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Genetic subtyping of *Listeria monocytogenes* via multiple-locus sequence typing using *iap, sigB* and *actA*

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ABSTRACT. Pulse field gel electrophoresis (PFGE) is widely used for listeriosis surveillance. Although this technique is effective for epidemiology, the data among laboratories are inconsistent. We previously reported a method for *Listeria monocytogenes* subtyping combined with sequence analysis of partial *iap* and whole genome restriction fragment length polymorphism (RFLP) using *Xba*I, *Cla*I (*Ban*III) and *Pst*I. However, distinguishing subtypes was challenging, because the output comprised complicated fragment patterns. In this study, we aimed to establish a simple genotyping method that does not depend on visual observation, rather it focuses on multi-locus sequence typing (MLST) using three genes, *iap*, *sigB* and *actA*. Sixty-eight strains of *L. monocytogenes* including EGD-e as a reference strain were investigated to ensure consistency with previous data on the genetic characterization. All strains were grouped into 29 types by both analyses. Although there are some differences in classification, major clades included the same strains. Simpson's indices of diversity (SID) by MLST and *iap*-RFLP-based typing were 0.967 (95% confidence interval [CI]: 0.955/0.978) and 0.967 (95% CI: 0.955/0.979), respectively. The discriminatory power of both methods can be considered almost identical. Compared with the results of 38 selected strains, the strains within the MLST clusters in this study coincided with those obtained using PFGE. Thus, the MLST strategy could help differentiate among *L. monocytogenes* isolates during epidemiological studies.

KEY WORDS: genotyping, *Listeria monocytogenes*, multi-locus sequence typing, pulse field gel electrophoresis, restriction fragment length polymorphism

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Listeria monocytogenes, a gram-positive, motile, facultatively anaerobic and non-spore-forming bacillus, causes listeriosis. This bacterium is ubiquitous in nature; thus, food-mediated listeriosis has acquired attention, because of the outbreaks, in particular in Europe and the United States [5, 27, 29]. The high-risk populations, including children, the elderly, immunocompromised individuals and pregnant women, tend to exhibit severe symptoms accompanied with septicemia, meningitis, abortion and stillbirth, resulting in high mortality [18]. In total, 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e and 7) of *L. monocytogenes* have been identified based on reactions to somatic and flagellar antigens. *L. monocytogenes* strains belonging especially to serotypes 1/2a, 1/2b, 1/2c and 4b cause over 98% of all human listeriosis infections. At present, four genetic lineages have been described for *L. monocytogenes* [13]. Lineage I includes group serotypes 1/2b, 3b, 4b, 4d and 4e; lineage II includes serotypes 1/2a, 1/2c, 3a and 3c; and lineage III, including serotypes 4a, 4c and some strains belonging to serotype 4b, represents three distinct subgroups, IIIA, IIIB and IIIC. Lineage IIIB was recently reclassified as lineage IV [13].

Genetic surveillance of pathogens is required to determine the route of infection from sources to susceptible hosts in an attempt to prevent further spread of contamination and infection. Various types of molecular analysis, including pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) using polymerase chain reaction (PCR) products or genomic DNA, ribotyping and comparison of nucleotide sequences, have been developed for the classification of *L. monocytogenes* [38]. We have performed surveillance for *L. monocytogenes* contamination of food and the environment in Japan since 1996. Additionally, we have reported identical genetic profiles and serotypes among strains isolated from retail meats and human patients [36]. Consistent with lineage, we have shown that *L. monocytogenes* isolated in Japan can be classified roughly into three groups using the *iap* sequence [34, 35]. We proposed that phylogenetic analysis combined with *iap* sequencing and whole genome RFLP (*iap*-RFLP) is a useful method to genetically differentiate among *L. monocytogenes* isolates [15, 24, 25, 31, 33]. This method revealed that domestic

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meat is contaminated by strains of epidemic clone 1 that has been associated with several widespread outbreaks in Europe and the United States, though the frequency of isolation seems to be low [15]. However, deciphering the fragment pattern obtained from *iap*-RFLP followed by classification of subtypes is a challenge. In this study, we aimed to develop a simple, multiple-locus sequence typing (MLST) method that references previous data and is based on the nucleotide sequences of only three genes: *iap*, *sigB* and *actA*. The products of *iap* and *actA* are known virulence factors, whereas *sigB* is a housekeeping gene that encodes one of the sigma factors, Sigma B. We ascertained whether the discriminatory ability of this simple MLST was equal to that of our *iap*-RFLP method using *L. monocytogenes* strains isolated from meat (domestic or imported), skin of beef cattle and patients with listeriosis. Thereafter, we compared phylogenic clustering using MLST versus the gold standard subtyping method, PFGE.

MATERIALS AND METHODS

Bacterial strains: In this study, we used 67 *L. monocytogenes* strains [15, 31, 34, 35]. These strains were isolated from skin of beef cattle from a Japanese farm (five strains), Japanese patients with listeriosis (seven strains) and meat produced in Japan (37 strains) or imported to Japan from other countries (18 strains) (Table 1). Serotypes of these strains included 1/2a (34 isolates), 1/2b (16 isolates), 1/2c (three isolates), 3b (one isolate) and 4b (13 isolates). EGD-e strain (serotype: 1/2a; GenBank accession no. AL591824) was used as the reference strain.

RFLP analysis: Genomic DNA from *L. monocytogenes* was extracted and purified as previously described [24, 25, 31, 33]. For RFLP analysis, genomic DNA was digested with restriction enzymes *Xba*I, *Cla*I (*Ban*III) or *Pst*I according to the manufacturer's instructions (Takara Bio, Otsu, Japan). The reactants were separated on 0.8% agarose gels. DNA fragments were stained with ethidium bromide (Nacalai Tesque, Kyoto, Japan) and visualized using an ultraviolet transilluminator (UVP, Upland, CA, U.S.A.). RFLP patterns were analyzed, and the strains were classified accordingly. RFLP analysis was repeated more than three times for each genomic DNA. RFLP patterns with less than five differences were considered to be of the same genotype.

