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RAPD- and *actA* Gene-Typing of *Listeria monocytogenes* Isolates of Human Listeriosis, the Intestinal Contents of Cows and Beef

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***Abstract*: Seventy-five *L. monocytogenes* isolates of human listeriosis, the intestinal contents of cows and beef were divided into 5 major clusters, 17 sub-clusters and 28 minor clusters by typing using random amplification of polymorphic DNA (RAPD). According to their major RAPD category, *L. monocytogenes* isolates serotyped as 1/2b and 4b were distinguished from *L. monocytogenes* isolates of serovars 1/2a and 1/2c. Moreover serovar 4b was distinguished from serovar 1/2b by a difference in the RAPD sub-cluster category. All *L. monocytogenes* were found to possess either *actA* gene Type I or II, and only one *actA* gene type was detected in each RAPD minor cluster. *actA* gene Type II was observed in 32.0%, 38.5% and 18.9% of isolates from humans, cows and beef, respectively, and was detected more frequently in serovar 4b (46.9%) than in serovars 1/2a (22.2%), 1/2b (7.7%) and 1/2c (0.0%). Twenty (80%) of 25 human isolates fell within three minor RAPD types (II-d (16%), V-p-1 (36%), V-p-2 (28%)). Two isolates from humans and beef were found to have the same RAPD type (Type IV-k-1), *actA* gene type (Type I) and serovar (1/2b). Our results suggest that only a few genotypes of *L. monocytogenes* are predominant in human listeriosis in Japan, although the human isolates were collected over a broad span of time and a wide geographical range. Our results also suggest that RAPD-, *actA* gene- and sero-typing can be useful for epidemiological analysis.**

***Key words*: *Listeria monocytogenes*, Human listeriosis, RAPD, *actA* gene types**

Listeria monocytogenes (*L. monocytogenes*) is a gram-positive facultative intracellular bacterium, which causes listeriosis both in humans and animals. This bacterial pathogen is widespread in the environment; it has been found in food products, and can also be carried by almost any animal species including asymptomatic humans (7, 8, 17, 18, 22). Listeriosis has been recognized as a food-borne disease since the outbreaks of the 1980s, when contaminated food was shown to be the primary vehicle of *L. monocytogenes* (5, 18). In Japan, 10 to 60 sporadic cases of human listeriosis have been reported every year since 1958. However, no specific food sources have been identified (23).

Recently, the genetic analysis of *L. monocytogenes* in Japan has been reported. *L. monocytogenes* isolated from raw milk and a bulk tank on a dairy farm were found to share the same random amplified polymorphic DNA (RAPD) type (29). Pulsed-field gel electrophoresis (PFGE) analysis showed the spread of *Listeria* in cheese by cross-contamination in the cheese-processing environment (13). Three PFGE types of *L. monocytogenes* isolated from foods obtained in retail shops were also detected in isolates from Japanese patients with listeriosis (14).

Molecular genetic typing and identification of various virulence markers of *L. monocytogenes* should help to determine sources of human listeriosis (2–4, 9, 15, 16, 19, 25, 26, 28). The ActA protein is one of the virulence factors in *L. monocytogenes*; it is the crucial factor for actin-based movement in the cytosol of eukaryotic cells, for direct cell-to-cell spread and for escape from the defense mechanisms of host cells (10, 11, 20, 21). Previously, we reported the shortening of *actA* gene of *L. monocytogenes* resulting from the deletion of one proline-rich unit (LU) (12) and we designated *L. monocytogenes* strains with only one LU as *actA* gene Type II, and

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Abbreviations: DW, distilled water; LU, large unit and proline-rich unit; PBS, phosphate buffered saline; PFGE, pulsed-field gel electrophoresis; RAPD, random amplification of polymorphic DNA; TAE, Tris-acetate EDTA buffer.

