Table 1 (*continued*)

Isolate no.[a]	Origin	Isolation date (day/mo/yr)[c]	MLVA cluster	MLVA type (no. of repeats) for			Ribotype	MLST type[d]	PFGE type
				TR1	TR2	TR3			
Other isolates (1/2a, 1/2b)									
FSL F2-026 (1/2b)	Human sporadic	29-Jun-99	N/A	21	18	9	1043A	30	35
FSL F2-245 (1/2b)	Human sporadic	20-Jan-00	N/A	8	13	9	1052A	30	35
29-10-1 (1/2b)	Food (minced tuna)	17-Feb-05	N/A	14	15	6	1051D	31	36
FSL E1-039 (1/2b)	Animal (bovine)	Apr-00	N/A	16	16	9	1042B	36	37
FSL E1-041 (1/2b)	Animal (ovine)	Apr-00	N/A	16	11	9	1042C	29	38
FSL E1-058 (1/2b)	Animal (bovine)	May-00	N/A	20	15	9	1028A	30	39

[a] Isolates marked with a † were used for initial MLVA.
[b] Isolates FSL F2-525 and FSL J1-158 represent lineage III serotype 4b isolates.
[c] Isolation year, month, or date are given where available.
[d] For MLST types marked with a * and ** MLST data were obtained from a previous publication (Zhang et al., 2004 and Chen et al., 2007, respectively); for all other isolates MLST types were determined as part of the study reported here.
[e] N/A=not applicable; the lineage III serotype 4b strains did not have clear tandem repeat sequences in the TR1 and 3 amplicons.

(Jeffers et al., 2001), while ECII is represented by ribotype DUP-1044A (Gray et al., 2004). However, ribotyping has lower discriminatory power than PFGE for *L. monocytogenes*, in particular for serotype 4b strains (Aarnisalo et al., 2003; Grif et al., 2006). PFGE, on the other hand, generally provides for highly discriminatory subtyping of *L. monocytogenes* and is often considered the gold standard subtyping method for this organism. However, even with the standard PulseNet two-enzyme PFGE protocol, the ability to discriminate serotype 4b isolates and epidemic clones may be limited. For example, a recent study found that 15 ribotype DUP-1038B isolates from human cases, foods, animals and environmental sources in New York State as well as isolates from the 1985 listeriosis outbreak in Los Angeles and from the 1983–87 outbreak in Switzerland all shared the same PFGE type (Fugett et al., 2007). In addition, fragment-based methods like PFGE may be difficult to standardize and compare between laboratories and PFGE data do not allow for phylogenetic analyses (Wiedmann, 2002). MLST methods overcome many of the limitations of PFGE (Maiden et al., 1998), but MLST also only allows for limited discrimination of serotype 4b isolates and epidemic clones (Borucki et al., 2004; Zhang et al., 2004). For example, the MLST protocol established by Zhang et al. (2004) was not able to differentiate ECII isolates linked to two different outbreaks in the US (Chen et al., 2007), even though isolates from these outbreaks could be differentiated by PFGE (Chen et al., 2005).

Analysis of tandem repeat sequences has been utilized for molecular subtyping since there are potentially extensive polymorphisms in the number of repeats among individuals of the same species (Schlötterer and Tautz, 1992) or clonal group. A subtyping approach that targets multiple variable-number of tandem repeat (VNTR) loci, often called multiple-locus VNTR analysis (MLVA), has been used successfully for discriminatory subtyping of a number of bacterial pathogens, including *Bacillus anthracis* (Keim et al., 2000; Le Flèche et al., 2001; Ryu et al., 2005), *Escherichia coli* (Lindstedt et al., 2004a; Noller et al., 2003), *Haemophilus influenzae* (van Belkum et al., 1997), *Mycobacterium tuberculosis* (Fronthingham and Meeker-O'Connell, 1998), *Neisseria meningitides* (Yazdankhah et al., 2005), *Salmonella enterica* (Lindstedt et al., 2004b; Ramisse et al., 2004), and *Yersinia pestis* (Klevytska et al., 2001). As MLVA has been shown to allow for sensitive subtype discrimination of highly clonal pathogens difficult to differentiate by other methods (e.g., *B. anthracis, Y. pestis*), we developed and validated a MLVA method to specifically discriminate *L. monocytogenes* serotype 4b strains and clonal groups. The MLVA method described here is easy to interpret and allows for rapid and sensitive subtyping of serotype 4b strains, using only 3 tandem repeat regions. The study reported here provides important data beyond those presented in a recent publication (Murphy et al., 2007), which investigated agarose gel-based MLVA for subtyping of *L. monocytogenes* using only food isolates, most of them representing serotype 1/2a.

2. Materials and methods

2.1. Bacterial isolates

A total of 60 *L. monocytogenes* lineage I, serotype 4b isolates were used in this study (Table 1). In addition to 39 isolates from the Cornell Food Safety Laboratory (FSL) collection, isolates were purchased from American Type Culture Collection (ATCC; Manassas, Va.), National Collection of Type Cultures (NCTC; London, United Kingdom), and Collection de l'Institut Pasteur (CIP; Paris, France). Two seafood isolates (Handa et al., 2005) and nine pork isolates were obtained from food products sampled in Japan. In total, 38 human isolates, 17 food isolates and 5 animal isolates representing lineage I and serotype 4b were included in this study. In addition to these 60 isolates, 6 serotype 1/2a isolates, 6 serotype 1/2b isolates, and two serotype 4b lineage III isolates were also characterized by MLVA; serotype 4b isolates in this lineage are rare and distinct from serotype 4b lineage I isolates (Roberts et al., 2006; Ward et al., 2004).

2.2. MLVA

Among more than 100 potential VNTRs identified by the Tandem Repeat Finder program (http://tandem.bu.edu/trf/trf.html) (Benson, 1999) in the genome sequence for *L. monocytogenes* F2365 (GenBank accession No. AE017262) (Nelson et al., 2004), three VNTR regions (TR1 to TR3), which showed the highest levels of allelic variation in initial studies among 26 of the isolates studied, were selected as targets for the final MLVA scheme (Table 2). These 26 isolates were included in the 60 isolates used for the further study. The predicted ORF designations and their functions were obtained from The Institute for Genomic Research Comprehensive Microbial Resource website (http://www.tigr.org) (Peterson et al., 2001).

L. monocytogenes DNA for PCR amplification of TR1 to TR3 was prepared using a DNA extraction kit (Mag Extractor-Genome; Toyobo

Table 2
Characteristics of VNTR loci used for MLVA

TR	Repeat motif	No. of alleles	Diversity[a]	Location in F2365[b]	No. of repeats in F2365[b]
TR1	CCGGTAGAT	17	0.924	1150428–1150552	14
TR2	CATCGG	11	0.798	1849463–1849564	17
TR3	TCA	4	0.686	196959–196985	9

[a] Diversity (expressed as Simpson's Index of Discrimination) was calculated based on MLVA data for the 60 L. monocytogenes lineage I serotype 4b isolates characterized here.
[b] Location and No. of repeats are based on the genome sequence for L. monocytogenes isolate F2365.

Co. Ltd., Tokyo, Japan) (Takahashi et al., 2003). PCR was performed in 20-μl mixtures containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 20 pmol of each primer, 0.2 mM of each dNTP, 10 ng of template DNA and 0.5 U of Takara Taq DNA polymerase (Takara Bio, Otsu, Japan) using a Gene Amp 9700 thermal cycler (Applied Biosystems, Foster City, Calif.). PCR primers (Table 3) were designed from sequences flanking the TR1 to TR3 regions. PCR conditions included an initial denaturation step (94 °C for 5 min), followed by 30 cycles at 94 °C for 30 s, 60 °C for 40 s, 72 °C for 1 min, and a final extension of 72 °C for 4 min. The PCR amplification products were sequenced on an ABI PRISM 310 genetic analyzer (Applied Biosystems) with the Big Dye terminator v.3.0 cycle sequencing kit (Applied Biosystems).

To facilitate rapid typing, fragment size analysis of the TR1 to TR3 PCR products was also performed. For this purpose, the TR1 to TR3 forward primers were labeled with 6-carboxy-4,7,2',4',5',7'-hexachlorofluorescein (HEX), 6-carboxy-4,7,2',7'-tetrachloro-fluorescein (TET) and 5-carboxyfluorescein (FAM), respectively (Table 3). PCR amplification for fragment size analysis was performed in a 10-μl mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1 pmol of fluorescently labeled forward primer and non-labeled reverse primer, 0.2 mM of each dNTP, 5 ng of template DNA and 0.25 U of Takara Taq DNA polymerase. A 1-μl aliquot of each PCR product was mixed with 10 μl of Hi-Di formamide (Applied Biosystems) and 1 μl of GeneScan-500 ROX size standard (Applied Biosystems). Capillary electrophoresis of PCR products was performed on an ABI PRISM 310 Genetic Analyzer with POP4-polymer using the standard GeneScan module with filter set C. MLVA types were assigned to TR1 through TR3 based on the number of repeats present for a given TR, e.g., TR1 type 22 for isolate CIP 103322 (Table 1) indicates that 22 TR1 repeats were found in this isolate.

2.3. Stability of MLVA patterns

To test the stability of *L. monocytogenes* MLVA patterns, three epidemiologically distinct isolates (Lma5, CIP103322, and CIP103575; Table 1) were passaged daily for 70 days in BHI broth. MLVA was performed on isolates obtained after every 10th passage.

2.4. MLST

Since *L. monocytogenes* serotype 4b isolates are highly clonal, we used a MLST scheme previously described by Zhang et al. (2004), which is based on DNA sequencing of 6 *L. monocytogenes* virulence and virulence-associated genes, to further characterize all serotype 4b isolates that were characterized by MLVA. Zhang et al. (2004) referred to this approach as multi-virulence-locus sequence typing (MVLST). PCR amplification and DNA sequencing was performed essentially as described by Zhang et al. (2004); sequencing reactions were run using an ABI PRISM 310 Genetic Analyzer. For each locus, DNA sequences that differed by at least one nucleotide were assigned different arbitrary allele numbers (Maiden et al., 1998).

Table 3
PCR primers and amplification conditions for selected VNTR loci[a]

Locus	Primer	Sequence (5'-3')[b]	PCR products	
			Location in F2365	Length
TR1	TR-1f	HEX-ACATGGGAAGGGTTGCAA	1150293–1150591	299
	TR-1r	GGATTTACTTGATTTGACGGGT		
TR2	TR-2f	TET-CCATCGAAGACTACTGTTTGTA	1849739–1849272	468
	TR-2r	GACGGTACTGTTATCGGAAA		
TR3	TR-3f	FAM-GAAGGTAAAAACGGCGAAAAA	196776–197158	383
	TR-3r	ATTGCTTCTCCGTATCCCTCA		

[a] The primers outlined, synthesized without fluorescent dyes, here were also used for sequencing analysis.
[b] Primers were designed using the genome sequence for *L. monocytogenes* isolate F2365; HEX, TET, and FAM indicate the fluorescent dyes used to label a given primer.

