis from humans in 1993 [22], showed identical or very similar PFGE patterns with those observed among isolates from independent outbreaks in Japan and travellers to Southeast Asia, a single clonal line of PT4 may have spread over England, Southeast Asia and Japan, suggesting a global spread of Enteritidis PT4.

PFGE patterns among isolates of PT22 and PT13a from outbreaks appeared to be identical respectively. Although PT22 was first reported in 1994, isolates of this PT from sporadic cases and broilers showed different PFGE patterns. Isolation of different clonal strains of PT22 from poultry products may suggest a possibility of prevalence of this PT in the future. On the other hand, sporadic isolates of PT13a showed the same PFGE pattern. However, these results may be a reflection of the limited number of cases reported in Japan. Alternatively, they may indicate that whereas PT22 appears heterogeneous PT13a was homogeneous.

In combination with an established method like phage typing, PFGE with *Bln*I appears to be of value in the epidemiological investigation of Enteritidis. Phage types of isolates from outbreaks in Japan have become more diverse over the past 5 Years [2]. It would therefore be of interest to determine whether or not genetic diversity exists within Enteritidis and if so to what extent.

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