Table 1a. Characteristics of *L. monocytogenes* isolates from humans

Strain	Serovar	*actA* type	RAPD type	Year	Place	Age	Sex	Status
H8	1/2a	I	II-c	1988	Kyoto	72	Male	Meningitis
H9	1/2a	I	II-d	1988	Yamanashi	62	Male	Meningitis
H11	1/2a	I	II-d	1988	Nagasaki	40	Male	Bacteraemia
H24	1/2a	I	II-d	1988	Akita	0	Male	Meningitis
H28	1/2a	I	II-d	1989	Kyoto	32	Female	Bacteraemia
H7	1/2a	I	IV-l-1	1988	Fukuoka	0	Female	Bacteraemia
H27	1/2b	I	IV-k-1	1987	Nagasaki	77	Female	Bacteraemia
H53	4b	I	V-p-1	1986	Tokyo	70	Female	Bacteraemia
H54	4b	I	V-p-1	1987	Tokyo	88	Female	Meningitis
H50	4b	I	V-p-1	1988	Oita	0.1	Male	Meningitis
H52	4b	I	V-p-1	1988	Osaka	51	Male	Meningitis
H56	4b	I	V-p-1	1989	Hyogo	1.1	Female	Meningitis
H57	4b	I	V-p-1	1989	Tokyo	91	Male	Bacteraemia
H42	4b	I	V-p-1	1991	Fukushima	5	Male	Meningitis
H43	4b	I	V-p-1	1991	Aomori	1.1	Male	Meningitis
H45	4b	I	V-p-1	1991	Aomori	2	Male	Meningitis
H37	4b	I	V-o	1991	Yamagata	0	Female	Bacteraemia
H48	4b	II	V-p-2	1988	Shizuoka	2	Male	Bacteraemia
H49	4b	II	V-p-2	1988	Fukuoka	2.1	Female	Meningitis
H51	4b	II	V-p-2	1988	Aomori	72	Male	Bacteraemia
H58	4b	II	V-p-2	1989	Wakayama	1	Female	Meningitis
H39	4b	II	V-p-2	1991	Tochigi	1	Male	Meningitis
H40	4b	II	V-p-2	1991	Ehime	56	Male	M and B
H41	4b	II	V-p-2	1991	Saga	62	Male	Meningitis
H55	4b	II	V-q	1989	Shizuoka	62	Male	Bacteraemia

(M and B): meningitis and bacteraemia.

strains with two LUs as *actA* gene Type I (12).

PCR and RAPD typing are rapid, simple and inexpensive methods that can be used to find sources of human listeriosis in Japan. A virulence marker for *L. monocytogenes* would also be useful in the epidemiological investigation of these sources. In this study, we compared *L. monocytogenes* isolates of human listeriosis, the intestinal contents of cows and beef in Japan with RAPD typing, *actA* gene typing and serovar analysis.

Materials and Methods

Bacterial strains. A total of 75 *L. monocytogenes* strains were obtained from Dr. M. Terao, Niigata Prefectural Research Laboratory for Health and Environment, Niigata, and from Dr. T. Iida and Dr. Y. Kokubo, Tokyo Metropolitan Research Laboratory of Public Health. These strains were isolated from 1986 until 1996. Twenty five isolates were collected from patients with listeriosis, 13 from the intestinal contents of cows, and 37 from beef. The beef was collected from various markets in Tokyo. The characteristics of the *L. monocytogenes* isolates are described in Table 1.

DNA preparation. *L. monocytogenes* isolates were cultured in 500 µl of BHI broth (brain heart infusion medium; Difco, Detroit, Mich., U.S.A.) at 37 C for 18 hr. The cultures were centrifuged at 2,000×*g* for 10 min, and the pelleted organisms were suspended in 500 µl of PBS(−) (phosphate buffered saline). After the suspensions were boiled for 10 min, bacterial DNA in 300 µl of each suspension was extracted by phenol/chloroform treatment. DNA was precipitated with ethanol and the DNA pellet was dissolved in 300 µl of DW (distilled water), then used as template DNA for PCR.