Sequence analysis: Each strain was incubated in brain heart infusion broth (BD, Franklin Lakes, NJ, U.S.A.) at 37°C for 18 hr. After incubation, bacterial cells were harvested by centrifugation, washed with sterilized MilliQ water and suspended in 400 μl TE solution (10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0). The bacterial suspensions were boiled for 15 min to lyse the cells, followed by centrifugation at 15,000 ×*g* for 10 min at 4°C to remove denatured proteins and bacterial membranes. The supernatant containing DNA was obtained and stored at −80°C until use. In addition, DNA for the *iap* sequencing was extracted and purified as previously described [24, 25, 31, 33]. To determine the nucleotide sequence, partial *iap*, *sigB* and *actA* were amplified using specific primer pairs, SI3A/SI4B [24, 25, 31, 34, 36], LMsigB15/LMsigB16 [39] and massF/massR [12, 41], respectively (Table 2). The size of *iap*, *sigB* and *actA* amplicons (810, 841 and 827 bp, respectively) were confirmed by 1.0% agarose gel electrophoresis. Cycle sequencing using *iap* amplicons was performed with Hitachi DNA Sequencer 5500 (Hitachi, Tokyo, Japan) as previously described [24, 25, 31, 33]. Sequence analyses of *sigB* and *actA* were carried out at Eurofins Genomics (Tokyo, Japan). The comparative sequences of *iap*, *sigB* and *actA* in the reference strain, EGD-e, were located at 1,116–1,522 (407 bp), 41–702 (662 bp) and 1,357–1,917 (561 bp) positions, respectively. The sequence data were edited and aligned using DNAsis pro (Hitachi software, ver. 2.0). Phylogenetic analyses were conducted using MEGA, version 7.0 [11] and the unweighted-pair group method with arithmetic mean (UPGMA). All sequence data were registered at the DNA Data Bank of Japan (Mishima, Japan); accession numbers are indicated in Table 1. Unfortunately, the strains belonging to *iap* group C described in the previous report [34] were not tested for MLST, because their partial *actA* was not amplified using a massF/massR primer pair. In addition to 68 strains used in this study, 211 strains registered in the Food Microbe Tracker database (www.pathogentracker.net) maintained by Cornell University were analyzed *in silico* for the classification of nucleotide sequences of *sigB* (179 strains) and *actA* (194 strains) (Supplementary Table 1). Serotypes included 1/2a (57 strains), 1/2b (35 strains), 1/2c (seven strains), 3a (four strains), 3b (six strains), 3c (one strain), 4a (19 strains), 4b (50 strains) and 4c (10 strains). Additionally, 20 and two strains, whose serotypes were designated as unspecified and untypeable, respectively.

PFGE analysis: Molecular subtyping of *L. monocytogenes* strains by PFGE was performed based on standardized laboratory protocol, PulseNet (https://www.cdc.gov/listeria). Bacterial suspensions solidified with SeaKem Gold agarose (Lonza, Rockland, NY, U.S.A.) were lysed, washed and digested with the restriction enzymes, *Apa*I and *Asc*I (New England BioLab Japan, Tokyo, Japan). The digested samples were separated by electrophoresis [16].

Diversity *index*: Simpson's index of diversity (SID) was recommended to evaluate the discriminative ability of genotyping methods [17, 30]. The SID and the 95% confidence intervals (CI) are presented in the following equations:

$$SID = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} nj(nj-1)$$

$$\sigma^2 = \frac{4}{N}\left[\sum \pi j^3 - \left(\sum \pi j^2\right)^2\right]$$

$$CI = \left[D - 2\sqrt{\sigma^2}, D + 2\sqrt{\sigma^2}\right]$$

Where N is the total number of sample strains, S is the total number of different types described, n_j is the number of strains belonging to the jth type, and π_j is the frequency n_j/N.