2.5. PFGE

Single enzyme PFGE with ApaI, instead of the standard PulseNet two-enzyme PFGE protocol with ApaI and AscI, was performed as described by Graves and Swaminathan (2001) with minor modification. DNA plugs for PFGE were made using CHEF bacterial plug kit (Bio-Rad, Hercules, Calif.). Briefly, *L. monocytogenes* strains were grown overnight in BHI medium, followed by addition of chloramphenicol to a final concentration of 180 μg/ml and subsequent incubation for up to 1 h at 30 °C. Bacterial cells were then pelleted by centrifugation at 10,000 rpm for 5 min, resuspended in 100 μl of cell suspension buffer (10 mM Tris pH 7.2, 20 mM NaCl, 50 mM EDTA), and equilibrated to 50 °C. Bacterial suspensions were mixed with equal volumes of CleanCut agarose (2%, 50 °C) and poured into a mold to form agarose plugs (15 min, 4 °C). Plugs with immobilized bacteria were incubated for 2 h at 37 °C in a lysozyme solution, rinsed with sterile water, and subsequently incubated overnight at 50 °C in proteinase K solution. Plugs were then washed four times (1 h each) with 1 ml of 1 × wash buffer with gentle agitation at room temperature for 1 h, followed by overnight incubation with 30 U of ApaI (New England Biolabs, Beverly, MA) in 300 μl of 1 × enzyme buffer. Following the restriction digest, the plugs were incubated in 1 ml of 1 × wash buffer for 30 min with gentle agitation. One-third of each plug was placed in 1.0% agarose (Seakem Agarose Gold; FMC Bioproducts, Rockland, ME) gel in 0.5 × TBE buffer. PFGE was performed for 22 h at 14 °C with the CHEF-DR II system (Bio-Rad) at 6 V/cm with switch times ranging from 4 to 40 s. Gels were stained with ethidium bromide and photographed while transilluminated with UV light.

2.6. Ribotyping

Ribotyping data were already available for all isolates obtained from the Cornell strain collection (Table 1). For all other isolates, automated EcoRI ribotyping was carried out using the RiboPrinter microbial characterization system (Qualicon Inc., Wilmington, Del.) according to manufacturer's instructions, using isolates grown overnight at 30 °C on BHI agar plates; this represents the same procedure that was used to characterize all isolates obtained from Cornell. The RiboPrinter instrument automatically performs cell lysis and restriction enzyme digestion followed by gel electrophoresis and Southern hybridization with an rRNA gene probe. Images are analyzed using the RiboPrinter analysis software, which normalizes fragment pattern data for band intensity and band size relative to molecular weight markers. Patterns were then compared to the RiboPrinter database for characterization; a DuPont ID (e.g., DUP-1038) is automatically assigned to a ribotype pattern that matches a DuPont ID pattern with a similarity of 0.85 or above. If visual inspection observed more than one distinct ribotype pattern within a given Dupont ID, an alphabetical letter was assigned at the end of each Dupont ID (e.g. DUP-1038A, DUP-1038B); assignment of these refined IDs was performed by comparing ribotype patterns to patterns in the Cornell University PathogenTracker database (http://www.pathogentracker.net). If a given ribotype pattern did not match a DuPont ID pattern with a similarity > 0.85 (and thus was not assigned a DuPont ID), we assigned a unique type designation based on the "Ribogroup" that had been assigned by the instrument (e.g., ribogroup 172-75-S-1) after visual inspection and confirmation.

2.7. Data analysis

MLVA and MLST data (i.e., numerical types for TR1 to 3 [representing the number of repeats in a given TR] and MVLST allelic types for all 6 genes) were analyzed using the BioNumerics v.4.0 software (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis using the numerical profiles for each isolate was performed using the categorical coefficient and the UPGMA method. For PFGE, gel

images in TIFF format were processed with BioNumerics software; cluster analysis was performed by UPGMA using Dice coefficient with 1.5% tolerance (Graves and Swaminathan, 2001).

2.8. Discriminatory index (DI)

Discriminatory power, i.e., the ability to distinguish between unrelated strains, was calculated using Simpson's index of diversity (DI, Hunter and Gaston, 1988). As the value approaches 1, the power of the method to discriminate unrelated strains increases. For MLVA, DI was calculated separately for each TR region as well as based on the combined MLVA type, which considered that data for all three TR regions.

3. Results

3.1. VNTR profiles

Among more than 100 potential TRs initially identified in the genome of *L. monocytogenes* F2365, seven were selected for initial analysis using a set of 26 isolates. Subsequently, three repeat regions, which were found to be highly variable and could consistently be amplified in all serotype 4b isolates tested, were selected for analysis in 60 serotype 4b isolates, including the initial 26 isolates (Table 1). All three VNTR regions are located within ORFs, including two ORFs encoding surface anchored cell wall proteins (TR1 and TR2; TIGR annotation 1144 and 1826, respectively), and one ORF encoding a conserved hypothetical protein (TR3; TIGR annotation 0197). DNA sequencing of the PCR products for TR1 to TR3 identified 17, 11, and 4 alleles of different lengths for TR1, TR2, and TR3, respectively (Tables 1 and 2). When analyzed separately, TR1 showed the highest discriminatory power (DI=0.924), followed by TR2 (DI=0.798) and TR3 (DI=0.686). The number of repeats for a given TR varied from 9 to 27 for TR1, 8 to 22 for TR2, and 5 to 9 for TR3 (Table 1). Although the numbers of nucleotides in each repeat unit were stable for each TR (9, 6 and 3 nucleotides in each repeat unit of TR1, 2 and 3, respectively), repeat sequences occasionally showed small variations within a given isolate. For example, in isolate FSL C1-132 most TR1 repeat units had a sequence of CCGGTAGAT, while some repeats represented sequence variants, such as CCGGTAGAC. TR 2 and 3 showed similar sequence variations among repeat sequences within a given isolate. When all three TR types were analyzed together a total of 37 MLVA types could be differentiated among the 60 serotype 4b isolates.

Since a sequencing based approach to characterization of VNTRs (as detailed in the previous paragraph) requires each TR region to be analyzed separately, we also developed and validated a capillary electrophoresis-based analysis approach that allows for all 3 VNTR loci to be characterized simultaneously. With this approach, electropherograms could be obtained within 30 min after completion of PCR amplification. For each isolate, three peaks (corresponding to TR1, 2, and 3) were clearly identifiable. However, the apparent sizes of the obtained fragments differed slightly from the sizes determined by DNA sequencing (i.e., up to 2 nt differences in TR1 and TR2, and 1 nt difference in TR3). These size differences may be due to secondary structure formation in the PCR products (Schouls et al., 2004). However, these discordances are small relative to the number of nucleotides in one repeat unit, and they thus do not affect the ability of capillary gel electrophoresis to accurately determine the repeat numbers in TR1 to TR3. Gel electrophoretic determination of TR repeat numbers was confirmed to be highly reproducible (i.e., each of the three runs yielded the same repeat numbers for all 60 serotype 4b isolates).

Some of the food isolates tested were obtained from the same plants. In three instances, isolates from the same plant had identical MLVA patterns, including (i) isolates Lma5 and Lma7 (plant A), these isolates showed identical MLST and ribotypes, but different PFGE patterns (Table 1); (ii) isolates Lmb15 and Lmb17 (plant B), these isolates showed identical ribotypes, but different PFGE types and MLST patterns (Table 1); and (iii) isolates Lmc1 and Lmc39 (plant F), these isolates showed identical PFGE types and ribotypes but different MLST patterns (Table 1).

3.2. Stability test of MLVA patterns

MLVA on three isolates that were passaged daily for 70 days and tested after each 10 passages showed no variation in repeat numbers for TR1, 2, or 3.

3.3. MLVA of selected L. monocytogenes isolates representing serotypes and lineage other than lineage I serotype 4b

Whereas TR2 and 3 were amplified successfully in6 serotype 1/2a and 6 serotype 1/2b isolates, no PCR product was initially obtained for these isolates for TR1 (Table 3). When primers targeting TR1 that had been reported by Murphy et al. (2007) were used on these 12 isolates, successful amplification of TR1 was obtained though. However, the lengths of the regions flanking TR1 in these isolates were different from the length of the TR1 flanking regions in isolates of serotype 4b, making it difficult to identify the repeat number for the serotype 1/2a and 1/2b isolates based on fragment size analysis. Thus, the TR1 region does not appear to be an appropriate for MLVA of *L. monocytogenes* isolates of different serotypes when fragment sizing is used to identify the number of repeats.

In addition, we also tested two serotype 4b isolates that grouped into *L. monocytogenes* lineage III. These two lineage III isolates showed 14 repeats in TR2 (Table 1), but did not have clear tandem repeat sequences in the TR1 and 3 amplicons, supporting the previous observations that lineage III serotype 4b strains are genetically distinct from lineage I serotype 4b strains and have a number of unique genetic characteristics (Liu et al., 2006; Roberts et al., 2006).

3.4. Ribotyping

The 60 serotype 4b isolates characterized by MLVA represented ribotypes DUP-1038B ($n=20$), DUP-1042B ($n=18$), DUP-1044A ($n=10$), and DUP-1042A ($n=6$) as well as five ribotypes that occurred only once or twice (Table 1).

3.5. PFGE

Single enzyme ApaI PFGE discriminated 27 subtypes among the 60 serotype 4b isolates. Isolates from a given outbreak showed the identical PFGE types, including FSL J1-119 and FSL J1-110 (representing isolates from the 1985 listeriosis outbreak in Los Angeles; Table 1) as well as isolates FSL J1-116 and FSL N3-013 (representing an isolate from the 1988–90 outbreak in the United Kingdom). Both of these isolate pairs also showed identical MLST types (Chen et al., 2007 Table 1). While the two isolates from the outbreak in the United Kingdom showed different MLVA types, the two isolates from the Los Angeles outbreak showed the same MLVA types.

3.6. Discriminatory ability of MLVA, PFGE, MLST, and ribotyping

MLVA, single enzyme PFGE, MLST, and ribotyping discriminated the 60 serotype 4b isolates tested into 37, 27, 25, and 9 different subtypes. Calculation of Simpson index for discrimination also showed that MLVA is more discriminatory (DI=0.980) than PFGE (DI=0.932), MLST (DI=0.874), and ribotyping (DI=0.760). Even when the 26 isolates that were used for initial MLVA analysis set were excluded in DI value calculation (yielding only 34 isolates), the DI value for MLVA (0.957) was still higher than the DI values for PFGE (DI=0.898), MLST (DI=0.843), and ribotyping (DI=0.765). The improved discriminatory power of MLVA is also illustrated by the observation that ten isolates that all showed the same MLST and PFGE