PCR amplification of the proline-rich region. The region encoding the two LUs (large units) was amplified

Table 1b. Characteristics of *L. monocytogenes* isolates from cows

Strain	Serovar	*actA* type	RAPD type	Year	Place
C2	1/2a	I	I-b-1	1988	Tokyo
C3	1/2a	I	I-a	1989	Tokyo
C4	1/2a	I	I-b-2	1990	Toyama
C6	1/2b	I	I-a	1990	Shiga
C5	1/2b	II	IV-k-5	1990	Fukuoka
C7	1/2c	I	I-b-2	1988	Tokyo
C8	1/2c	I	I-b-2	1990	Osaka
C9	1/2c	I	I-b-2	1990	Aomori
C10	1/2c	I	I-b-2	1991	Tokyo
C11	4b	II	IV-l-2	1990	Tokyo
C12	4b	II	IV-l-2	1990	Tokyo
C13	4b	II	IV-l-2	1990	Tokyo
C14	4b	II	IV-l-2	1990	Tokyo

Table 1c. Characteristics of *L. monocytogenes* isolates from beef

Strain	Serovar	*actA* type	RAPD type
M4	1/2a	I	III-f-1
M3	1/2a	I	III-f-2
M5	1/2a	I	III-f-2
M8	1/2a	I	III-f-2
M9	1/2a	I	III-f-2
M6	1/2a	II	III-e-1
M2	1/2a	II	III-e-1
M1	1/2a	II	III-e-2
M10	1/2a	II	III-e-3
M16	1/2b	I	IV-h
M15	1/2b	I	IV-i
M20	1/2b	I	IV-i
M13	1/2b	I	IV-j
M14	1/2b	I	IV-j
M12	1/2b	I	IV-k-1
M11	1/2b	I	IV-k-2
M17	1/2b	I	IV-k-3
M18	1/2b	I	IV-k-4
M19	1/2b	I	IV-l-1
M21	1/2c	I	III-f-2
M22	1/2c	I	III-f-2
M23	1/2c	I	III-f-2
M25	1/2c	I	III-f-2
M26	1/2c	I	III-f-2
M27	1/2c	I	III-f-2
M28	1/2c	I	III-f-2
M24	1/2c	I	III-g
M29	4b	I	IV-l-1
M31	4b	I	IV-l-1
M34	4b	I	IV-l-1
M35	4b	I	IV-l-1
M37	4b	I	IV-l-1
M38	4b	I	IV-l-1
M36	4b	I	IV-n
M30	4b	II	IV-l-3
M32	4b	II	IV-l-3
M33	4b	II	IV-m

Beef was obtained from various markets in Tokyo from 1989 to 1991.

by PCR with the primer pair PR5, 5′-TGA AGA GGT AAA TGC TTC GGA CTT-3′, and PR3, 5′-CGC TTA TTT TCG GTA CCT TTG GA-3′ (12). PCR amplification was carried out in a 50 μl reaction mixture consisting of 5 μl of template DNA (15–30 ng), 50 pM each of the two primers, 2.5 units of Ex *Taq* polymerase (TaKaRa, Japan) in Tris-HCl (pH 8.0), 0.2 mM of each dNTP, 50 mM KCl, and 2.0 mM $MgCl_2$. The PCR mixture was denatured at 92 C (5 min), and then subjected to 25 cycles of amplification (denaturation at 92 C for 1 min, annealing at 45 C for 1 min, extension at 74 C for 2 min). The oligonucleotide primers used for PCR were synthesized by Nihon Genosys Biotechnologies, Inc., Japan.

PCR amplification of randomly amplified polymorphic DNA (RAPD). RAPD analysis of *L. monocytogenes* was based on PCR fingerprinting using a short primer HLWL85 (5′-ACA ACT GCT C-3′) (27). The PCR amplifications were carried out in a reaction mixture (50 μl) consisting of 5 μl of template DNA (15–30 ng), 100 pmol primer, 2.5 units of Ex *Taq* polymerase (TaKaRa) in Tris-HCl (pH 8.0), 0.2 mM of each dNTP, 50 mM KCl, and 2.0 mM $MgCl_2$. After an initial denaturation step at 92 C for 3 min, 45 PCR cycles (each cycle consisting of 94 C for 2 min, 35 C for 2 min and 72 C for 2 min) were performed, with a final incubation at 72 C for 10 min.

