

Original Article

Genetic Classification of *Listeria monocytogenes* Serotype 4b Strains, Including Epidemic Clones, Isolated from Retail Meat in the Tokyo Metropolitan Area

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SUMMARY: A food-borne pathogen, *Listeria monocytogenes* serotype 4b, has been frequently isolated from patients with listeriosis, and numerous outbreaks of listeriosis are associated with this serotype. In the present study, we performed subtyping of *L. monocytogenes* serotype 4b strains on the basis of genetic analyses. Thirty-four isolates of serotype 4b were classified into 8 genotypes, namely genotypes 12, 15, 16, 17, 18, 23, 24, and 25, on the basis of the sequence for the partial *iap* gene. Genetic analyses revealed that genotype 16 and genotypes 24 and 25 belong to epidemic clone I (ECI) and ECII, respectively, which have been frequently associated with listeriosis outbreaks in the United States and Europe. The genotype isolated most frequently from retail meats in the Tokyo metropolitan area was genotype 12 (52%), followed by genotype 16 (29%), which belongs to ECI. We suggest that ECI is a common subtype of *L. monocytogenes* in retail meat in the area under investigation. On the other hand, ECII isolates were confirmed to be present in retail meat in Japan but were rare.

INTRODUCTION

Listeria monocytogenes is recognized as an important food-borne pathogen. Listeriosis is associated with severe manifestations of invasive diseases, including neurological infections such as encephalitis and meningitis, septicemia, and abortion, which primarily occur in immunocompromised populations. Food-borne outbreaks of listeriosis have been documented in the United States and Europe since the 1980s (1). Approximately 2,500 cases of human listeriosis, with 500 deaths, have been estimated to annually occur in the United States (2). In contrast, sporadic cases of human listeriosis have generally been reported in Japan, although a food-borne outbreak of listeriosis was documented in 2001 (3). The incidence of listeriosis in Japan has been reported to be 1.00 to 1.60 cases per million individuals, which is lower than that in the United States and Europe (4); however, the prevalence of *L. monocytogenes* in food items in Japan is almost comparable with that in the United States and Europe (5).

L. monocytogenes is categorized into 13 serovars; 33% to 50% of the isolates from patients with sporadic listeriosis are of serotype 4b, and numerous outbreaks of listeriosis are associated with this serovar (6). Therefore, elucidation of the characteristics of *L. monocytogenes* serotype 4b is required for the control of listeriosis. In addition to serotyping, molecular typ-

ing methods, which have considerable ability to discriminate microorganisms have been applied for the subtyping of *L. monocytogenes*. Strains isolated from several outbreaks have been found to possibly share unique genetic characteristics (7). A group of such strains is designated an epidemic clone (EC). Among ECs, ECI and ECII are the major ECs of serotype 4b (7). ECI was first documented in Nova Scotia, Canada, in 1981 (8), and since then, ECI strains have been reported to be associated with numerous outbreaks in the United States and Europe (7). Genomic DNA extracted from the ECI strains has been reported to be resistant to digestion with *Sau3AI*, and genetic markers specific to ECI strains have been identified (9–15). ECII emerged during a multistate outbreak involving hot dogs in the United States in 1998 and 1999 (16,17) and was subsequently associated with another multistate outbreak associated with turkey deli meat in the United States (9,18). Genetic markers specific to ECII strains are available for genetic classification of outbreak-associated strains (9,11,19). However, the ecology of EC in food items and food-processing environments is poorly understood (7).

We previously have investigated the prevalence of *L. monocytogenes* in retail meat collected from the Tokyo metropolitan area over the period 1998–2003 and identified serotype 4b as one of the major serovars frequently isolated from meat samples (20). In addition, our previous study on the genetic variation of this pathogen revealed that certain strains of serotype 4b, which were classified within genotypes on the basis of partial nucleotide sequence of the *iap* gene, were considered to belong to ECI (21). The study also revealed that ECI

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strains had been isolated from domestic retail meat as well as patients with listeriosis in Japan, although this clone was rare. ECII, on the other hand, was not isolated from any of the samples. In contrast, a high prevalence of ECI and ECII strains has been reported in the United States and Europe (11,14,22). The frequent outbreaks caused by EC are attributable to their ability to persist in food items or food-processing environments (23,24). In support of this hypothesis, ECII and ECIII, ECs of serotype 1/2a, have been found to persist in the same food processing environments (7,24). This raises the possibility that the ECs have the ability to expand their niche in food items and food processing environments in Japan. Further analysis involving the subtyping of serotype 4b strains and estimation of the prevalence of the subtypes, including ECI and ECII, is therefore required.