Table 1. Genetic classification of *L. monocytogenes* strains used in this study

Strain	Source	Country	Serotype	MLST										RFLP				PFGE		
				iap			*sigB*			*actA*			MLST type	*Xba*I	*Cla*I (*Ban*III)	*Pst*I	*iap*-RFLP type	*Apa*I	*Asc*I	Pulsotype
				bp	Acc. #	*iap* type	bp	Acc. #	*sigB* type	bp	Acc. #	*actA* type								
EGD-e	Rabbit	England	1/2a	407	AL591975	0	662	AL591977	1	561	AL591974	1	1	X1	C1	P1	1	1	1	1
3E1	Skin of beef cattle	Japan	1/2a	407	AB294575	0	662	LC158691	34	561	LC158758	18	7	X1	C2	P2	2	16	13	2
3E2	Skin of beef cattle	Japan	1/2a	407	AB294576	0	662	LC158692	34	561	LC158759	18	7	X1	C2	P2	2	16	13	2
H3	Patient	Japan	1/2a	395	AB365680	1	662	LC158693	12	561	LC158760	67	10	X2	C3	P3	3	10	7	3
76P1	Pork	Japan	1/2c	413	AB365666	2	657	LC158694	36	561	LC158761	1	6	X1	C4	P1	4	4	10	4
78P1	Pork	Japan	1/2c	413	AB365667	2	662	LC158695	1	561	LC158762	1	2	X1	C4	P1	4	N.D.[a)]	N.D.	N.D.
173B3	Beef	Japan	1/2a	413	AB365647	2	662	LC158696	1	561	LC158763	1	2	X1	C4	P1	4	N.D.	N.D.	N.D.
23C1	Chicken	Japan	1/2c	413	AB365669	3	662	LC158697	1	561	LC158764	68	9	X3	C5	P4	5	2	17	5
76P2	Pork	Japan	1/2a	389	AB365649	4	662	LC158698	12	561	LC158765	21	15	X4	C6	P5	6	6	4	6
78P5	Pork	Japan	1/2a	389	AB365652	4	662	LC158699	12	561	LC158766	21	15	X4	C6	P5	6	7	4	7
89C5	Chicken	Japan	1/2a	389	AB365650	4	662	LC158700	12	561	LC158767	21	15	X4	C6	P5	6	N.D.	N.D.	N.D.
YC35P1	Pork	Ireland	1/2a	389	AB365695	4	662	LC158701	12	561	LC158768	21	15	X4	C6	P5	6	N.D.	N.D.	N.D.
80C1	Chicken	Japan	1/2a	395	AB365655	5	662	LC158702	1	561	LC158769	1	16	X5	C7	P6	7	12	16	8
H1	Patient	Japan	1/2a	395	AB365682	5	662	LC158703	1	561	LC158770	1	16	X5	C7	P6	7	N.D.	N.D.	N.D.
HM1	Patient	Japan	1/2a	395	AB365653	5	662	LC158704	1	561	LC158771	1	16	X5	C7	P6	7	11	15	9
HM2	Patient	Japan	1/2a	395	AB365656	5	662	LC158705	1	561	LC158772	1	16	X5	C7	P6	7	N.D.	N.D.	N.D.
265C1	Chicken	Japan	1/2a	395	AB365657	6	662	LC158706	12	561	LC158773	27	13	X6	C8	P7	8	19	8	10
268C1	Chicken	Japan	1/2a	401	AB365658	7	662	LC158707	1	561	LC158774	1	14	X7	C9	P8	9	17	3	11
104P5	Retail pork	Japan	1/2a	395	AB517745	8	662	LC158708	1	561	LC158775	1	3	X7	C9	P8	10	17	2	12
221C1	Chicken	Japan	1/2a	395	AB365659	8	662	LC158709	1	561	LC158776	1	3	X7	C9	P8	10	N.D.	N.D.	N.D.
YC39B1	Beef	U.S.A.	1/2a	395	AB365699	8	662	LC158710	1	561	LC158777	1	3	X7	C9	P8	10	18	5	13
223C3	Chicken	Japan	1/2a	407	AB365662	9	662	LC158711	1	561	LC158778	18	11	X8	C10	P9	11	N.D.	N.D.	N.D.
YC35P6	Pork	Ireland	1/2a	407	AB365702	9	662	LC158712	1	561	LC158779	18	11	X8	C10	P9	11	N.D.	N.D.	N.D.
YC35P8	Pork	Ireland	1/2a	407	AB365770	9	662	LC158713	1	561	LC158780	18	11	X8	C10	P9	11	N.D.	N.D.	N.D.
YC35P12	Pork	Ireland	1/2a	407	AB365701	9	662	LC158714	1	561	LC158781	18	11	X8	C10	P9	11	5	14	14
12H	Patient	Japan	1/2a	401	AB365663	10	662	LC158715	1	561	LC158782	1	5	X10	C12	P11	12	13	11	15
186C1	Chicken	Japan	1/2a	401	AB365664	10	662	LC158716	1	561	LC158783	1	5	X10	C12	P11	12	N.D.	N.D.	N.D.
188C3	Chicken	Japan	1/2a	401	AB365665	10	662	LC158717	1	561	LC158784	1	5	X10	C12	P11	12	14	12	16
YC4P12	Pork	Denmark	1/2a	389	AB365703	11	662	LC158718	1	561	LC158785	18	8	X11	C13	P12	13	N.D.	N.D.	N.D.
YC51P12	Pork	Denmark	1/2a	389	AB365704	11	662	LC158719	1	561	LC158786	18	8	X11	C13	P12	13	3	6	17
YC51P13	Pork	Denmark	1/2a	389	AB365780	11	662	LC158720	1	561	LC158787	18	8	X11	C13	P12	13	N.D.	N.D.	N.D.
72C1	Chicken	Japan	1/2b	389	AB365670	12	662	LC158722	5	561	LC158789	12	21	X13	C15	P14	15	25	21	18
74C1	Chicken	Japan	1/2b	389	AB365721	12	662	LC158723	5	561	LC158790	12	21	X13	C15	P14	15	N.D.	N.D.	N.D.
42C1	Chicken	Japan	4b	389	AB365719	12	662	LC158721	2	561	LC158788	16	24	X12	C14	P13	14	N.D.	N.D.	N.D.
82B1	Beef	Japan	4b	389	AB365726	12	662	LC158724	2	561	LC158791	16	24	X12	C14	P13	14	N.D.	N.D.	N.D.
338B2	Beef	Japan	4b	389	AB457597	12	662	LC158725	2	561	LC158792	16	24	X12	C14	P13	14	N.D.	N.D.	N.D.
468B1	Beef	Japan	4b	389	AB365725	12	662	LC158726	2	561	LC158793	16	24	X12	C14	P13	14	33	25	19
YC20C9	Chicken	China	1/2b	395	AB365784	13	662	LC158738	5	561	LC158805	3	17	X15	C17	P16	19	20	24	27
YC36C2	Chicken	Canada	1/2b	395	AB365742	13	662	LC158739	5	561	LC158806	3	17	X15	C17	P16	19	N.D.	N.D.	N.D.
YC50C3	Chicken	China	3b	395	AB365786	13	662	LC158740	5	561	LC158807	3	17	X15	C17	P16	19	N.D.	N.D.	N.D.
1E1	Skin of beef cattle	Japan	1/2b	395	AB294570	13	662	LC158727	37	561	LC158794	3	18	X15	C17	P17	16	21	20	20
100P3	Pork	Japan	1/2b	395	AB365762	13	662	LC158733	3	561	LC158800	8	19	X16	C18	P17	18	26	18	24
112P3	Pork	Japan	4b	395	AB365737	13	662	LC158734	3	561	LC158801	8	19	X16	C18	P17	18	N.D.	N.D.	N.D.
114P3	Pork	Japan	1/2b	395	AB365744	13	662	LC158735	3	561	LC158802	8	19	X16	C18	P17	18	N.D.	N.D.	N.D.
66C3	Chicken	Japan	1/2b	395	AB365756	13	662	LC158728	5	561	LC158795	9	22	X14	C16	P15	17	N.D.	N.D.	N.D.
69C3	Chicken	Japan	1/2b	395	AB365764	13	662	LC158729	5	561	LC158796	9	22	X14	C16	P15	17	30	23	21
79C1	Chicken	Japan	1/2b	395	AB365675	13	662	LC158730	5	561	LC158797	9	22	X14	C16	P15	17	30	22	22
91C3	Chicken	Japan	1/2b	395	AB365747	13	662	LC158731	5	561	LC158798	9	22	X14	C16	P15	17	29	22	23
93C1	Chicken	Japan	1/2b	395	AB365748	13	662	LC158732	5	561	LC158799	9	22	X14	C16	P15	17	N.D.	N.D.	N.D.
116C1	Chicken	Japan	1/2b	395	AB365735	13	662	LC158736	5	561	LC158803	9	22	X14	C16	P15	17	30	22	25
YC20C12	Chicken	China	1/2b	395	AB365752	13	662	LC158737	5	561	LC158804	9	22	X14	C16	P15	17	27	19	26
63P1	Pork	Japan	1/2b	404	AB365676	14	662	LC158741	5	561	LC158808	14	20	X17	C19	P18	20	24	26	28
H2	Patient	Japan	4b	389	AB365691	15	662	LC158742	2	561	LC158809	2	28	X18	C20	P19	21	22	30	29
11H	Patient	Japan	4b	389	AB365707	16	662	LC158743	35	561	LC158810	2	26	X18	20	P19	22	N.D.	N.D.	N.D.
229C1	Chicken	Japan	4b	389	AB365708	16	662	LC158744	2	561	LC158811	2	27	X18	C20	P19	22	23	29	30
393P1	Pork	Japan	4b	389	AB457603	16	662	LC158745	2	561	LC158812	2	27	X18	C20	P19	22	N.D.	N.D.	N.D.
499C5	Retail chicken	Japan	4b	389	AB517764	16	662	LC158746	2	561	LC158813	2	27	X19	C20	P19	23	23	28	31
1E3	Skin of beef cattle	Japan	1/2a	401	AB294572	20	662	LC158747	1	561	LC158814	1	4	X1	C25	P24	24	8	9	32
YC13C10	Chicken	U.S.A.	1/2a	401	AB365711	20	662	LC158748	1	561	LC158815	1	4	X1	C25	P24	24	N.D.	N.D.	N.D.
YC13C11	Chicken	U.S.A.	1/2a	401	AB365710	20	662	LC158749	1	561	LC158816	1	4	X1	C25	P24	24	9	9	33
YC17P13	Pork	Ireland	1/2a	401	AB365712	20	662	LC158750	1	561	LC158817	1	4	X10	C12	P11	25	15	11	34
YC21P8	Pork	Canada	1/2a	407	AB365714	21	662	LC158751	1	561	LC158818	21	12	X23	C26	P25	26	N.D.	N.D.	N.D.
YC21P12	Pork	Canada	1/2a	407	AB365713	21	662	LC158752	1	561	LC158819	21	12	X23	C26	P25	26	N.D.	N.D.	N.D.
YC21P14	Pork	Canada	1/2a	407	AB365788	21	662	LC158753	1	561	LC158820	21	12	X23	C26	P25	26	N.D.	N.D.	N.D.
2E1	Skin of beef cattle	Japan	1/2b	401	AB294574	22	662	LC158754	7	561	LC158821	10	23	X24	C27	P26	27	28	27	35
241C1	Retail chicken	Japan	4b	407	AB517776	24	662	LC158755	2	561	LC158822	69	25	X25	C28	P27	28	31	31	36
505C7	Chicken	Japan	4b	371	AB457607	25	662	LC158756	6	561	LC158823	11	29	X26	C29	P28	29	N.D.	N.D.	N.D.
508C6	Retail chicken	Japan	4b	371	AB517769	25	662	LC158757	6	561	LC158824	11	29	X26	C29	P28	29	32	32	37