Detection of actA *gene types and RAPD types.* Amplified PCR products were separated by gel electrophoresis on 1.5% (w/v) agarose gels in TAE (Tris-acetate EDTA buffer). DNA was stained with 0.1 $\mu g \cdot ml^{-1}$ ethidium bromide, and visualized using a UV-transilluminator (302 nm). The patterns of the amplified products were recorded and analyzed using a Fluor-S TM Multi-Imager (Bio-Rad, U.S.A.). The type of each *actA* gene product was determined by the size of the PCR product reflecting the nucleotide sequence of the *actA* gene (Type I with two LUs corresponds to a 623 bp product and Type II, lacking one LU, corresponds to a 518 bp product) (12). RAPD profiles were obtained following the general protocol described in the WHO Multicenter Study on *L. monocytogenes* subtyping (27).

Serovars. Serotyping of *L. monocytogenes* was performed according to the method of Iida using *Listeria* antiserum "SEIKEN" (Denka Seiken Co., Ltd., Japan) (7).

Results

RAPD Typing

The primer HLWL85 was used for RAPD typing, and the profiles obtained are shown in Fig. 1. The RAPD patterns of 75 *L. monocytogenes* strains (isolates) formed 28 minor clusters. Based on the similarity of strong band patterns, the 75 isolates were separated into 5 major clusters (Type I to Type V) or 17 subclusters (Type a to Type q). Sub-clusters of Types b, f and p were divided into two minor clusters according to the presence of fine constant bands. In addition, sub-clusters of e, k and l were divided into 3, 5 and 3 minor clusters, respectively.

The results of RAPD typing are summarized in Fig. 2. Twenty of the 25 human isolates (80.0%) were classified within 3 RAPD types; 4 isolates were Type II-d (16.0%, 4/25), 9 were Type V-p-1 (36.0%, 9/25) and 7 were Type V-p-2 (28.0%, 7/25). Nine of 13 isolates from the intestinal contents of cows (69.2%) were classified