In the present study, we performed molecular subtyping of 34 isolates of *L. monocytogenes* serotype 4b and classified them into genetic lineages on the basis of partial nucleotide sequences of the *iap* gene, as described previously (21). The results were used for further evaluation of genetic variation within *L. monocytogenes* serotype 4b. Resistance of genomic DNA to *Sau3AI* digestion and detection of ECI- or ECII-specific genetic markers were also employed for the detection of EC. The obtained results were used for investigating the frequency of *L. monocytogenes* 4b subtypes, including ECI and ECII, in retail meat collected from the Tokyo metropolitan area.

MATERIALS AND METHODS

Bacterial strains: Twenty-eight strains of *L. monocytogenes* serotype 4b were previously isolated from different classes of retail meat, including beef, chicken and pork, from the Tokyo metropolitan area (Tokyo, Chiba, Kanagawa, Saitama) during the period 1998–2003 (Table 1) (20). In addition, 1 and 3 strains of serotype 4b, which were isolated from retail meat from the same area in 1989 and 1997, respectively, were included in the present study. All these strains were isolated from domestic products in Japan. Furthermore, 2 strains of serotype 4b (strains H2 and H11), which were isolated from Japanese patients with listeriosis, were also included. Of the 34 strains under investigation, the partial nucleotide sequences of the *iap* gene had been previously determined for 14 isolates (21). The corresponding sequences from the ECI (strain F2365) and ECII (strain H7858) reference strains were employed for genetic classification.

Gene amplification: Nine genetic regions of the *L. monocytogenes* isolates were amplified by polymerase chain reaction (PCR). The partial *iap* gene sequence, including the region encoding the threonine-asparagine repeat units (25), was amplified using the primer pair SI3A and SI3B, as described previously (26). The amplification of *gltA*, which is a genetic marker specific to serotype 4b, and of the 17B, 85M, and 133 regions, which are genetic markers specific to ECI, was performed using the primer pairs described by Yildirim et al. (14). Three ECII-specific genetic markers, 4bSF7, 4bSF18, and ORF1365–1366, were amplified using primer pairs reported by Evans et al. (19). Another

Table 1. *L. monocytogenes* isolates used in the present study

Strain	Meat class	Purchased date	Store	Source or reference
28C1	Chicken	6/15/1997	A	21
42C1	Chicken	6/16/1997	B	21
82B1	Beef	2/22/1998	C	21
125C1	Chicken	6/29/1998	B	21
133B1	Beef	6/28/1998	A	21
143C1	Chicken	6/29/1998	E	This study
174P1	Pork	6/13/1999	F	This study
229C1	Chicken	10/6/1997	D	21
241C1	Chicken	6/12/2000	G	This study
249C3	Chicken	6/10/2000	H	This study
270P1	Pork	6/11/2000	I	This study
295C1	Chicken	6/19/2000	C	21
338B2	Beef	6/10/2001	H	This study
339C6	Chicken	6/10/2001	H	This study
340C1	Chicken	6/10/2001	I	This study
353BP2	Beef & pork	6/10/2001	J	This study
355C4	Chicken	6/11/2001	D	21
379P4	Pork	6/16/2001	H	This study
380C1	Chicken	6/16/2001	H	This study
392B1	Beef	6/22/2001	D	21
393P1	Pork	6/19/2002	F	This study
468B1	Beef	5/31/2003	D	21
489P1	Pork	5/31/2003	D	21
499C4	Chicken	5/30/2003	K	This study
502C1	Chicken	6/1/2003	L	This study
505C1	Chicken	6/1/2003	C	This study
505C7	Chicken	6/1/2003	C	This study
508C4	Chicken	6/1/2003	M	This study
514C4	Chicken	5/31/2003	N	This study
528C4	Chicken	6/21/2003	K	This study
549C1	Chicken	6/22/2003	C	This study
H2	Patient	1989		21
H11	Patient	2000		21
SC32	Chicken	1989	O	21

ECII-specific genetic marker, LMOh7858_0487.8, was amplified using the primer pair described by Chen and Knabel (10). The PCR assay for the *iap* gene was performed as described by Ueda et al. (26), and PCR assays for the ECI- and ECII-specific genetic markers, except for LMOh7858_0487.8, were performed as described by Yildirim et al. (14). The amplification conditions for the LMOh7858_0487.8 marker included an initial denaturation step at 95°C for 15 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, followed by extension at 72°C for 1 min and a final extension step at 72°C for 8 min. The amplified products were separated by electrophoresis using 1% agarose gels. Clear DNA bands of the expected sizes, viewed under a UV transilluminator, were considered to indicate positive results for the amplification targeting serotype 4b- and ECI-specific genetic markers as well as LMOh7858_0487.8. However, in the PCR assay for the markers 4bSF7, 4bSF18, and ORF1365–1366, the absence of amplified products was considered to be a positive result. All amplification experiments were independently repeated at least twice.