a) N.D.: Not done.

Table 2. Primers used in this study

Target gene	Primer name	Sequence (5′ to 3′)
iap	SI3A	ACTGGTTTCGTTAACGGTAAA
	SI4B	TTTAGTGTAACCAGAGCAATC
sigB	LMsigB15	AATATATTAATGAAAAGCAGGTG
	LMsigB16	ATAAATTATTTGATTCAACTGCC
actA	massF	GCTGATTTAAGAGATAGAGGAAC
	massR	TTTATGTGGTAATTTGCTGTC

RESULTS

Classification by sequence of iap *and RFLP analysis of L. monocytogenes genome* c*ombined subtyping method*: To establish the DNA sequence-based subtyping method, we first performed *iap*-RFLP assay using 67 *L. monocytogenes* isolates and EGD-e (Table 1). The target sequence was located at the nucleotide position of *iap* in *L. monocytogenes*, from 1,116 to 1,522 bp in the reference strain, EGD-e. So far, 26 *iap* types have been designated to 0 through 25 based on a comparison of nucleotide sequences (Supplementary Table 2) [15, 34, 35]. Sixty-eight strains were classified into 22 *iap* types (Table 1). RFLP patterns of *L. monocytogenes* genomes digested with *Xba*I, *Cla*I or *Pst*I were classified into 26 (X1 to X26), 29 (C1 to C29) and 28 (P1 to P28) patterns, respectively (Supplementary Fig. 1) [15, 36]. All strains were classified into 22, 25 and 23 types according to genomic RFLP analyses, digested with *Xba*I, *Cla*I and *Pst*I, respectively (Table 1). RFLP analysis was repeated more than three times for each isolate, and its pattern did not change depending on the year of experiment or researcher. Five RFLP patterns, X1, X7, X10, X18 and X20, contained multiple *iap* types determined by the *iap* sequences, and *iap* types 9, 12, 13, 16 and 20 were found to have more than two RFLP patterns using *Xba*I. Strains assigned to pattern X1 were further classified into three (P1, P2 and P24) and four (C1, C2, C4 and C25) patterns by *Pst*I and *Cla*I, respectively (Table 1 and Supplementary Fig. 1). Consequently, *L. monocytogenes* strains used in this study were classified into 29 *iap*-RFLP types as a result of a combination of nucleotide sequencing for partial *iap* and RFLP analyses digested with *Xba*I, *Cla*I and *Pst*I. SID of this *iap*-RFLP method was 0.967 (95% CI: 0.955/0.979).