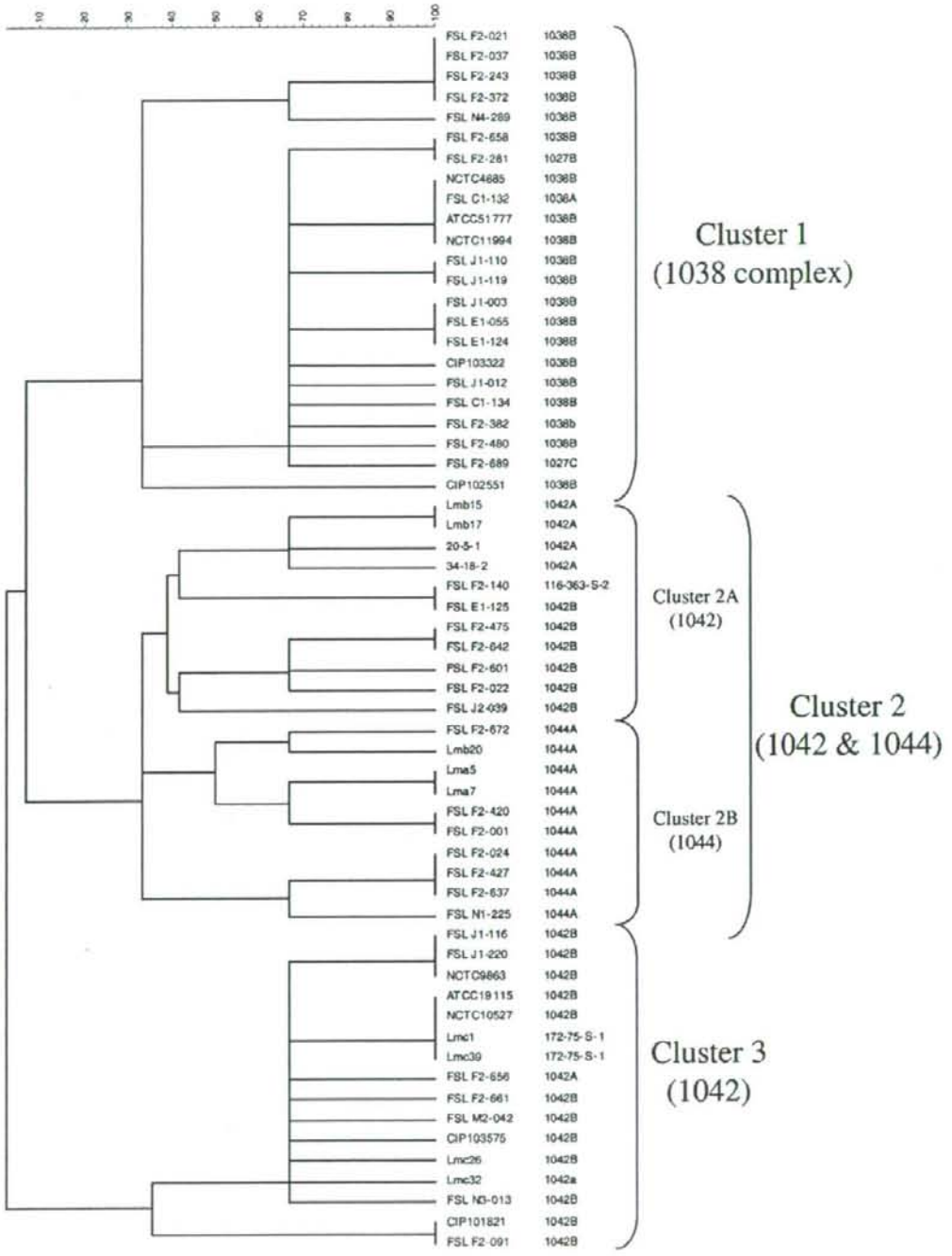

Fig. 1. MLVA-based dendrogram for 60 *L. monocytogenes* serotype 4b isolates. The dendrogram was constructed based on repeat numbers for TR1, 2, and 3 (as determined by DNA sequencing) using the categorical coefficient and the UPGMA method. Similarity percentages are shown above the dendrogram (extremely low similarities occur because some isolates may differ in repeat numbers for all three TRs). A dendrogram constructed based on fragment size obtained by capillary electrophoresis would be identical since the same allelic variants were defined by this method. The tree displayed is a dendrogram based on fragment size data and cannot be construed as a phylogenetic tree.

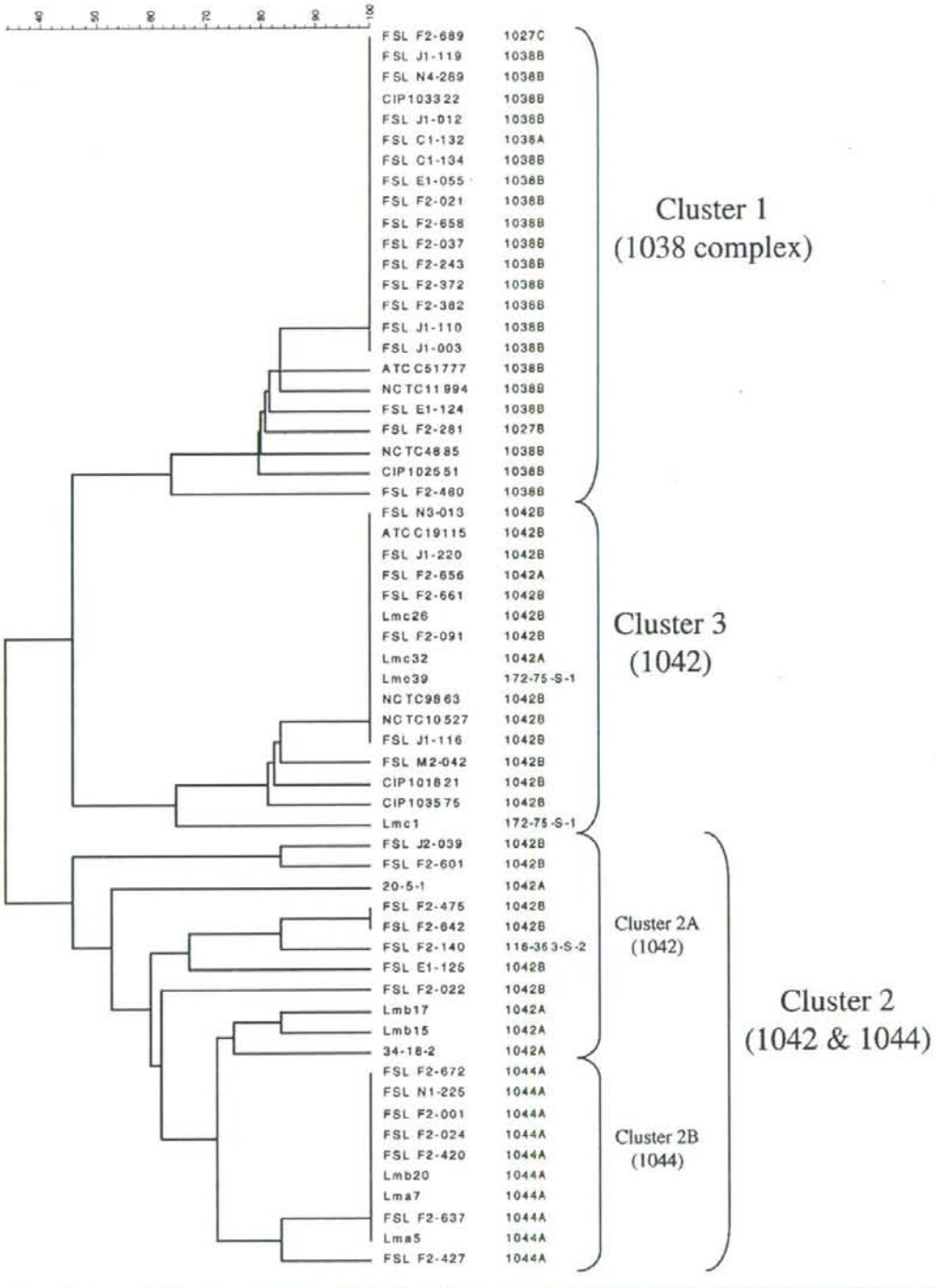

Fig. 2. MLST-based dendrogram for 60 *L. monocytogenes* serotype 4b isolates. The dendrogram was constructed based on allelic types for the 6 genes sequenced using the categorical coefficient and the UPGMA method.

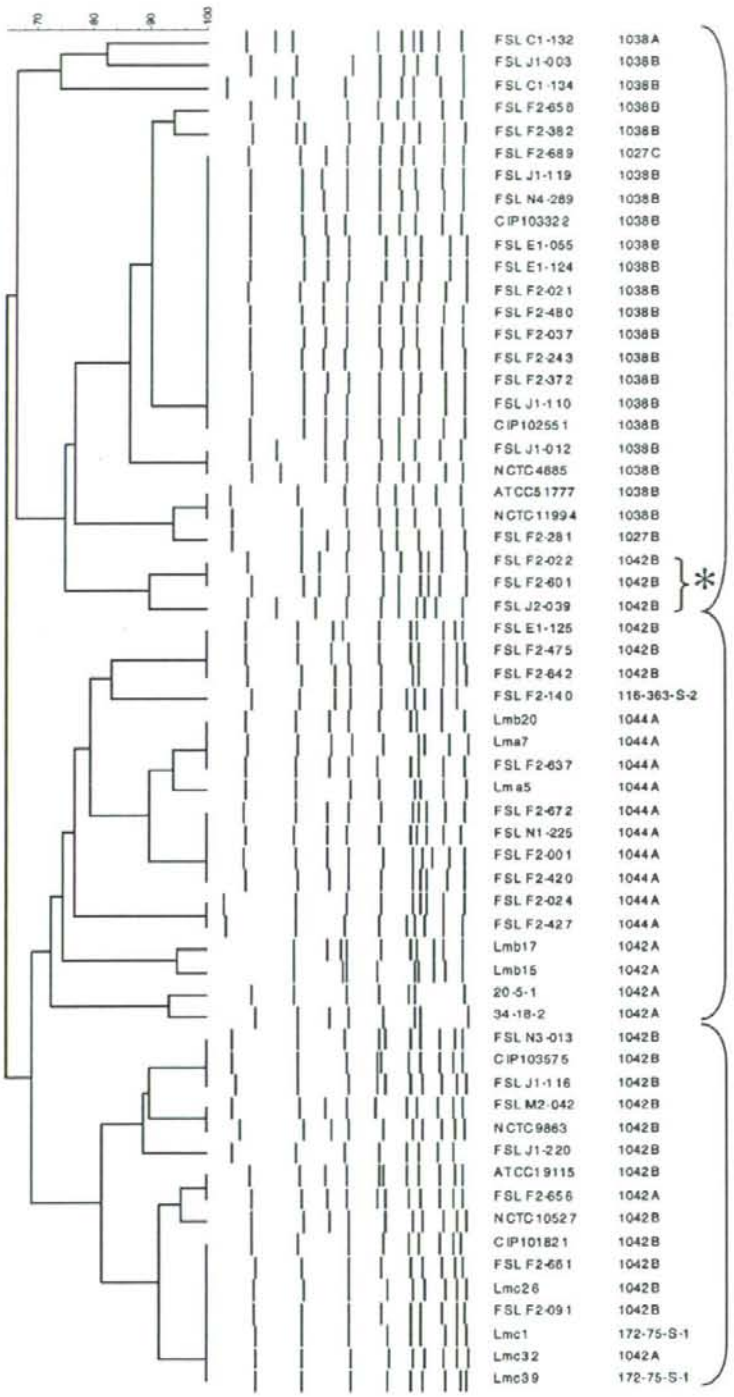

Fig. 3. ApaI PFGE-based dendrogram for 60 *L. monocytogenes* serotype 4b isolates. The dendrogram was constructed using the Dice coefficient and UPGMA method. Band positions were assigned using the BioNumerics software. The three isolates marked by an asterisk represent ribotype DUP-1042B isolates that clustered different based on PFGE types as compared to clustering based on MLVA and MLST types.

types (i.e., MLST type 1 and PFGE type 6; see Table 1), represented 6 different MLVA types; MLVA also differentiated two animal isolates from the human isolates in this group, indicating the ability of this MLVA protocol to differentiate apparently unrelated isolates that group together by PFGE and MLST.