Genetic classification on the basis of partial nucleotide sequences of the *iap* gene: Partial nucleotide se-

quences of the *iap* gene, including 1116–1522 nt of the EGD-e strain (GenBank accession no.: X52268), were determined as described previously (26). The following sequences were compared: sequences determined in the present study, previously reported sequences from strains of serotypes, 1/2a, 1/2b, and 1/2c (21), and the corresponding region from strains EGD-e, F2365, and H7858 (GenBank accession nos.: X52268, NC002973, and AADR01000029, respectively). The partial nucleotide sequences of the *iap* gene were edited and subsequently aligned using DNAsis Pro (version 2.0; Hitachi Software Engineering, Tokyo, Japan).

Digestion of genomic DNA with restriction enzymes: Genomic DNA from the isolates of *L. monocytogenes* serotype 4b was digested with *Sau3AI* and *MboI* (Takara Bio Inc, Shiga, Japan), according to the manufacturer’s instructions. The resultant DNA fragments were separated on 0.8% agarose gels. All restriction digestion experiments were independently repeated at least twice.

Statistical analysis: Comparisons were statistically analyzed using the chi-square test, with the level of sig-

nificance set at a *P* value of 0.05.

Nucleotide sequence accession numbers: Partial nucleotide sequences of the *iap* gene determined in the present study have been deposited in the DDBJ bank database under accession numbers AB457592–AB457611.

RESULTS

Genetic classification of *L. monocytogenes* 4b isolates on the basis of partial nucleotide sequences of the *iap* gene: Isolates of *L. monocytogenes* serotype 4b investigated in the present study were assigned to 8 genotypes on the basis of partial nucleotide sequences of the *iap* gene (Table 2). Five of these genotypes (genotypes 12, 15, 16, 17, and 18) had been previously identified (21,27), whereas the remaining 3 genotypes (genotypes 23, 24, and 25) were identified in the present study. The ECI reference strain F2365 was classified as genotype 16, as described previously (21,27). Strains of genotype 25, however, were found to have identical *iap* gene sequences with the ECII reference strain H7858.

Table 2. Results of PCR and restriction enzyme digestion

Strain	Genotype by <i>iap</i> gene	Digested by		4b <i>GltA</i>	ECI-specific marker			ECII-specific marker				Epidemic clone
		<i>MboI</i>	<i>Sau3AI</i>		17B	85M	133	SF7	SF18	1365–66 ¹⁾	487.8 ¹⁾	
28C1	12	+	+	+	–	–	–	+	+	+	–	
42C1	12	+	+	+	–	–	–	+	+	+	–	
82B1	12	+	+	+	–	–	–	+	+	+	–	
125C1	17	+	+	–	–	–	+	+	+	–	–	
133B1	18	+	–	–	–	–	+	+	+	–	–	
143C1	23	+	+	–	–	–	+	+	+	–	–	
174P1	12	+	+	+	–	–	–	+	+	+	–	
229C1	16	+	–	+	+	+	+	+	+	+	–	ECI
241C1	24	+	+	+	–	–	–	–	–	–	+	ECII
249C3	12	+	+	+	–	–	–	+	+	+	–	
270P1	16	+	–	+	+	+	+	+	+	+	–	ECI
295C1	16	+	–	+	+	+	+	+	+	+	–	ECI
338B2	12	+	+	+	–	–	–	+	+	+	–	
339C6	12	+	+	+	–	–	–	+	+	+	–	
340C1	16	+	–	+	+	+	+	+	+	+	–	ECI
353BP2	12	+	+	+	–	–	–	+	+	+	–	
355C4	12	+	+	+	–	–	–	+	+	+	–	
379P4	12	+	+	+	–	–	–	+	+	+	–	
380C1	12	+	+	+	–	–	–	+	+	+	–	
392B1	12	+	+	+	–	–	–	+	+	+	–	
393P1	16	+	–	+	+	+	+	+	+	+	–	ECI
468B1	12	+	+	+	–	–	–	+	+	+	–	
489P1	12	+	+	+	–	–	–	+	+	+	–	
499C4	16	+	–	+	+	+	+	+	+	+	–	ECI
502C1	12	+	+	+	–	–	–	+	+	+	–	
505C1	16	+	–	+	+	+	+	+	+	+	–	ECI
505C7	25	+	+	+	–	–	–	–	–	–	+	ECII
508C4	25	+	+	+	–	–	–	–	–	–	+	ECII
514C4	12	+	+	+	–	–	–	+	+	+	–	
528C4	16	+	–	+	+	+	+	+	+	+	–	ECI
549C1	16	+	–	+	+	+	+	+	+	+	–	ECI
H2	15	+	–	+	+	+	+	+	+	+	–	ECI
H11	16	+	–	+	+	+	+	+	+	+	–	ECI
SC32	15	+	+	+	–	–	–	+	+	+	–	