MLST analysis using iap, sigB and actA: In order to develop a DNA sequence-based subtyping method that can refer to the data of *iap*-based RFLP analyses, we focused on two genes, *sigB* and *actA*, in addition to *iap*. To investigate the characteristics of *sigB* and *actA*, the nucleotide sequences of these genes in strains registered in the Food Microbe Tracker database were compared to the EGD-e sequence *in silico*. Next, we evaluated whether the method developed in the present study showed a high discriminatory ability in the classification of *L. monocytogenes*. The nucleotide sequences for partial *sigB*, approximately 660 bp in length, were determined and used for genetic classification of 247 strains, which consisted of 68 strains used in this study and 179 Food Microbe Tracker strains (Table 1 and Supplementary Table 1). The number and type of point mutations in partial *sigB* are presented in Table 3. Thirty-seven *sigB* types were determined using *sigB* sequences (Table 1 and Supplementary Table 3). In total, 112 point mutations were found in partial *sigB* of 246 strains as compared with that of EGD-e. There were no insertions, however, deletion of five nucleotides was found in 76P1. In addition to 76P1, nonsense mutation in *sigB* was detected in 1E1. In comparison, partial *actA*, 562 bp in length, was analyzed using the nucleotide sequences of 262 isolates, including 68 strains used in this study and 194 Food Microbe Tracker strains. *In silico* assay was used for classification into 69 *actA* types (Table 4). In total, 152 point mutations were identified, compared with EGD-e sequence. No insertions or deletions were observed. The 67 strains isolated in Japan and 194 Food Microbe Tracker strains were classified into 17 and 65 *actA* types, respectively (Table 1 and Supplementary Table 1). Consequently, all strains used in this study were divided into 29 MLST types using *iap*, *sigB* and *actA* sequences (Table 1 and Fig. 1). SID of MLST was 0.967 (95% CI: 0.955/0.978). Strains assigned to certain *iap* types (0, 2, 12 and 16) and *iap* type 13 were further classified into two and four MLST types, respectively (Table 1). The phylogenic tree of MLST types indicated that strains were roughly clustered in two groups (MLST Clusters A and B). MLST Clusters A and B were consistent with lineages II and I, respectively.

PFGE analysis using Apa*I and* Asc*I*: To compare the MLST classification with that of PFGE, 38 strains were selected. PFGE patterns obtained using PulseNet protocol with restriction enzymes, *Asc*I and *Apa*I, could be distinguished into 32 types. Finally, the 38 strains were separated into 37 pulsotypes (Table 1 and Supplementary Fig. 2). The analyses of both PFGE patterns represented two major clusters (PFGE Clusters A and B) associated with their lineages as well as the results from MLST.

DISCUSSION

Compared with the EGD-e sequence, the *iap* target region sequence was used for classification into 26 types and three groups based on total point mutations. The *iap* mutation leads to the reduction of virulence, but systemic infections are caused [3]. It is thought that the virulence-promoting function of Iap protein (p60) has been due to its cell wall hydrolysis ability [28]. The Iap contains a C-terminal endopeptidase domain, two N-terminal Lysin motif (LysM) domains and a single N-terminal Src homology 3 (SH3)-like domain [28]. The *iap* region used in this study is other than these domains. Group A contained less than nine places of mutations, including 14 *iap* types (0–11, 20 and 21). Eight *iap* types (12–16, 22, 24 and 25), which contained 22–25 places of mutations, belonged to group B. Group C (four *iap* types; 17–19 and 23) contained more than 50 places of mutation (Supplementary Table 2). As described previously [15, 34], groups A and B were suggested to correspond to lineages II and I, respectively [20, 21, 37]. Unfortunately, no isolate was classified into lineage III, which is supposed to consist of serotype 4a according to Rasmussen *et al*. [20]. In contrast,