3.7. Cluster analysis of MLVA, MLST, and PFGE patterns

A MLVA dendrogram constructed based on repeat number variation observed by sequence analysis (Fig. 1) yielded three major clusters that correlated well with ribotype as well as epidemic clone assignments of isolates. Cluster 1 included all isolates classified as ribotypes DUP-1038A, DUP-1038B, and DUP-1027. All isolates in this cluster had nine TR3 repeats, while none of the isolates in clusters 2 or 3 had nine TR3 repeats. All 4 ECI isolates included in the isolate set tested here (i.e., FSL J1-003, CIP103322, FSL J1-110 and FSL J1-119) grouped into cluster 1; these isolates represent the strains linked to the human listeriosis outbreaks from Nova Scotia (1981), Switzerland (1983–87), and Los Angeles (1985). Cluster 2 included isolates with DUP-1042B (n=6), DUP-1042A (n=4; all representing one cluster within cluster 2), as well as all the isolates classified as DUP-1044A. One isolate with a unique ribotype (FSL F2-140) also grouped into this cluster. Within cluster 2, isolates with ribotype DUP-1044A clustered separately from the isolates with other ribotypes. Cluster 2 included the ECII isolate FSL N1-225, a human isolate from the multistate human listeriosis outbreak that occurred in the US in 1998/99. All isolate in cluster 2 had five TR3 repeats, presence of 5 TR3 repeats was unique to isolates in this cluster.

Cluster 3 included isolates with ribotypes DUP-1042B (12 isolates), DUP-1042A (2 isolates), and 172-75-S-1 (2 isolates); all isolates in this cluster had 12 TR2 repeats. This cluster included the ECIa isolates FSL J1-116 and FSL N3-013 (isolates from the 1988–90 outbreak in the United Kingdom), FSL J1-220 (an isolate from the 1979 outbreak in Boston) and CIP103575 (an isolate from the 1983 outbreak in Massachusetts).

Cluster analysis based on MLST data yielded a dendrogram that provided very similar clustering to that observed in the MLVA dendrogram. The same three main clusters that were identified in the MLVA dendrogram were also observed in the MLST dendrogram (Fig. 2); grouping of isolates into these three clusters was the same for MLVA and MLST. Cluster analysis based on PFGE types (Fig. 3) also yielded essentially the same three clusters defined by MLVA and MLST. Grouping of isolates to clusters in the PFGE dendrogram was basically identical to the grouping found in MLVA and MLST dendrograms, with the exception of three ribotype DUP-1042 isolates, which were grouped into cluster 1 (which contained only DUP-1038B and DUP-1027 isolates in the MLVA and MLST dendrograms).

4. Discussion

We characterized 60 *L. monocytogenes* serotype 4b isolates, which represent a difficult to discriminate *L. monocytogenes* clonal group that is commonly involved in human listeriosis outbreaks and cases (Farber and Peterkin, 1991; Schuchat et al., 1991) using MLVA as well as MLST, PFGE, and ribotyping. Our data show that the MLVA method described here (i) provides for highly discriminatory subtyping of *L. monocytogenes* serotype 4b strains, (ii) identifies three major groups within the serotype 4b, which are consistent with the groups identified by other subtyping methods, and (iii) provides for rapid and reliable results when repeat variation is detected using a capillary electrophoresis-based approach.

4.1. MLVA provides for highly discriminatory subtyping of L. monocytogenes serotype 4b strains

Consistent with previous studies that have shown MLVA to be useful for typing closely related strains (Coletta-Filho et al., 2001; Keys et al., 2005; Klevytska et al., 2001), we have shown here that MLVA also provides for highly discriminatory subtyping of *L. monocytogenes* serotype 4b isolates. As MLVA targets highly variable genomic regions, concerns exist that MLVA markers may be unstable and may thus rapidly change during passage in the laboratory, natural environments, or infected hosts (van Belkum et al., 1998). This would potentially lead to different MLVA patterns for epidemiologically linked isolates, complicating surveillance and epidemiological investigations. To address this concern with the specific MLVA protocol developed here, lab passage experiments were performed, which showed that the MLVA markers used were stable during 70 passages in rich medium. While the serotype 4b isolate set used here generally represented unrelated isolates, we also included some epidemiologically related isolates in our study to initially evaluate the ability of the MLVA scheme described here to provide epidemiologically relevant subtype data. For example, human and food isolates from the 1985 listeriosis outbreak in Los Angeles showed the same MLVA type. Identical MLVA patterns were also found among food isolates obtained from each of three plants, providing initial support that MLVA can group together epidemiologically related food isolates; the fact that some of these isolates were differentiated by PFGE and/or the MLST protocol used here may even indicate that PFGE and MLST can sometimes detect differences that may not be epidemiologically relevant (i.e., epidemiologically related isolates may be grouped into different subtypes). In this context it is important to emphasize that our MLVA scheme targets ORFs, while PFGE can detect differences in *L. monocytogenes* prophage regions that can be highly variable and unstable, this possibly providing different subtypes for isolates from the same outbreak (Kathariou et al., 2006). Overall, our data indicate that MLVA generally groups epidemiologically related isolates into the same MLVA type, even though we identified one incidence (1983–87 human listeriosis outbreak in the United Kingdom) where epidemiologically linked human and food isolates represented different MLVA types (due to variation in one of the three TRs tested). As these food and human isolates represented an outbreak that occurred 20 years ago and as no information on the number of lab passages isolates were exposed to is available, it is possible that the MLVA (and MLST) differences between these isolates occurred during laboratory passages. Further MLVA studies using a larger set of, ideally low passage, epidemiologically related and unrelated serotype 4b isolates will be necessary to further define the likelihood that MLVA correctly classifies epidemiologically related and unrelated isolates into subtypes. In addition, MLVA of isolates passaged in animals may need to be performed to further assess the stability of the chosen markers, particularly since two of them are in surface molecules, which may be under selective pressure in an host, e.g., if they are targeted by host antibodies or cytotoxic T cells.

4.2. MLVA identifies three major groups within the serotype 4b, which are consistent with the groups identified by other subtyping methods

A considerable numbers of studies have indicated that *L. monocytogenes* strains differ in their virulence characteristics and/or in their associations with human listeriosis cases and outbreaks (Barbour et al., 2001; Franciosa et al., 2005; Jacquet et al., 2004; Olier et al., 2002; Roche et al., 2001). Consequently, a number of subtyping-based studies have classified *L. monocytogenes* into distinct lineages and clonal groups, including specific epidemic clones (Aarts et al., 1999; Brosch et al., 1994; Call et al., 2003; Ducey et al., 2007; Graves et al., 1994; Piffaretti et al., 1989; Rasmussen et al., 1995; Ward et al., 2004; Wiedmann et al., 1997). In general, most subtyping methods have identified two major, common *L. monocytogenes* lineages; one lineage contains the vast majority of serotype 1/2b and 4b isolates (classified as lineage I by some and division II by others [reviewed by Wiedmann, 2002]), while the other lineage contain predominantly serotype 1/2a and 1/2c isolates (Wiedmann, 2002). A third lineage, which is rarely

isolated from foods or human cases, includes serotype 4a, 4c, as well as unusual serotype 4b isolates (Roberts et al., 2006; Ward et al., 2004; Wiedmann, 2002). A number of groups have also identified clonal groups within lineage I (as well as lineage II) that appear to be overrepresented among human listeriosis outbreaks and have thus been classified as epidemic clones. In particular, lineage I, serotype 4b isolates appear to contain three epidemic clones, including ECI, ECIa (Kathariou, 2003), and ECII (Evans et al., 2004). Interestingly, separate cluster analyses based on MLVA, MLST and PFGE data yielded three major clusters, with each cluster, with a few exceptions, containing the same isolates regardless of the typing method used. Importantly, these three clusters appear to represent the three main lineage I serotype 4b epidemic clones, based on clustering of outbreak isolates previously classified to epidemic clone. Clusters 1, 2, and 3 defined here included isolates representing ECI, ECII, and ECIa. Isolates in cluster 1 were predominantly ribotype DUP-1038B, consistent with previous observation that ECI isolates represent this ribotype (Fugett et al., 2006). Isolates in cluster 3 were predominantly ribotype DUP-1042B, also consistent with observations that ECIa isolates represent this ribotype (Fugett et al., 2006). Cluster 2 not only included ribotype DUP-1044A isolates, consistent with observations that ECII isolates represent this ribotype (Fugett et al., 2006), but also included number of DUP-1042B isolates. As ECII, ribotype DUP-1044A isolates represented a separate cluster (2B), within cluster 2, our data suggest that MLVA also provides for rapid classification of serotype 4b isolates into epidemic clones, including improved classification and discrimination over EcoRI ribotyping, particularly as ribotype DUP-1042 appears to represent two distinct *L. monocytogenes* subgroups.

4.3. MLVA provides for rapid and reliable subtyping results for L. monocytogenes serotype 4b strains when repeat variation is detected using a capillary electrophoresis-based approach

While Murphy et al. (2007) previously reported an MLVA protocol for *L. monocytogenes* that included fragment size determination based on agarose-gel electrophoresis, exact determination of amplicon sizes on agarose gels can be difficult, potentially causing problems with MLVA where small size difference can be critical. Our method here, which used a capillary electrophoresis approach for fragment separation, provides for more exact as well as more rapid determination of amplicon sizes (and thus repeat numbers). Our data furthermore showed that the length of flanking regions at the both ends of the TR1 region was different between isolates of serotype 4b and those of serotype 1/2a and 1/2b; this variation may interfere with fragments sizing, suggesting that the TR1 region may not be an appropriate target for an MLVA protocol for subtyping of all *L. monocytogenes* serotypes.

5. Conclusions

The MLVA scheme reported here allows for rapid and sensitive subtype discrimination of *L. monocytogenes* serotype 4b strains, which represents a *L. monocytogenes* subgroup which has been difficult to discriminate with many other subtyping methods (Graves et al., 1994; Mereghetti et al., 2002; O'Donoghue et al., 1995; Ridley, 1995). While the current MLVA scheme reported here seems highly suitable as a secondary typing method for serotype 4b isolates that are difficult to differentiate by other subtyping methods, further validation of the reported MLVA protocol against the standard two-enzyme *L. monocytogenes* PFGE protocol will be necessary. Our study also suggests that further development of *L. monocytogenes* MLVA schemes with the goal of developing a highly discriminatory MLVA method that can differentiate as well as identify all *L. monocytogenes* serotypes is warranted. Further development of *L. monocytogenes* MLVA will provide researchers as well as public health and food microbiology laboratories with a rapid and discriminatory subtyping method, which also provides data that are more easily compared and digitally shared between laboratories than other fragment-based methods such as AFLP, PFGE and RFLP (Noller et al., 2003).

Acknowledgements

This work was partly supported by the Food Safety Commission of Japan (0605), Japanese Ministry of Health, Labour and Welfare (H20-011), the National Food Research Institute of Japan (project: Development of evaluation and management methods for supply of safe, reliable and functional food and farm produce), and Grant-in-Aid for Scientific Research (B 20380121) from the Ministry of Education, Science, Sports and Culture of Japan. This work was also supported in part by USDA Special Research Grant 2004-34459-14296 (to Martin Wiedmann). We thank Esther Fortes for technical support.