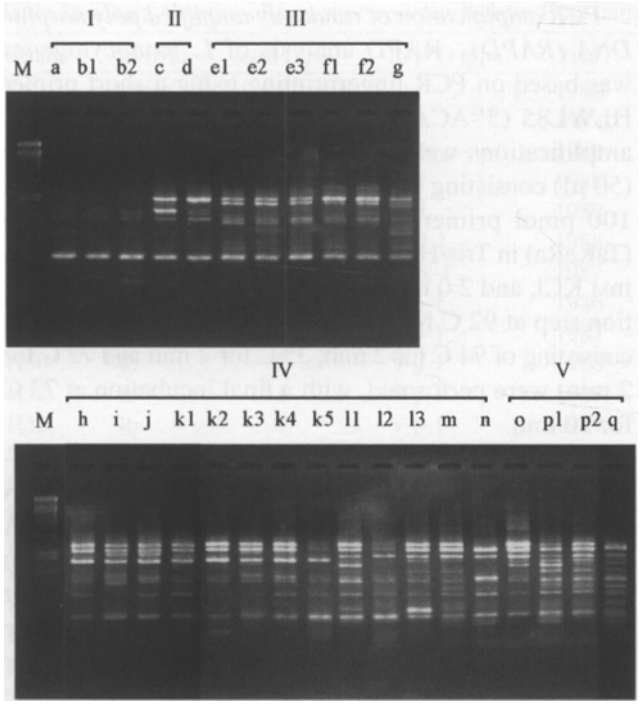

Fig. 1. Representative band patterns of 28 minor RAPD types of *L. monocytogenes* identified using primer HLWL85. Seventy-five isolates were classified into 5 major clusters (Types I to V). Major RAPD Type I was comprised of 2 sub-RAPD types (Type I-a and I-b), and sub-RAPD Type b was divided into 2 minor types by fine constant bands (Type I-b-1 and I-b-2). Major RAPD Type II was comprised of 2 sub-RAPD types (Type II-c and II-d). Major RAPD Type III was comprised of 3 sub-RAPD types (Type III-e, f and g). Both sub-RAPD Type III-e and III-f were divided into 3 and 2 minor types, respectively, by fine constant bands (Type III-e-1, III-e-2 and III-e-3, and Type III-f-1 and III-f-2). Major RAPD Type IV was comprised of 7 sub-RAPD types (Type IV-h, i, j, k, l, m and n), and sub-RAPD Type IV-k and IV-l were divided into 5 and 3 minor types, respectively, by fine constant bands (Type IV-k-1, k-2, k-3, k-4 and k-5, and Type IV-l-1, l-2 and l-3). Major RAPD Type V was comprised of 3 sub-RAPD types (Type V-o, p and q), and sub-RAPD Type p was divided into 2 minor types by fine constant bands (Type V-p-1 and V-p-2). Marker: M=λ Eco-T14 ladder (TaKaRa).

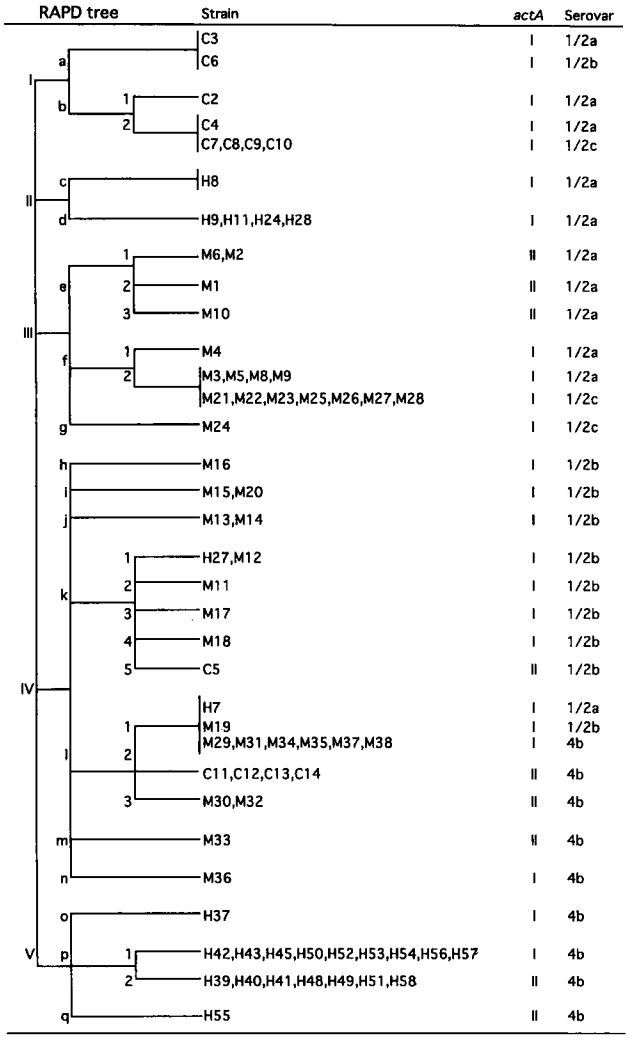

Fig. 2. Diagram of 28 clusters of 75 *L. monocytogenes* isolates classified by RAPD profiles using primer HLWL85. The 75 isolates were classified into 5 major RAPD types, 17 sub-RAPD types and 28 minor RAPD types. The 28 minor RAPD types were sub-classified according to *actA* gene types and serovars. Strains H, C and M were *L. monocytogenes* isolated from human listeriosis, intestinal contents of cows and beef, respectively.

within 2 RAPD types; 5 isolates were Type I-b-2 (38.5%, 5/13) and 4 were Type IV-l-2 (30.8%, 4/13). Eighteen (48.6%) of 37 isolates from beef were classified within 2 RAPD types; 11 isolates were Type III-f-2 (29.7%, 11/37) and 7 were Type IV-l-1 (18.9%, 7/37).

actA *Gene Typing*

The frequencies of *actA* gene types are summarized in Table 2. Fifty-five (73.3%) of 75 isolates were identified as *actA* gene Type I, and 20 isolates (26.7%) as *actA* gene Type II. Eight (32.0%) of the 25 human isolates, 5 (38.5%) of the 13 cow isolates, and 7 (18.9%) of the 37 beef isolates were designated as *actA* gene Type II (Table 2). All isolates were classified as either *actA* gene Type I or II.