¹⁾: 1365-66, ORF1365-1366; 487.8, LMOh7858_0487.8.

A phylogenetic tree previously constructed from partial *iap* gene sequences revealed that *L. monocytogenes* was roughly classified into 3 groups and that serotype 4b strains were assignable to 2 of them, namely groups B and C (21,27). The present study revealed that 31 strains assigned to the genotypes 12, 15, 16, 24, and 25 were classified as group B, which was recognized as lineage 1 and predominantly composed of serotypes 1/2b and 4b (25,28); the remaining 3 strains assigned to genotypes 17, 18, and 23 were classified as group C.

Detection of ECI- and ECII-specific genetic markers: The results of PCR analysis are summarized in Table 2. The serotype 4b-specific genetic marker *gltA* was detected in all the serotype 4b strains assigned to group B. However, the 3 strains assigned to group C were considered to be negative because agarose gel electrophoresis revealed faint DNA bands or amplified products of different sizes from these isolates. These results suggest differences in the *gltA* gene sequences in these 3 strains. Amplification of 17B, 85M, and 133 regions related that isolates of genotype 16 possessed all the ECI-specific genetic markers. In contrast, amplified products for any ECI-specific genetic marker were not detectable from the other serotype 4b isolates, except for strain H2. PCR targeting 4bSF7, 4bSF18, and ORF1365–1366 revealed the absence of corresponding PCR products from the strains 505C7 and 508C1, which were classified as genotype 25. In addition, PCR for the 3 ECII-specific genetic markers failed to amplify any product from the strain 241C1, which was classified as genotype 24. PCR targeting LMOh7858_0487.8 amplified products only in these 3 isolates.

Digestion of genomic DNA with *Sau3AI*: Restriction digest analyses revealed that genomic DNA from isolates assigned to genotype 16 as well as 2 other isolates (133B1 and H2) was resistant to digestion with *Sau3AI* (Table 2). In contrast, genomic DNA from all the isolates under investigation was sensitive to digestion with *MboI*.

Comparison of genetic classification on the basis of the *iap* gene sequence and genetic analyses for the detection of EC: The genetic classifications on the basis of the *iap* gene sequence, PCR, and restriction digest analyses were compared for identifying the subtypes of *L. monocytogenes* serotype 4b. Sixteen isolates assigned to genotype 12 were negative in all PCR experiments targeting genetic markers specific for EC (Table 2). In addition, the genomic DNA of these isolates was sensitive to digestion with *Sau3AI*. Consequently, these isolates cannot be categorized as either ECI or ECII. Nine isolates from retail meat samples and 1 from a patient with listeriosis, which were assigned to genotype 16, revealed the presence of all ECI-specific genetic markers as well as *Sau3AI*-resistance of genomic DNA; they were thus considered to represent ECI. Strain H2, which did not share sequence similarity with F2365, was also categorized as ECI, as described previously (21). On the other hand, the genetic characteristics of strain SC32, which was assigned to the same genotype as strain H2, were identical to those of the strains of genotype 12 but not ECI. Amplification of the 4 genetic markers 4bSF7, 4bSF18, ORF1365–1366, and LMOh7858_0487.8 revealed that strains 241C1, 505C7, and 508C1, which were assigned to genotypes 24 or 25, could be identified

as ECII. Three isolates assigned to genotypes 17, 18, and 23 could not be categorized as either ECI or ECII because the genetic characteristics of ECI or ECII were detectable in these isolates.