References

Aarnisalo, K., Autio, T., Sjöberg, A.-M., Lundén, J., Korkeala, H., Suihko, M.-L., 2003. Typing of *Listeria monocytogenes* isolates originating from the food processing industry with automated ribotyping and pulsed-field gel electrophoresis. Journal of Food Protection 66, 249–255.

Aarts, H.J., Hakemulder, L.E., Van Hoef, A.M.A., 1999. Genomic typing of *Listeria monocytogenes* strains by automated laser fluorescence analysis of amplified fragment length polymorphism fingerprint patterns. International Journal of Food Microbiology 49, 95–102.

Barbour, A.H., Rampling, A., Hormaeche, C.E., 2001. Variation in the infectivity of *Listeria monocytogenes* isolates following intragastric inoculation of mice. Infection and Immunity 69, 4657–4660.

Benson, G., 1999. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Research 27, 573–580.

Borucki, M.K., Kim, S.H., Call, D.R., Smole, S.C., Pagotto, F., 2004. Selective discrimination of *Listeria monocytogenes* epidemic strains by a mixed-genome DNA microarray compared to discrimination by pulsed-field gel electrophoresis, ribotyping, and multilocus sequence typing. Journal of Clinical Microbiology 42, 5270–5276.

Brosch, R., Chen, J., Luchansky, J.B., 1994. Pulsed-field fingerprinting of listeriae: identification of genomic divisions for *Listeria monocytogenes* and their correlation with serovar. Applied and Environmental Microbiology 60, 2584–2592.

Cai, S., Kabuki, D.Y., Kuaye, A.Y., Cargioli, T.G., Chung, M.S., Nielsen, R., Wiedmann, M., 2002. Rational design of DNA sequence-based strategies for subtyping *Listeria monocytogenes*. Journal of Clinical Microbiology 40, 3319–3325.

Call, D.R., Borucki, M.K., Besser, T.E., 2003. Mixed-genome microarrays reveal multiple serotype and lineage-specific differences among strains of *Listeria monocytogenes*. Journal of Clinical Microbiology 41, 632–639.

Chen, Y., Zhang, W., Knabel, S.J., 2005. Multi-virulence-locus sequence typing clarifies epidemiology of recent listeriosis outbreaks in the United States. Journal of Clinical Microbiology 43, 5291–5294.

Chen, Y., Zhang, W., Knabel, S.J., 2007. Multi-virulence-locus sequence typing identifies single nucleotide polymorphisms which differentiate epidemic clones and outbreak strains of *Listeria monocytogenes*. Journal of Clinical Microbiology 45, 835–846.

Coletta-Filho, H.D., Takita, M.A., de Souza, A.A., Aguilar-Vildoso, C.I., Machado, M.A., 2001. Differentiation of strains of *Xylella fastidiosa* by a variable number of tandem repeat analysis. Applied and Environmental Microbiology 67, 4091–4095.

Ducey, T.F., Page, B., Usgaard, T., Borucki, M.K., Pupedis, K., Ward, T.J., 2007. A single-nucleotide-polymorphism-based multilocus genotyping assay for subtyping lineage I isolates of *Listeria monocytogenes*. Applied and Environmental Microbiology 73, 133–147.

Evans, M.R., Swaminathan, B., Graves, L.M., Altermann, E., Klaenhammer, T.R., Fink, R.C., Kernodle, S., Kathariou, S., 2004. Genetic markers unique to *Listeria monocytogenes* serotype 4b differentiate epidemic clone II (hot dog outbreak strains) from other lineages. Applied and Environmental Microbiology 70, 2383–2390.

Farber, J.M., Peterkin, P.I., 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiology and Molecular Biology Reviews 55, 476–511.

Franciosa, G., Maugliani, A., Floridi, F., Aureli, P., 2005. Molecular and experimental virulence of *Listeria monocytogenes* strains isolated from cases with invasive listeriosis and febrile gastroenteritis. FEMS Immunology and Medical Microbiology 43, 431–439.

Fronthingham, R., Meeker-O'Connell, W.A., 1998. Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. Microbiology 144, 1189–1196.

Fugett, E., Fortes, E., Nnoka, C., Wiedmann, M., 2006. International life science institute North America *Listeria monocytogenes* strain collection: development of standard *Listeria monocytogenes* strain sets for research and validation studies. Journal of Food Protection 69, 2929–2938.

Fugett, E.B., Schoonmaker-Bopp, D., Dumas, N.B., Corby, J., Wiedmann, M., 2007. Pulsed field gel electrophoresis (PFGE) analysis of temporally matched *Listeria monocytogenes* isolates from human clinical cases, foods, ruminant farms, and urban and natural environments reveals source associated as well as widely distributed PFGE types. Journal of Clinical Microbiology 45, 865–873.

Graves, L.M., Swaminathan, B., 2001. PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. International Journal of Food Microbiology 65, 55–62.

Graves, L.M., Swaminathan, B., Reeves, M.H., Hunter, S.B., Weaver, R.E., Plikaytis, B.D., Schuchat, A., 1994. Comparison of ribotyping and multilocus enzyme electrophoresis for subtyping of *Listeria monocytogenes* isolates. Journal of Clinical Microbiology 32, 2936–2943.

Gray, M.J., Zadoks, R.N., Fortes, E.D., Dogan, B., Cai, S., Chen, Y., Scott, V.N., Gombas, D.E., Boor, K.J., Wiedmann, M., 2004. *Listeria monocytogenes* isolates from foods and humans form distinct but overlapping populations. Applied and Environmental Microbiology 70, 5833–5841.

Grif, K., Heller, I., Wagner, M., Dierich, M., Würzner, R., 2006. A comparison of *Listeria monocytogenes* serovar 4b isolates of clinical and food origin in Austria by automated ribotyping and pulsed-field gel electrophoresis. Foodborne Pathogens and Disease 3, 138–141.

Handa, S., Kimura, B., Takahashi, H., Koda, T., Hisa, K., Fujii, T., 2005. Incidence of *Listeria monocytogenes* in raw seafood products in Japanese retail stores. Journal of Food Protection 68, 411–415.

Hunter, P.R., Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. Journal of Clinical Microbiology 26, 2465–2466.

Jacquet, C., Doumith, M., Gordon, J.I., Martin, P.M.V., Cossart, P., Lecuit, M., 2004. A molecular marker for evaluating the pathogenic potential of foodborne *Listeria monocytogenes*. Journal of Infectious Diseases 189, 2094–2100.

Jeffers, G.T., Bruce, J.L., McDonough, P.L., Scarlett, J., Boor, K.J., Wiedmann, M., 2001. Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases. Microbiology 147, 1095–1104.

Kathariou, S., 2003. Foodborne outbreaks of listeriosis and epidemic-associated lineages of *Listeria monocytogenes*. In: Torrence, M.E., Isaacson, R.E. (Eds.), Microbial food safety in animal agriculture. Iowa State University Press, Ames, Iowa, pp. 243–256.

Kathariou, S., Graves, L., Buchrieser, C., Glaser, P., Siletzky, R.M., Swaminathan, B., 2006. Involvement of closely related strains of a new clonal group of *Listeria monocytogenes* in the 1998–99 and 2002 multistate outbreaks of foodborne listeriosis in the United States. Foodborne Pathogens and Disease 3, 292–302.

Keim, P., Price, L.B., Klevytska, A.M., Smith, K.L., Schupp, J.M., Okinaka, R., Jackson, P.J., Hugh-Jones, M.E., 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. Journal of Bacteriology 182, 2928–2936.

Keys, C., Kemper, S., Keim, P., 2005. Highly diverse variable number tandem repeat loci in the *E. coli* O157:H7 and O55:H7 genomes for high-resolution molecular typing. Journal of Applied Microbiology 98, 928–940.

Klevytska, A.M., Price, L.B., Schupp, J.M., Worsham, P.L., Wong, J., Keim, P., 2001. Identification and characterization of variable-number tandem repeats in the *Yersinia pestis* genome. Journal of Clinical Microbiology 39, 3179–3185.

Le Flèche, P., Hauck, Y., Onteniente, L., Prieur, A., Denoeud, F., Ramimsse, V., Sylvestre, P., Benson, G., Ramisse, F., Vergnaud, G., 2001. A tandem repeats database for bacterial genomes: application to the genotyping of *Yersinia pestis* and *Bacillus anthracis*. BMC Microbiology. 1, 2.

Lindstedt, B.A., Vardund, T., Kapperud, G., 2004a. Multiple-locus variable-number tandem-repeats analysis of *Escherichia coli* O157 using PCR multiplexing and multicolored capillary electrophoresis. Journal of Microbiological Methods 58, 213–222.

Lindstedt, B.A., Vardund, T., Aas, L., Kapperud, G., 2004b. Multiple-locus variable-number tandem-repeats analysis of *Salmonella enterica* subsp. *enterica* serovar Typhimurium using PCR multiplexing and multicolor capillary electrophoresis. Journal of Microbiological Methods 59, 163–172.

Liu, D., Lawrence, M.L., Gorski, L., Mandrell, R.E., Ainsworth, A.J., Austin, F.W., 2006. *Listeria monocytogenes* serotype 4b strains belonging to lineages I and III possess distinct molecular features. Journal of Clinical Microbiology 44, 214–217.

Maiden, M.C.J., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M., Spratt, B.G., 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proceedings of the National Academy of Sciences of the United States of America 95, 3140–3145.

Mereghetti, L., Lanotte, P., Savoye-Marczuk, V., Marquet-Van Der Mee, N., Audurier, A., Quentin, R., 2002. Combined ribotyping and random multiprimer DNA analysis to probe the population structure of *Listeria monocytogenes*. Applied Environmental Microbiology 68, 2849–2857.

Murphy, M., Corcoran, D., Buckley, J.F., O'Mahony, M., Whyte, P., Fanning, S., 2007. Development and application of multiple-locus variable number of tandem repeat analysis (MLVA) to subtype a collection of *Listeria monocytogenes*. International Journal of Food Microbiology 187–194.

Nelson, K.E., Fouts, D.E., Mongodin, E.F., Ravel, J., DeBoy, R.T., Kolonay, J.F., Rasko, D.A., Angiuoli, S.V., Gill, S.R., Paulsen, I.T., Peterson, J., White, O., Nelson, W.C., Nierman, W., Beanan, M.J., Brinkac, L.M., Daugherty, S.C., Dodson, R.J., Durkin, A.S., Madupu, R., Haft, D.H., Selengut, J., Aken, S.V., Khouri, H., Fedorova, N., Forberger, H., Tran, B., Kathariou, S., Wonderling, L.D., Uhlich, G.A., Bayles, D.O., Luchansky, J.B., Fraser, C.M., 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. Nucleic Acids Research 32, 2386–2395.

Noller, A.C., McEllistrem, M.C., Pacheco, A.G.F., Boxrud, D.J., Harrison, L.H., 2003. Multilocus variable-number tandem repeat analysis distinguishes outbreak and sporadic *Escherichia coli* O157:H7 isolates. Journal of Clinical Microbiology 41, 5389–5397.

O'Donoghue, K., Bowker, K., McLauchlin, J., Reeves, D.S., Bennett, P.M., MacGowan, A.P., 1995. Typing of *Listeria monocytogenes* by random amplified polymophic DNA (RAPD) analysis. International Journal of Food Microbiology 27, 245–252.