The Comparison of RAPD Types, actA *Gene Types and Serovars*

Isolates of RAPD Types I, II, III and V were from cows, humans, beef and humans, respectively (Fig. 2).

All isolates classified as RAPD Type I and Type II were *actA* gene Type I (Fig. 2). RAPD Type III isolates were comprised of *actA* gene Type I and Type II. However, all strains of RAPD Type III-f and Type III-g were *actA* gene Type I, and all strains of RAPD Type III-e were *actA* gene Type II. RAPD Types IV and V were

Table 2. The incidence of *actA* gene types and serovars of *L. monocytogenes*

Serovar	*actA* gene type (%)		
	Type I	Type II	Total
1/2a	14 (77.8)	4 (22.2)	18 (100)
1/2b *	12 (92.3)	1 (7.7)	13 (100)
1/2c **	12 (100.0)	0 (0.0)	12 (100)
4b	17 (53.1)	15 (46.9)	32 (100)
Total	55 (73.3)	20 (26.7)	75 (100)

Significant differences in the ratios of *actA* gene Types I and II were found between serovar 4b and serovars 1/2b or 1/2c. [*: $\chi^2=(1, N=45)=4.60$, $P<0.005$; **: $\chi^2=(1, N=44)=6.58$, $P<0.01$].

also comprised of *actA* gene Type I and Type II. However, *actA* gene Type I and Type II were never detected in the same RAPD minor cluster.

Twenty (26.7%) of 75 isolates were classified as *actA* gene Type II (Table 2). Fifteen (75.0%), 1 (5.0%) and 4 (20.0%) of 20 strains classified as *actA* gene Type II were serovars 4b, 1/2b and 1/2a, respectively. On the other hand, 17 (30.9%), 12 (21.8%), 12 (21.8%) and 14 (25.5%) of 55 strains classified as *actA* gene Type I were typed as serovars 4b, 1/2c, 1/2b and 1/2a, respectively. All of serovar 1/2c isolates were classified as *actA* gene Type I.

All isolates of serovars 4b and 1/2b were classified as RAPD Type IV and Type V, except strain C6 (serovar 1/2b) which was classified as RAPD Type I-a (Fig. 2). Moreover, serovar 4b was distinguished from serovar 1/2b by a difference in the sub-cluster category. However, strain H7 (serovar 1/2a) and strain M19 (serovar 1/2b) were included in the same Type IV-l-1 group with six strains of serovar 4b (M29, M31, M34, M35, M37 and M38). All other isolates of serovars 1/2a and 1/2c were classified as RAPD Type I, II or III.

RAPD Type IV-k-1 was represented by a human isolate (H27, serovar 1/2b) and a beef isolate (M12, serovar 1/2b) (Fig. 2). One human isolate (H7, serovar 1/2a) and seven beef isolates (M19, serovar 1/2b, and M29, M31, M34, M35, M37 and M38, serovar 4b) were RAPD Type IV-1-1.

Characteristics of L. monocytogenes *Isolated from Human Listeriosis*

L. monocytogenes isolated from human listeriosis is characterized in Table 1a. Twenty-five isolates were obtained from 14 cases of meningitis, 10 cases of bacteraemia, and one case of both meningitis and bacteraemia. All of the human isolates were *actA* gene Type I or II, and comprised eight RAPD types and three serovars. However, these types were not associated with the year or place of isolation, or with the age, sex, or disease status of the patient.

Discussion

Seventy-five isolates of *L. monocytogenes* from listeriosis patients, intestinal contents of cows and beef were divided into 5 major clusters, 17 sub-clusters and 28 minor clusters by typing using random amplification of polymorphic DNA (RAPD) (Fig. 1). The results indicated a broad genetic heterogeneity of *L. monocytogenes* in Japan. This heterogeneity was also revealed by pulsed-field gel electrophoresis (PFGE) in a report by Nakama et al (14).