Genotype frequencies of *L. monocytogenes* 4b isolates obtained from retail meat in the Tokyo metropolitan area: The frequency of isolation of the various genotypes was investigated using 31 strains isolated from retail meat obtained in the Tokyo metropolitan area from 1997 to 2003 (Table 1). Genotype 12 had the highest isolation frequency; approximately half (52%) of the isolates under examination were assigned to this genotype. These strains were isolated from retail meat purchased from 9 stores over the entire study period, except 2002. Genotype 16, which belongs to ECI, had the second highest isolation frequency (29%). The strains of genotype 16 were isolated from meat obtained from 5 stores and were continuously detected from 2000 to 2003. Genotypes 24 and 25 included 1 and 2 isolates, respectively, showing that 10% of the isolates under examination belonged to ECII. This frequency was significantly lower than that of ECI ($P = 0.02$). ECII emerged in 2000 and was subsequently isolated in 2003. In addition, only 1 isolate was assigned to the genotype 17, 18, or 23.

DISCUSSION

In general, the genetic classification of *L. monocytogenes* isolates on the basis of partial nucleotide sequences of the *iap* gene was in agreement with the classification on the basis of PCR for EC markers and genomic DNA digestion with *Sau3AI*, with a few exceptions. Strain SC32 was assigned to the same genotype as strain H2, which was identified as ECI in a previous study (21); however, the PCR and restriction enzyme analyses revealed that this strain could not be categorized as either ECI or ECII. Therefore, the classification of strain SC32 requires further analysis. With regard to ECII, the *iap* gene sequences of 2 strains assigned to genotype 25 were found to be identical to that of the ECII reference strain, H7858. Moreover, as expected, all ECII-specific genetic markers were detected in these strains. Strain 241C1, assigned to genotype 24, did not show a close genetic relationship with the ECII reference strain; however, amplification targeting 4bSF7, 4bSF18, and ORF1365–1366 failed to yield products, suggesting that this strain belongs to ECII. Further evidence confirming the categorization of this strain as ECII was obtained by PCR experiments targeting LMOh7858_0487.8, which was developed as a positive genetic marker for ECII. Amplified fragments could be detected from strain 241C1; this strain was thus considered to belong to ECII in the present study. The genetic variation in the *iap* gene of ECII strains suggests that horizontal gene transfer and recombination had occurred in strain 241C1.

In a previous study, we revealed the presence of ECI strains, which belong to genotype 16, in retail meat samples and patients with listeriosis from the Tokyo metropolitan area; however, the results suggested a rare presence of ECI in food items (21). In the present study, however, almost 30% of the serotype 4b isolates from retail meat samples collected in the area belonged to ECI. In addition, we previously showed that almost

25% of *L. monocytogenes* strains isolated from retail meat samples belonged to serotype 4b (20). Taken together, the frequency of ECI among the strains isolated from retail meat samples has been estimated to be approximately 7.5%. This isolation frequency appears to be higher than that from food items in Italy (11), but lower than that from food items and food-processing environments in the United States (14). These findings suggest that this EC is a common subtype of *L. monocytogenes* in retail meat samples from Tokyo metropolitan area.

ECII strains, which belong to genotypes 24 and 25, were also detected in retail meat samples, although they were not identified in our previous study (21). Statistical analysis revealed that the isolation frequency of ECII from retail meat was significantly lower than that of ECI, suggesting a rare distribution of ECII in retail meat and meat-processing environments. The isolation frequency of ECII from retail meat in the Tokyo metropolitan area is lower than the corresponding frequency in the United States and Italy, where the frequencies of ECII and ECI are roughly comparable (11,24). The difference in the relative frequencies of ECI and ECII in the present study suggests niche differentiation, although both these ECs have been isolated from a wide range of food items (11,24). Moreover, the absence of ECI and ECII strains from ready-to-eat raw seafood products in Japan has been previously reported (31,32), suggesting a limited distribution of these ECs in food items. Continuous monitoring for these ECs in retail meat and other food items is required for testing this hypothesis.

In our previous study, 3 pairs of strains were isolated from different meat classes simultaneously purchased from the same store (338B2 and 339C6, 379P4 and 380C1, 468B1 and 489P1), which suggested cross-contamination (20). All these strains suspected of cross-contamination were assigned to genotype 12 in the present study, which was also the genotype with the highest isolation frequency. On the other hand, in 3 other instances, the same subtypes were isolated from the same meat class purchased from the same store but on different sampling dates spaced 1–3 weeks apart (339C6 and 380C1, 499C4 and 528C4, 505C1 and 549C1) (20); these strains were suspected to show persistence, and 2 of them belonged to ECI. Taken together, these findings suggest that ECI contamination has spread in the area under investigation.

Conflict of interest None to declare.

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