Olier, M., Pierre, F., Lemaitre, J.P., Divies, C., Rousset, A., Guzzo, J., 2002. Assessment of the pathogenic potential of two *Listeria monocytogenes* human faecal carriage isolates. Microbiology 148, 1855–1862.

Peterson, J.D., Umayam, L.A., Dickinson, T., Hickey, E.K., White, O., 2001. The comprehensive microbial resource. Nucleic Acids Research 29, 123–125.

Piffaretti, J.C., Kressebuch, H., Aeschbacher, M., Bille, J., Bannerman, E., Musser, J.M., Selander, R.K., Rocourt, J., 1989. Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. Proceedings of the National Academy of Sciences of the United States of America 86, 3818–3822.

Ramisse, V., Houssu, P., Hernandez, E., Denoeud, F., Hilaire, V., Lisanti, O., Ramisse, F., Cavallo, J.D., Vergnaud, G., 2004. Variable number of tandem repeats in *Salmonella enterica* subsp. *enterica* for typing purposes. Journal of Clinical Microbiology 42, 5722–5730.

Rasmussen, O.F., Skouboe, P., Dons, L., Rossen, L., Olsen, J.E., 1995. *Listeria monocytogenes* exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes. Microbiology 141, 2053–2061.

Revazishvili, T., Kotetishvili, M., Stine, O.C., Kreger, A.S., Morris Jr., J.G., Sulakvelidze, A., 2004. Comparative analysis of multilocus sequence typing and pulsed-field gel electrophoresis for characterizing *Listeria monocytogenes* strains isolated from environmental and clinical sources. Journal of Clinical Microbiology 42, 276–285.

Ridley, A.M., 1995. Evaluation of a restriction fragment length polymorphism typing method for *Listeria monocytogenes*. Research in Microbiology 146, 21–34.

Roberts, A., Nightingale, K., Jeffers, G., Fortes, E., Kongo, J.M., Wiedmann, M., 2006. Genetic and phenotypic characterization of *Listeria monocytogenes* lineage III. Microbiology 152, 685–693.

Roche, S.M., Velge, P., Bottreau, E., Durier, C., Marquet-van der Mee, N., Pardon, P., 2001. Assessment of the virulence of *Listeria monocytogenes*: agreement between a plaque-forming assay with HT-29 cells and infection of immunocompetent mice. International Journal of Food Microbiology 68, 33–44.

Ryu, C., Lee, K., Hawng, H.J., Yoo, C.K., Seong, W.K., Oh, H.B., 2005. Molecular characterization of Korean *Bacillus anthracis* isolates by amplified fragment length polymorphism analysis and multilocus variable-number tandem repeat analysis. Applied and Environmental Microbiology 71, 4664–4671.

Salcedo, C., Arreaza, L., Alcalá, B., de la Fuente, L., Vázquez, J.A., 2003. Development of a multilocus sequence typing method for analysis of *Listeria monocytogenes* clones. Journal of Clinical Microbiology 41, 757–762.

Schlötterer, C., Tautz, D., 1992. Slippage synthesis of simple sequence DNA. Nucleic Acids Research 20, 211–215.

Schouls, L.M., van der Heide, H.G.J., Vauterin, L., Vauterin, P., Mooi, F.R., 2004. Multiple-locus variable-number tandem repeat analysis of Dutch *Bordetella pertussis* strains reveals rapid genetic changes with clonal expansion during the late 1990s. Journal of Clinical Microbiology 186, 5496–5505.

Schuchat, A., Swaminathan, B., Broome, C.V., 1991. Epidemiology of human listeriosis. Clinical Microbiology Reviews 4, 169–183.

Takahashi, H., Kimura, B., Yoshikawa, M., Fujii, T., 2003. Cloning and sequencing of the histidine decarboxylase genes of gram-negative, histamine-producing bacteria and their application in detection and identification of these organisms in fish. Applied and Environmental Microbiology 69, 2568–2579.

van Belkum, A., Scherer, S., van Leeuwen, W., Willemse, D., van Alphen, L., Verbrugh, H., 1997. Variable number of tandem repeats in clinical strains of *Haemophilus influenzae*. Infection and Immunity 65, 5017–5027.

van Belkum, A., Scherer, S., van Alphen, L., Verbrugh, H., 1998. Short-sequence DNA repeats in prokaryotic genomes. Microbiology and Molecular Biology Reviews 62, 275–293.

Ward, T.J., Gorski, L., Borucki, M.K., Mandrell, R.E., Hutchins, J., Pupedis, K., 2004. Intraspecific phylogeny and lineage group identification based on the *prfA* virulence gene cluster of *Listeria monocytogenes*. Journal of Bacteriology 186, 4994–5002.

Wiedmann, M., 2002. Molecular subtyping methods for *Listeria monocytogenes*. Journal of Association of Official Analytical Chemists International 85, 524–531.

Wiedmann, M., Bruce, J.L., Keating, C., Johnson, A.E., McDonough, P.L., Batt, C.A., 1997. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. Infection and Immunity 65, 2707–2716.

Yazdankhah, S.P., Lindstedt, B.A., Caugant, D.A., 2005. Use of variable-number tandem repeats to examine genetic diversity of *Neisseria meningitides*. Journal of Clinical Microbiology 43, 1699–1705.

Zhang, W., Jayarao, B.M., Knabel, S.J., 2004. Multi-virulence-locus sequence typing of *Listeria monocytogenes*. Applied and Environmental Microbiology 70, 913–920.

Food Sci. Technol. Res., 14 (6), 557 – 564, 2008

Subtyping of *Listeria monocytogenes* Based on Nucleotide Polymorphism in the *clpC*, *inlA*, *hlyA*, and *plcA* Genes and Rapid Identification of *L. monocytogenes* Genetically Similar to Clinical Isolates

Ken-ichi Honjoh[1], Kumiko Fujihara[1], Takahiro Haraguchi[1], Yukari Ono[1], Hiroshi Kobayashi[1], Hiroshi Hiwaki[2], Hideaki Kamikado[3], Sung Sik Jang[4], Sangryeol Ryu[4] and Takahisa Miyamoto[1]*

[1] *Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, 6-10-1, Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan*

[2] *Fukuoka City Institute for Hygiene and the Environment, 2-1-3, Jigyohama, Chuo-ku, Fukuoka 810-0065, Japan*

[3] *Research and Development Center, Meiji Dairies Corporation, 540, Naruda, Odawara, Kanagawa 250-0862, Japan*

[4] *Department of Food and Animal Biotechnology, School of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Korea*

Received April 23, 2008; Accepted July 8, 2008

To develop a new method for identification of *Listeria monocytogenes* genetically similar to clinical isolates, single-nucleotide polymorphism (SNP) typing and multi-locus sequence typing (MLST) of 126 isolates of *L. monocytogenes* from clinical and environmental samples were performed based on sequence analysis of parts of four genes (*hlyA*, *clpC*, *inlA*, and *plcA*). Based on the sequences of the isolates in this study, SNP typing showed that *hlyA*, *clpC*, *inlA*, and *plcA* genes were categorized into 9, 14, 17, and 21 types, respectively. MLST showed that the isolates were grouped into 35 types including 12 types of clinical isolates. Out of those, four MLST types were found in food or environmental and clinical isolates. In particular, all clinical isolates with serotype 1/2a were grouped into the same *hlyA* SNP A5 type. A method using real-time PCR combined with Cycling Probe Technology was developed for rapid identification of SNP type of *L. monocytogenes* genetically similar to the clinical isolates. By using this method, the 1/2a clinical isolates showing MLST-2 were successfully identified with a specific primer set and a cycling probe designed on the basis of sequence of *hlyA*. Furthermore, clinical isolates of serotype 4b showing MLST-4 or -35 were successfully identified by a method using cycling probes based on sequences of *clpC* and *inlA*.

Keywords: cycling probe technology, *Listeria monocytogenes*, real-time PCR, SNP typing

Introduction

Listeria species are widely distributed in the environment. As they are found in soil and in mammals, they are often contaminants in various types of food, mainly meats and dairy products (Vázquez-Boland *et al.*, 2001). *Listeria monocytogenes* is a significant food-borne pathogen and causes an infectious disease known as listeriosis. In the food industry, contamination of food by this bacterium may lead to serious problems since it can grow even at low temperatures and high salt concentrations during storage of ready-to-eat foods such as unsterilized dairy products and raw vegetables.

In Japan, although sporadic cases of listeriosis have been reported, no serious epidemic has occurred. However, *L. monocytogenes* is often detected in foodstuffs, which may lead to a potential outbreak of listeriosis (Okutani *et al.*, 2004). Listeriosis may result in mortality for pregnant women, infants, immunocompromised individuals, and elderly individuals (Vázquez-Boland *et al.*, 2001).

For identification and subtyping of *L. monocytogenes*, several techniques, such as phenotypic typing (serological

*To whom correspondence should be addressed.
Email: tmiyamot@agr.kyushu-u.ac.jp

typing, phage typing, and multilocus enzyme electrophoresis) and molecular typing techniques (ribotyping, restriction enzyme analysis, PCR-based typing, and DNA sequencing) have been used in epidemiology (Gasanov *et al.*, 2005). Furthermore, multilocus sequence typing (MLST) based on DNA sequences has extremely high and accurate discriminatory power (Gasanov *et al.*, 2005). MLST has been applied to subtyping of *L. monocytogenes* (Cai *et al.*, 2002; Salcedo *et al.*, 2003). However, because it includes sequence analysis, it is time-consuming. For identification of *Campylobacter jejuni*, real-time PCR using Taqman technology has been applied to single nucleotide polymorphisms (SNP) typing based on MLST (Best *et al.*, 2005). A rapid and reliable method is desired for SNP typing of *L. monocytogenes*.

Four genes (*hlyA*, *clpC*, *inlA*, and *plcA*), whose products are involved in adhesion and infection of cells, were used as targets for identification and subtyping of pathogenic isolates of *L. monocytogenes* in the present paper. The *hlyA* gene encodes listeriolysin O, which is an essential virulence factor (Mengaud *et al.*, 1988). The *clpC* gene encodes *ClpC* ATPase, which is involved in stress tolerance and survival of *L. monocytogenes* (Rouquette *et al.*, 1996) and in adhesion and invasion of host cells (Nair *et al.*, 2000). The *inlA* gene encodes internalin, which is involved in the entry of the bacteria into cells (Gaillard *et al.*, 1991). The *plcA* gene encodes phosphatidyl-inositol-specific phospholipase C, which is involved in escaping from a vacuole after invasion of host cells (Alberti-Segui *et al.*, 2007).

In this study, we determined the nucleotide sequences of parts of the *hlyA*, *clpC*, *inlA*, and *plcA* genes of 126 isolates from food, environmental, and clinical sources. By multiple alignment analysis of the DNA sequence of the respective genes, DNA polymorphisms were found, and SNP typing and MLST were performed. A novel, rapid SNP typing method was developed by using real-time PCR combined with Cycling Probe Technology (Bekkaoui *et al.*, 1996) to identify *L. monocytogenes* of the same genotype as the clinical isolates.

Materials and methods

Bacterial strains and medium A total of 126 isolates of *L. monocytogenes* from food or environmental samples ($n=111$) and clinical samples ($n=15$) were used. The serotypes of the isolates are listed in Table 1. Isolation and identification of *L. monocytogenes* were carried out according to the standard method described by the Pharmaceutical Society of Japan (2000). Isolates were serotyped with a commercially available serotyping kit (Denka Seiken Co., Tokyo, Japan) according to the manufacturer's instructions. Tryptic soy broth (TSB: Difco Co., Ltd., Detroit, MI, USA) was used for enrichment of the bacterium.