L. monocytogenes isolates typed as serovars 4b and 1/2b were clearly distinguished from those of serovars 1/2a and 1/2c with respect to the category of the major RAPD type (Fig. 2). Moreover, serovar 4b was distinguished from serovar 1/2b by a difference in the category of their RAPD sub-cluster. Similar distinctions in genomic divisions among serovars have been made in other countries by PFGE analysis, electrophoretic typing and determination of restriction fragment lengths (4, 15, 25). However, our results with RAPD typing showed sharper genomic divisions among serovars. This association between RAPD types and serovars was also detected in *L. monocytogenes* isolated from different animals and on dairy farms in Japan (29).

In our study, the incidence of *actA* gene Type II was 32.0%, 38.5% and 18.9% in isolates of human listeriosis, intestinal contents of cows and beef, respectively. Wiedmann et al (28) reported that the incidence of a unique *actA* gene sequence, which corresponded to *actA* gene Type II in our typing, was isolated from 38% of animals in New York state and adjacent states between 1986 and 1996. They also reported that the *actA* gene Type II sequence was detected in the ribotype dd0647 of *L. monocytogenes* implicated in at least four human foodborne listeriosis outbreaks. Their report suggests that further epidemiological and pathogenic investigation of *L. monocytogenes* of *actA* gene Type II may demonstrate the significance of the *actA* gene in the development of listeriosis (12, 28).

Our previous report did not show an association between the ratios of *actA* gene types and serovars in human isolates. However, in this study, *actA* gene Type II was detected more frequently in serovar 4b (46.9% (15/32)) than in serovars 1/2a (22.2% (4/18)), 1/2b (7.7% (1/13)) and 1/2c (0.0% (0/12)) (Table 2). Moreover, significant differences in the incidence of *actA* gene types were found between serovar 4b and serovars 1/2b ($P<0.05$) or 1/2c ($P<0.01$). All of the *L. monocytogenes* isolates in this study were *actA* gene Type I or II. In a RAPD minor cluster, either *actA* gene type was

found (Fig 2). These findings suggest that the *actA* gene type can be a virulence marker if associations revealed with RAPD typing and serotyping are taken into account.

Interestingly, 80% (20/25) of isolates of human listeriosis were clustered into only three minor RAPD types (II-d: 16%(4/25), V-p-1: 36%(9/25) and V-p-2: 28%(7/25)) (Fig. 2). This indicates that a few genotypes of *L. monocytogenes* may be involved in human listeriosis in Japan, although these isolates did not show any significant correlation with sex, age or disease status of the patient, or the place or year of collection (Table 1a).

Specific sources of human listeriosis have not been identified in Japan as yet (23). However, *L. monocytogenes* is frequently isolated from beef products obtained at retail shops, from the fecal and intestinal contents of healthy cows, and on dairy farms where listeriosis has occurred in cows (6, 7, 17, 22, 24). Two isolates from a patient (H27) and a beef sample (M12) were found to have the same RAPD type (Type IV-k-1), *actA* gene type (Type I) and serovar (1/2b) (Fig. 2). PFGE analysis showed that the molecular varieties related to the large food-borne outbreaks in California and Switzerland were also found in *L. monocytogenes* isolated from both listeriosis patients and foods in Japan (13, 14). Genotyping is a useful tool for determining detailed lineages of *L. monocytogenes* isolated from various sources. The accumulation of this type of data may lead to the discovery of specific sources of human listeriosis infection in Japan (1, 2, 4, 15, 28).

This study is the first comparative genetic analysis by RAPD fingerprinting, *actA* gene typing and serotyping of *L. monocytogenes* isolated from humans, cows and beef in Japan. The use of these three techniques may provide a quick and convenient epidemiological means of investigating particular genomic strains of *L. monocytogenes* isolated from patients and original sources of listeriosis. However, the genetic lineages elucidated by these analyses cannot be used to account for the pathogenic characteristics of human isolates. For considerations of public health, further investigations might focus on the understanding of a particular genomic strain of *L. monocytogenes* frequently associated with human listeriosis as well as on the identification of potential sources of sporadic listeriosis infection in Japan.

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