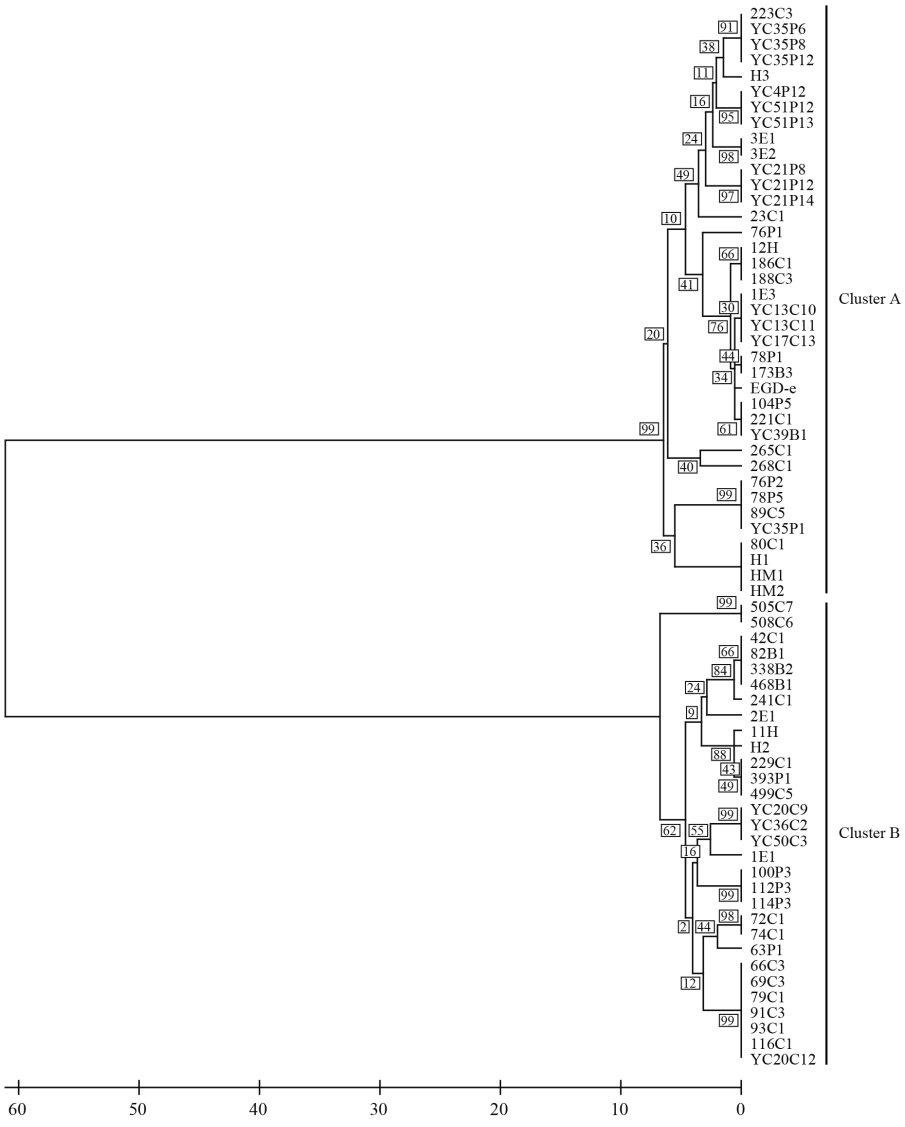

Fig. 1. Phylogenic classification using MLST. MLST profiles of 68 *L. monocytogene*s strains were based on partial sequences of *iap*, *sigB* and *actA*. Phylogenic analysis was performed using unweighted-pair group method analysis with arithmetic mean (UPGMA). The distances were calculated using the number of differences method based on the number of nucleotide differences per target sequence. The number in the square indicates the bootstrap rate (%). The percent value was obtained from 1,000 replications.

we preserved images of RFLP patterns of genomic DNA obtained from *L. monocytogenes* isolated since 1998. *L. monocytogenes* strains, including EGD-e, were classified into 26, 29 and 28 types using whole genomic RFLP analyses digested with *Xba*I, *Cla*I and *Pst*I, respectively. The classification based on partial *iap* sequences agreed with the RFLP-based classification. These results support our previous suggestion that *iap*-RFLP subtyping is useful for detailed differentiation of isolates for epidemiological purposes [24, 25, 31, 36]. However, certain drawbacks remain in RFLP analysis of genomic DNA with regard to distinction of RFLP patterns and inter-laboratory sharing of data. PFGE classification is a valuable investigation tool to recognize common sources of food-borne outbreaks [2]. However, PFGE is hard to determine the evolutionary relatedness of isolates [38], because PFGE patterns are influenced by changes in the accessory genome, including transient bacteriophages [42].

Several techniques for genetic classification of *L. monocytogenes* using DNA sequences have been developed. Repetitive-sequence-based PCR (Rep-PCR) targets noncoding short repetitive sequences [7]. This method is also robust across varying experimental conditions [8]. Multiple-locus variable-number tandem repeat analysis (MLVA) is a size analysis of amplified regions of DNA containing variable numbers of tandem repeats [4]. MLVA has been increasingly used as a complement tool for PFGE [32]. This method

requires normalization of sizing discrepancies for accurate and standardized MLVA on capillary electrophoresis [22]. In comparison with DNA size-based subtyping methods including PFGE and RFLP, the DNA sequence-based subtyping approach, such as MLST, is an informative tool for epidemiology and studies involving evolutionary relationships between strains [19]. The purpose of the present study was not to strict classification using MLST. The data obtained from our MLST can be used to compare with or refer to previous information.

Traditional MLST is based on several housekeeping genes, because these are non-susceptible to horizontal gene transfer and selection [14]. *L. monocytogenes* MLST database (http://bigsdb.web.pasteur.fr/listeria) maintained by the Pasteur Institute (Paris, France) is based on seven housekeeping genes: *abcZ*, *bglA*, *cat*, *dapE*, *dat*, *ldh* and *lhkA* [26, 40]. However, the evolution of virulence genes, which represent well-characterized pathogenicity of *L. monocytogenes,* is considered important. Previous MLST studies were performed using i) three housekeeping genes (*recA*, *prs* and *sigB*), two virulence genes (*actA* and *inlA*) and two intergenic regions (*hly-mpl* and *plcA-hly*) [1], ii) four housekeeping genes (*betL*, *dat*, *recA* and *sigB*) and three virulence genes (*actA*, *inlA* and *inlB*) [10] and iii) five housekeeping genes (*gap*, *prs*, *purM*, *ribC* and *sigB*) and two virulence genes (*actA* and *inlA*) [14]. These suggest that the nucleotide sequences of *sigB*, *actA* and *inlA* are useful for genetic classification. It was reported that there are 19 different mutations leading to premature stop codons in *inlA* and these mutations occur commonly in *L. monocytogenes* lineages I and II [13]. Therefore, we chose *sigB* and *actA* in addition to *iap* for MLST analysis in this study.