Preparation of genomic DNA Boiling method or DNeasy Tissue kit (Qiagen, Tokyo, Japan) was used for preparation of genomic DNA from *L. monocytogenes*. A 5-ml culture containing TSB was incubated at 37°C overnight. The cells were collected from 1 ml of the culture by centrifugation at 8,000 × g for 5 min. The pellet was suspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), boiled for 10 min, and centrifuged at 8,000 × g for 5 min. The supernatant was used as genomic DNA solution. The genomic DNA was also extracted by using the DNeasy Tissue kit with 1 ml of the bacteria culture according to the manufacturer's instruction.

Primer design for amplification of parts of the target genes To amplify parts of the *hlyA*, *clpC*, *inlA*, and *plcA* genes, oligonucleotides were designed based on the conserved regions of the respective genes, which were identified by comparing nucleotide sequences of the genes between *L. monocytogenes* EGD-e (serotype 1/2a; Glaser *et al.*, 2001) and F2365 (serotype 4b; Nelson *et al.*, 2004) (Table 2).

PCR PCR was performed with a 30-µl mixture containing 1 × Ex Taq buffer, 200 µM of each deoxynucleotide triphosphate, 20 pmol of each of the primers, 150 ng of template DNA, and 0.75 U of Takara ExTaq DNA polymerase (Takara Bio) using thermal cyclers (model Dice; Takara Bio, Inc., Kyoto, Japan, or model PCR Express; Hybaid, Ashford, UK). The reactions were incubated at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, and elongation at 72°C for 1 min.

PCR products were analyzed by agarose gel electrophoresis. After visually confirming that the products showed a single band, the products were purified with QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions and used for DNA sequencing.

DNA sequencing and sequence analysis Nucleotide sequences of the PCR products were determined using the Big Dye-Terminator Cycle Sequencing kit and an Applied Biosystems 3730 sequencer (Perkin-Elmer, Wallesley, MA, USA). DNA sequences were analyzed in both directions and were submitted to DDBJ database (http://www.ddbj.nig.ac.jp/Welcome-e.html).

Multiple sequence alignments of the *hlyA*, *clpC*, *inlA*, and *plcA* genes were constructed with the Clustal W program (Thompson *et al.*, 1994). Results of multiple sequence alignments were submitted to the EMBL-Align database (ftp://ftp.ebi.ac.uk/pub/databases/embl/align/).

Cycling probe assay for SNP typing Identification of isolates showing the same genotype as the clinical isolates of *L. monocytogenes* was performed by using real-time PCR and Cycling Probe Technology (Bekkaoui *et al.*, 1996) for

SNP typing. Primers and cycling probes were designed to detect SNP in the *hlyA*, *clpC*, and *inlA* genes (Table 3). All reagents for real-time PCR were from Takara Cycleave PCR Core kit (Takara Bio), which is optimized for real-time PCR. The amplification of respective parts of the *hlyA*, *clpC*, and *inlA* genes was carried out in a total volume of 10 µl. The reaction mixture contained 1 × Cycleave PCR buffer, 5 mM Mg^{2+}, 0.3 mM of each dNTP, 0.2 µM of each primer, 0.2 µM of probe, 1.2 ng of template DNA, 40 U of Tli Rnase HII, and 0.5 U of Takara Ex Taq HS. Amplification and fluorescence detection were performed using RealTime PCR system (model: Mx3000P, Stratagene, La Jolla, CA, USA). Cycle conditions were as follows: holding at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, primer

Table 1. *Listeria monocytogenes* isolates and their genetic properties used in this study.

Number	Culture collection	Origin	Serotype	SNP type[d]				MLST type
				hlyA	*clpC*	*inlA*	*plcA*	
1	LM1	chicken meat	1/2a	A6	B9	C3	D13	1
2	LM3	minced chicken	1/2a	A5	B2	C6	D16	2
3	LM4	chicken giblet	4b	A3	B10	C14	D8	3
4	LM7	minced chicken	1/2a	A5	B2	C6	D16	2
5	LM8	minced chicken	1/2a	A5	B2	C6	D16	2
6	LM9	chicken giblet	4b	A3	B13	C14	D8	4
7	LM10	chicken giblet	4b	A3	B13	C14	D8	4
8	LM11	chicken meat	1/2a	A6	B9	C3	D13	1
9	LM12	minced chicken	1/2a	A5	B2	C6	D16	2
10	LM13	minced chicken	1/2a	A5	B2	C6	D18	5
11	LM14	chicken meat	1/2a	A5	B2	C6	D17	6
12	LM15	chicken meat	1/2a	A5	B2	C6	D16	2
13	LM16	minced pork	1/2c	A8	B3	C1	D19	7
14	LM17	minced chicken	4b	A3	B11	C14	D8	8
15	LM18	minced chicken	4b	A3	B13	C14	D8	4
16	FCIHE LIS1[a]	Tokyo University VTU206 strains	4b	A3	B13	C14	D8	4
17	FCIHE LIS2	Strain from University of Occupational and Environmental Health	1/2a	A6	B3	C2	D19	9
18	FCIHE LIS3	horse meat	1/2b	A4	B12	C12	D8	10
19	FCIHE LIS7	chicken meat	1/2b	A1	B13	C10	D9	11
20	FCIHE LIS9	imported cheese	4b	A3	B14	C14	D3	12
21	FCIHE LIS10	karashimentaiko	1/2a	A9	B1	C8	D14	13
22	FCIHE LIS16	karashimentaiko	1/2b	A1	B13	C10	D9	11
23	FCIHE LIS19	karashimentaiko	3a	A5	B2	C6	D16	2
24	FCIHE LIS21	karashimentaiko	4c	A1	B13	C14	D1	14
25	FCIHE LIS28	karashimentaiko	3c	A5	B2	C6	D16	2
26	SNU1[b]	chicken meat	1/2b	A1	B13	C10	D9	11
27	SNU2	chicken meat	1/2b	A1	B13	C10	D9	11
28	SNU3	chicken meat	4b	A3	B13	C14	D8	4
29	SNU4	chicken meat	1/2b	A1	B13	C10	D9	11
30	SNU5	chicken meat	1/2b	A1	B13	C10	D9	11
31	SNU6	chicken meat	1/2b	A1	B13	C11	D9	15
32	SNU7	chicken meat	1/2c	A6	B3	C2	D19	9
33	SNU8	chicken meat	1/2a	A6	B3	C2	D19	9
34	SNU9	chicken meat	1/2a	A6	B4	C2	D19	16
35	SNU10	chicken meat	1/2a	A9	B1	C8	D14	13
36	SNU11	chicken meat	1/2a	A5	B2	C6	D16	2
37	SNU12	chicken meat	1/2a	A6	B8	C8	D20	17
38	SNU13	chicken meat	1/2a	A6	B8	C8	D20	17
39	SNU14	chicken meat	1/2a	A9	B1	C8	D14	13
42	SNU17	chicken meat	1/2b	A4	B12	C12	D8	10
44	SNU19	chicken meat	3b	A4	B13	C12	D2	18
45	MDC 144[c]	milk product	4b	A3	B14	C14	D3	12
46	MDC 145	milk product	4b	A3	B14	C14	D3	12
47	MDC 149	milk product environment	1/2b	A4	B12	C12	D8	10
48	MDC 150	milk product environment	3b	A4	B12	C12	D5	19
49	MDC 151	milk product environment	1/2b	A4	B12	C12	D8	10
50	MDC 152	milk product environment	1/2b	A4	B12	C12	D8	10
51	MDC 153	milk product	4b	A3	B14	C14	D3	12
52	MDC 154	milk product	4b	A3	B14	C14	D3	12
53	MDC 157	milk product	4b	A3	B14	C14	D3	12
54	MDC 159	processing milk product	4b	A3	B14	C14	D3	12
55	MDC 160	milk product material	4b	A3	B14	C14	D3	12
56	MDC 161	milk product	4b	A3	B14	C14	D3	12
57	MDC 164	milk product material	4b	A3	B14	C14	D3	12
58	MDC 165	milk product material	4b	A3	B14	C14	D3	12
59	MDC 167	milk product	4b	A3	B14	C14	D3	12
60	MDC 170	milk product	4b	A3	B14	C14	D3	12
61	MDC 171	milk product environment	4b	A3	B14	C14	D3	12
62	MDC 172	milk product environment	4c	A4	B14	C14	D3	20
63	MDC 173	milk product	3b	A3	B14	C14	D3	12
64	MDC 174	milk product material	4b	A3	B14	C14	D3	12
65	MDC 177	raw milk	1/2a	A7	B8	C4	D15	21
66	MDC 178	raw milk	1/2a	A7	B8	C4	D15	21

Table 1. *Listeria monocytogenes* isolates and their genetic properties used in this study (cont.).