The *sigB* sequence resulted in classification into 37 types and three groups by total point mutations as compared to that of EGD-e as well as *iap*. The number of point mutations in Groups A (*sigB* types 1, 11, 12, 34 and 36), B (*sigB* types 2–10, 13–26, 31–33, 35 and 37) and C (*sigB* types 27–30) was less than two, 25–30 and more than 50, respectively (Table 3). Although a partial sequence of *sigB* derived from 67 isolates did not show diversity in comparison with *iap*; the *iap* types 0, 2, 12 and 13 could be classified into two or three groups via *sigB* type. This suggests that the nucleotide sequence of *sigB* might be relatively conserved in *L. monocytogenes* regardless of the geographical distribution. Nonetheless, the *actA* sequences were classified into 69 types (Table 4). These types were further divided into two groups by total point mutations as compared to that of EGD-e. Group A (*actA* types 0, 18–38, 67 and 68) contained less than seven places of mutation, whereas group B (*actA* types 2–17, 39–66 and 69) contained more than 50 places of mutation. *actA* can be used as an evolutional indicator as it appears to have undergone positive selection [1]. The target sequence of *actA* was located at the C-terminal region of ActA protein. This region consists of the membrane anchor domain and the cell wall penetration domain. ActA is a natively unfolded protein, and the N-terminal region and central domain of ActA are responsible for its virulence [6]. Mutations in this region are unrelated to the virulence function for intracel-

Table 3. Sequence typing based on partial *sigB*

Mutation	Type																																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
A→C	0	2	2	2	2	2	2	2	2	2	0	0	2	2	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	2	0	2
A→G	0	5	5	5	5	5	6	5	5	3	1	0	6	6	5	5	5	5	5	5	5	5	6	5	5	5	10	10	10	10	5	5	5	1	5	0	5
T→C	0	4	4	4	4	4	4	4	4	4	0	0	4	4	5	5	6	4	4	4	5	5	4	3	5	4	9	8	9	8	4	3	3	0	4	0	4
T→G	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0
C→A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0
C→T	0	7	7	7	5	6	7	6	6	6	0	1	6	6	5	6	6	8	6	7	7	7	7	7	9	7	13	13	14	13	6	6	6	1	7	0	6
G→A	0	5	5	5	4	5	5	4	4	4	0	0	5	4	4	4	4	4	5	5	4	4	4	5	4	4	6	6	6	6	4	4	4	0	5	0	5
G→T	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	1	2	2	0	1	0	1
A→T	0	1	1	1	1	1	1	1	1	2	0	0	1	1	2	1	1	1	1	1	1	1	1	1	1	1	3	3	3	3	1	1	1	0	1	0	1
T→A	0	3	3	3	3	3	3	3	3	2	0	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3	2	2	2	0	3	0	3
C→G	0	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	0	0	0	0	1	0	1
G→C	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1
Total	0	29	28	30	26	28	30	27	27	25	1	1	28	27	27	27	28	28	27	28	28	28	28	27	30	27	54	53	55	53	26	26	26	2	30	0	29
AT→CG	0	11	11	12	11	11	12	11	11	9	1	0	12	12	11	12	13	11	11	11	12	12	12	10	12	11	22	21	22	21	11	10	10	1	11	0	11
CG→AT	0	12	12	12	9	11	12	10	10	11	0	1	12	11	11	11	11	13	12	13	12	12	12	13	14	12	22	22	23	22	11	12	12	1	13	0	12
A←→T	0	4	4	4	4	4	4	4	4	4	0	0	3	3	4	3	3	3	3	3	3	3	3	3	3	3	6	6	6	6	3	3	3	0	4	0	4
C←→G	0	2	1	2	2	2	2	2	2	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	4	4	4	4	1	1	1	0	2	0	2
Size (bp)	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	662	657	662

Table 4. Sequence typing based on partial *actA*

Mutation	Type																																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
A→C	0	3	3	3	3	3	3	3	3	3	3	3	3	3	4	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A→G	0	18	19	19	19	19	19	19	18	19	19	17	18	18	19	18	19	2	0	2	2	2	0	2	3	1	1	2	1	2	0	1	1	1	2
T→C	0	7	8	7	7	7	6	9	8	7	6	7	8	8	8	7	8	1	1	1	2	2	0	0	1	0	0	1	0	1	0	0	2	1	1
T→G	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
C→A	0	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0
C→T	0	7	7	10	8	7	7	7	7	7	7	8	7	7	7	7	7	0	0	2	0	0	0	0	0	0	1	0	1	1	1	0	0	0	0
G→A	0	10	11	10	10	10	10	10	10	10	10	10	9	10	10	9	9	1	1	1	1	1	1	0	1	0	1	2	0	1	0	0	1	1	1
G→T	0	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0
A→T	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
T→A	0	2	2	3	3	2	3	1	1	3	3	2	2	2	2	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C→G	0	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G→C	0	1	2	2	2	1	1	2	1	2	2	1	2	2	2	2	2	1	1	1	1	1	0	0	1	0	0	1	0	1	0	0	1	1	1
Total	0	57	60	63	61	57	57	59	56	59	59	56	57	58	60	56	58	5	3	7	6	6	1	2	7	1	5	6	4	6	1	3	6	5	6
AT→CG	0	29	31	30	30	30	29	32	30	30	29	28	30	30	32	28	31	3	1	3	4	4	0	2	5	1	1	3	1	3	0	1	3	2	4
CG→AT	0	23	24	26	24	23	23	23	23	23	23	24	22	23	23	22	22	1	1	3	1	1	1	0	1	0	4	2	3	2	1	0	2	2	1
A←→T	0	3	2	4	3	2	3	1	1	3	4	2	2	2	2	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
C←→G	0	2	3	3	4	2	2	3	2	3	3	2	3	3	3	3	3	1	1	1	1	1	0	0	1	0	0	1	0	1	0	0	1	1	1
Size (bp)	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561

Mutation	Type																																		
	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
A→C	0	0	0	2	2	2	3	2	2	1	2	1	2	2	2	2	2	2	2	2	5	5	6	5	5	5	5	2	3	2	2	0	0	3
A→G	2	0	0	16	16	17	17	18	16	16	16	18	16	16	15	17	16	15	16	16	23	22	24	24	23	23	23	16	17	16	17	2	3	18
T→C	1	1	0	7	7	7	5	6	6	6	6	6	6	7	7	6	7	6	6	6	6	7	7	7	7	7	6	8	8	8	8	1	1	7
T→G	0	0	0	1	1	1	2	2	1	1	2	1	1	2	1	1	1	2	1	2	2	2	2	2	2	2	2	1	1	2	2	0	1	1
C→A	0	0	0	3	3	3	3	3	3	3	3	3	3	3	3	4	3	4	3	3	4	4	4	4	4	4	4	3	3	3	3	0	0	3
C→T	1	0	1	9	9	9	8	8	9	8	8	8	9	10	9	7	8	9	9	8	10	10	10	10	10	11	11	12	9	11	11	0	0	7
G→A	1	0	0	10	11	12	10	13	11	13	12	10	10	12	10	12	10	12	11	11	10	10	10	10	10	10	11	10	10	11	10	1	1	10
G→T	0	0	0	2	2	2	3	3	3	3	3	4	3	2	2	2	2	3	3	3	3	3	4	3	3	3	3	3	2	3	3	0	0	3
A→T	0	0	1	1	1	2	2	2	2	2	2	2	1	1	1	1	2	2	2	2	2	0	2	2	1	1	1	0	1	1	0	0	0	0
T→A	0	0	0	1	1	2	3	3	2	3	3	3	2	2	1	2	2	2	2	3	2	3	3	2	3	3	3	2	2	3	2	0	0	3
C→G	0	0	0	1	1	1	1	1	1	2	1	1	1	1	1	1	1	0	1	2	0	0	0	0	0	0	0	2	1	2	2	1	0	1
G→C	1	0	0	1	2	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2
Total	6	1	2	54	56	59	58	62	57	59	59	58	55	59	53	57	55	58	57	59	68	67	73	70	69	70	70	60	58	63	61	6	7	58
AT→CG	3	1	0	26	26	27	27	28	25	24	26	26	25	27	25	26	26	25	25	26	36	36	39	38	37	37	36	27	29	28	29	3	5	29
CG→AT	2	0	1	24	25	26	24	27	26	27	26	25	25	27	24	25	23	28	26	25	27	27	28	27	27	28	29	28	24	28	27	1	1	23
A←→T	0	0	1	2	2	4	5	5	4	5	5	5	3	3	2	3	4	4	4	5	4	3	5	4	4	4	4	2	3	4	2	0	0	3
C←→G	1	0	0	2	3	2	2	2	2	3	2	2	2	2	2	3	2	1	2	3	1	1	1	1	1	1	1	3	2	3	3	2	1	3
Size (bp)	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561	561

lular motility of *L. monocytogenes*. Therefore, they are likely to identify the genetic character of gene sequence, because several *actA* mutations are found in a single strain alone. In total, 194 strains registered in Food Microbe Tracker were classified into 65 *actA* types, whereas 67 strains isolated in our laboratory were divided into 17 *actA* types. The number of *actA* types was less than that of *iap* type in our isolates. Additional studies may be necessary to verify the diversity in *actA* among strains, including the strains belonging to *iap* group C [34], derived from different sources, such as patients, environment and food. Taken together, we conclude that the *sigB* and *actA* are useful for genetic classification to detect certain characteristic mutations.

The discriminatory ability of MLST using *iap*, *sigB* and *actA* is the same as that of *iap*-RFLP method. Strains belonging to *iap*-RFLP types 4 and 22 were further classified into two MLST types (Table 1). In contrast, the *iap*-RFLP types 22 (except for 11H) and 23, 24 and 25 were integrated into the results from MLST. Strains of 229C1 (*iap*-RFLP type 22 and MLST type 27) and 499C5 (*iap*-RFLP type 23 and MLST type 27) shared the same PFGE patterns digested with *Apa*I. It is difficult to ascertain the reason for this discrepancy in the present data. These results suggest that MLST and *iap*-RFLP method have potential applications in epidemiology of *L. monocytogenes* to trace the source of human infection. Strains showing the same PFGE pattern were classified into a single MLST type. Although PFGE provides greater discrimination power than that of MLST, clustering and lineage distinction were consistent with the results from PFGE (Fig. 1). The PFGE patterns of 104P5 and 268C1 with regard to *Apa*I as well as 12H and YC17P13 with regard to *Asc*I were similar; however, a clear distinction was obtained in MLST results for these strains. The differences between 104P5 and 268C1 included two substitutions, A to G and T to C, in *iap*, whereas only one substitution (G to A) in *iap* differentiated 12H and YC17P13. This suggests that MLST analysis is suitable to detect single nucleotide polymorphisms. In the future, MLST analyses using whole genome sequence technology have global applications in subtyping of *L. monocytogenes* [9, 16, 23]. Our data in this study will be helpful as a reference.

The sequences of three genes in almost all the test strains were consistent with those of Food Microbe Tracker strains. The types of *sigB* and *actA*, which were not seen in the Food Microbe Tracker strains, have a single base substitution or nonsense mutation. Unfortunately, the *iap* target sequences of many Food Microbe Tracker strains are unspecified. Therefore, the specific character of the Japanese isolates could not be determined in this study. However, the strains that share the same type of genes with the foreign isolates derived from listeriosis patients are frequently isolated in Japan. It suggests that the risk of infection seems to routinely exist in Japan. In addition, almost 60% of food supply in Japan depends on imports from other countries. Consequently, there is an urgent need to develop effective countermeasures against *L. monocytogenes* infection, even though occurrences of human listeriosis in Japan are limited to sporadic infections. Simultaneous surveillance for *L. monocytogenes* contamination in food and environment along with listeriosis epidemiology is vital for maintenance of food hygiene. The results from this study include the strains isolated from imported meat; therefore, our MLST scheme can provide valuable epidemiological information during outbreaks caused by strains that have entered Japan from other countries.

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