Number	Culture collection	Origin	Serotype	SNP type				MLST type
				hlyA	*clpC*	*inlA*	*plcA*	
67	MDC 179	raw milk	3b	A3	B14	C14	D3	12
68	MDC 184	milk product	1/2b	A4	B12	C12	D8	10
69	MDC 185	milk product	1/2b	A4	B12	C12	D8	10
70	MDC 186	milk product	1/2b	A4	B12	C12	D8	10
71	MDC 187	milk product	1/2b	A4	B12	C12	D8	10
73	MDC 189	milk product	1/2b	A4	B12	C12	D8	10
74	MDC 190	milk product	1/2b	A3	B12	C12	D8	22
75	MDC 191	milk product	1/2b	A4	B12	C12	D8	10
76	MDC 192	milk product	1/2b	A4	B12	C12	D8	10
77	MDC 193	milk product	1/2b	A4	B12	C12	D8	10
78	MDC 194	processing milk product	1/2b	A4	B12	C12	D8	10
79	MDC 195	milk product	1/2b	A4	B12	C12	D8	10
80	MDC 196	milk product	1/2b	A4	B12	C12	D8	10
81	MDC 197	milk product	1/2b	A4	B12	C12	D8	10
82	MDC 198	milk product	1/2b	A4	B12	C12	D8	10
83	MDC 199	processing milk product	1/2b	A4	B12	C12	D8	10
84	MDC 201	processing milk product	1/2b	A4	B12	C12	D8	10
85	MDC 202	milk product	1/2b	A4	B12	C12	D8	10
86	MDC 203	milk product	1/2b	A4	B12	C12	D8	10
87	MDC 205	milk product	1/2b	A4	B12	C12	D8	10
88	MDC 206	processing milk product	1/2b	A4	B12	C12	D8	10
89	MDC 208	processing milk product	1/2b	A4	B12	C12	D8	10
90	MDC 209	milk product environment	1/2b	A4	B12	C12	D8	10
91	MDC 210	milk product environment	1/2b	A4	B12	C12	D8	10
92	MDC 211	milk product environment	1/2b	A4	B12	C12	D8	10
93	MDC 212	milk product	1/2b	A4	B12	C12	D8	10
94	MDC 213	milk product	1/2b	A4	B12	C12	D8	10
95	MDC 214	milk product environment	1/2b	A4	B12	C12	D10	23
96	MDC 215	milk product environment	1/2b	A4	B12	C12	D8	10
97	MDC 216	milk product	1/2b	A4	B12	C12	D8	10
98	MDC 217	milk product	1/2b	A4	B12	C12	D8	10
99	MDC 218	milk product	1/2b	A4	B12	C12	D8	10
100	MDC 219	milk product	1/2b	A4	B12	C12	D8	10
101	FCIHE LIS11	karashimentaiko	1/2a	A9	B1	C8	D14	13
102	FCIHE LIS12	karashimentaiko	1/2b	A1	B13	C10	D9	11
103	FCIHE LIS13	karashimentaiko	1/2b	A1	B13	C10	D9	11
104	FCIHE LIS14	karashimentaiko	1/2a	A9	B1	C8	D14	13
105	FCIHE LIS15	karashimentaiko	1/2b	A1	B13	C10	D9	11
106	FCIHE LIS17	karashimentaiko	1/2a	A9	B1	C8	D14	13
107	FCIHE LIS18	karashimentaiko	1/2b	A2	B13	C10	D9	24
108	FCIHE LIS20	karashimentaiko	UT	A5	B2	C6	D16	2
109	FCIHE LIS22	karashimentaiko	UT	A5	B2	C6	D16	2
110	FCIHE LIS23	karashimentaiko	UT	A7	B3	C5	D21	25
111	FCIHE LIS24	karashimentaiko	UT	A7	B3	C5	D21	25
112	FCIHE LIS25	karashimentaiko	1/2a	A9	B1	C8	D14	13
113	FCIHE LIS26	karashimentaiko	UT	A7	B3	C6	D21	26
114	FCIHE LIS27	karashimentaiko	UT	A9	B12	C12	D6	27
115	FCIHE LIS29	karashimentaiko	3a	A7	B3	C5	D21	25
117	FCIHE CLf-1	abortion (mother)	1/2a	A5	B2	C6	D16	2
118	FCIHE CLf-2	abortion (child)	1/2a	A5	B2	C6	D16	2
119	FCIHE CLm-1	Japanese patient	1/2a	A5	B7	C9	D16	28
120	FCIHE CLm-2	Japanese patient	1/2a	A5	B6	C7	D16	29
121	FCIHE CLm-3	Japanese patient	1/2a	A5	B5	C6	D16	30
122	FCIHE CLm-4	Japanese patient	1/2b	A3	B13	C17	D11	31
123	FCIHE CLm-5	Japanese patient	1/2b	A4	B12	C12	D8	10
124	FCIHE CLm-6	Japanese patient	1/2b	A3	B13	C12	D4	32
125	FCIHE CLm-7	Japanese patient	4b	A3	B13	C14	D8	4
126	FCIHE CLm-8	Japanese patient	4b	A3	B13	C16	D3	33
127	FCIHE CLm-9	Japanese patient	4b	A4	B12	C13	D7	34
128	FCIHE CLm-10	Japanese patient	4b	A3	B13	C15	D12	35
129	FCIHE CLm-11	Japanese patient	4b	A3	B13	C14	D8	4
130	FCIHE CLm-12	Japanese patient	4b	A3	B14	C14	D3	12
131	FCIHE CLm-13	Japanese patient	4b	A3	B13	C14	D8	4

[a]FCIHE: Fukuoka City Institute for Hygiene and the Environment, Fukuoka, Japan.

[b]SNU: Seoul University, Seoul, Korea.

[c]MDC: Meiji Dairies Corporation, Tokyo, Japan.

[d]Boxes with thick lines, dashed lines, thin lines, and dotted lines indicate SNP type detectable with *hlyA probe*, *clpC-M1 probe*, *clpC-M2 probe* and *inlA probe*, respectively.

annealing at 50°C for 15 sec, and elongation at 72°C for 20 sec.

Results

SNP typing and MLST of isolates based on nucleotide sequence determination of four genes To determine genetically common characteristics between clinical isolates and foodborne or environmental isolates of *L. monocytogenes*, parts of the *hlyA*, *clpC*, *inlA*, and *plcA* genes of 126 isolates (Table 1) were amplified by PCR. For amplification of the target genes, primers were designed based on the conserved regions in each gene for two strains (EGD-e and F2365) of *L. mono-*

cytogenes. The amplified fragments of the *hlyA*, *clpC*, *inlA*, and *plcA* genes were directly sequenced. The determined sequence regions of the respective genes were used for further analysis (Table 2). The region sizes of *hlyA*, *clpC*, *inlA*, and *plcA* were 402, 636, 608, and 713 bp, respectively. Multiple sequence alignment analysis showed that 20, 60, 48, and 45 SNP sites were found in the sequenced regions of *hlyA*, *clpC*, *inlA*, and *plcA*, respectively, among the 126 isolates (data not shown). Furthermore, the 126 isolates were categorized into 9 types (designated from Nos. A1 to A9), 14 (from Nos. B1 to B14), 17 (from Nos. C1 to C17), and 21 (from Nos. D1 to D21) based on the sequences of PCR products of *hlyA*, *clpC*, *inlA*, and *plcA*, respectively (Table 1). The sequences of the respective loci of the *hlyA*, *clpC*, *inlA*, and *plcA* genes have been deposited in DDBJ under accession numbers AB327187 through AB327195 (*hlyA*), AB327196 through AB327209 (*clpC*), AB327210 through AB327226 (*inlA*), and AB327227 through AB327247 (*plCA*). Data of multiple sequence alignments have been deposited in EMBL-Align database under accession numbers ALIGN_001157 (*hlyA*), ALIGN_001158 (*clpC*), ALIGN_001159 (*inlA*), and ALIGN_001160 (*plcA*).

Based on the SNP types of the four genes sequenced here, MLST was also carried out for categorization of the isolates. As shown in Table 1, the 126 isolates were categorized into 35 MLST types. The fifteen clinical isolates (Nos. 117-131) were categorized into 12 MLST types. Out of those, four types (MLST-2, -4, -10, and -12) were found in food or environmental isolates.

Probe design for real-time PCR using Cycling Probe Technology For the purpose of developing a rapid method for identification of *L. monocytogenes* genetically similar to clinical isolates of serotype 1/2a and 4b based on SNP in the four genes, a method using real-time PCR combined with Cycling Probe Technology was investigated. We first identified SNP sites specific to clinical isolates and then designed chimeric probes (DNA-RNA-DNA) to detect the specific SNP site, which was replaced by RNA in the probe. To identify *L. monocytogenes* genetically similar to clinical 1/2a isolates (MLST-2, -28, -29, and -30 in Table 1), *hlyA* was selected for real-time PCR because all clinical isolates showed the same nucleotide sequence of the gene and were categorized into SNP type No. A5. A probe (5'(FAM)-TTgGCTCATTTC

Table 2. Oligonucleotide sequences used for PCR.

Gene	Nucleotide sequence	Region and direction of gene sequence	Determined sequence region
hlyA	Forward: 5'-AAA TCA TCG ACG GCA ACC T-3' Reverse: 5'-ATT TCG GAT AAA GCG TGG TG-3'	1070 → 1558	1126 – 1527
clpC	Forward: 5'-TCT TGG TAT TAG TTT GAA TAA AGC TC-3' Reverse: 5'-TCA AAC GTA CTT TAG AAC CAG ATT-3'	378 → 1210	489 – 1124
inlA	Forward: 5'-TTT TTC TAT AAT AAC AAG GTA AGT GAC-3' Reverse: 5'-CTG TAT AGC TAT TGG CGC TAT-3'	1099 → 1918	1192 – 1799
plcA	Forward: 5'-ACT GGA ATA AGC CAA TAA AGA ACT C-3' Reverse: 5'-ATT GTT TGT TTT TCG GGG AAG T-3'	101 → 920	153 – 865

Table 3. Oligonucleotides for primers and probes for Cycling Probe Technology.

Gene	Primer or probe[a]	Nucleotide sequence	Position
hlA	Forward primer	5'-CCTGAAGGTAACGAAATTG-3'	
	Reverse primer	5'-TGCGTTACCTGGCAAATAG-3'	
	hlyA probe	5'(FAM[b])-TTg GCTCATTTC (Eclipse[c])-3'	1381-1392
clpC	Forward primer	5'-TCTAAAGAAATCCAACGTG-3'	
	Reverse primer	5'-TCATTACGAACAATTTGTTG-3'	
	clpC-M1 probe	5'(FAM)-GTa CGAAGAATA (Eclipse)-3'	593-604
	clpC-M2 probe	5'(FAM)-GAa GGACTAGC (Eclipse)-3'	652-662
inlA	Forward primer	5'-CCAAACACGGTGAAAAATG-3'	
	Reverse primer	5'-TACTTCATTGGTATAACTAG-3'	
	inlA probe	5'(FAM)-GCa TAACTACC (Eclipse)-3'	1351-1361

[a]Fluorescence- and quencher-labeled DNA/RNA chimera probe (cycling probe). Small letters indicate the RNA.
[b]Fluorescent dye.
[c]Quencher.

(Eclipse)-3'; small character indicates RNA), which is identical to a sequence found only in type No. A5, was designed and designated as *hlyA* probe (Table 3).

Secondly, we focused attention on identification of MLST-4 (Nos. 125, 129, and 131), which was abundant among the investigated clinical 4b isolates, and the *clpC* and *inlA* genes were selected as targets. A combination of detection of SNP type No. B13 for *clpC* and SNP type No. C14 for *inlA* would lead to identification of MLST-4 clinical isolates. For *clpC* SNP typing, two probes (5'(FAM)-GTaC-GAAGAATA (Eclipse)-3' and 5'(FAM)-GAaGGACTAGC (Eclipse)-3') were designed and designated as *clpC-M1 probe* and *clpC-M2 probe*, respectively (Table 3). The *clpC-M1 probe* detects a common SNP site found in four SNP types (Nos. B10-B13 in Table 1) and *clpC-M2 probe* detects another common site found in four SNP types (Nos. B10-B14, except for B12). By using *clpC-M1 probe* and *clpC-M2 probe*, SNP type No. B13 would be identified as well as Nos. B10 and B11. For *inlA* SNP typing (No. C14), a probe (5'(FAM)-GCaTAACTACC (Eclipse)-3'), which has a sequence identical to those of four SNP types (Nos. C8, C14, C15, and C17 in Table 1), was designed and designated as *inlA probe* (Table 3). The probe, in combination with the two *clpC probes*, would lead to identification of isolates similar to MLST-4 isolates.

Identification of L. monocytogenes genetically similar to the clinical 1/2a and 4b isolates For rapid identification of *L. monocytogenes* genetically similar to the clinical isolates based on SNP, real-time PCR using Cycling Probe Technology was performed (Fig. 1). The *hlyA probe* was used for identification of isolates similar to clinical 1/2a isolates (Fig. 1a). Isolates from food or environment (Nos. 1, 2, 3, 13, 19, 21, 44, 107, and 110) and clinical isolates (Nos. 118, 122, and 127) were analyzed as representatives of the respective SNP types (Nos. A1-A9) of the *hlyA* gene. The results showed that isolates of Nos. 2 and 118 (SNP type No. A5) were specifically identified by the present method. In contrast, other isolates were not identifiable. After real-time PCR, the reaction mixtures of all samples were analyzed by agarose gel electrophoresis and it was confirmed that the target products were amplified in all samples (data not shown). Furthermore, all isolates which were categorized into type No. A5 were identified by this method (data not shown), indicating that 6 MLST types (MLST-2, -5, -6, -28, -29, and

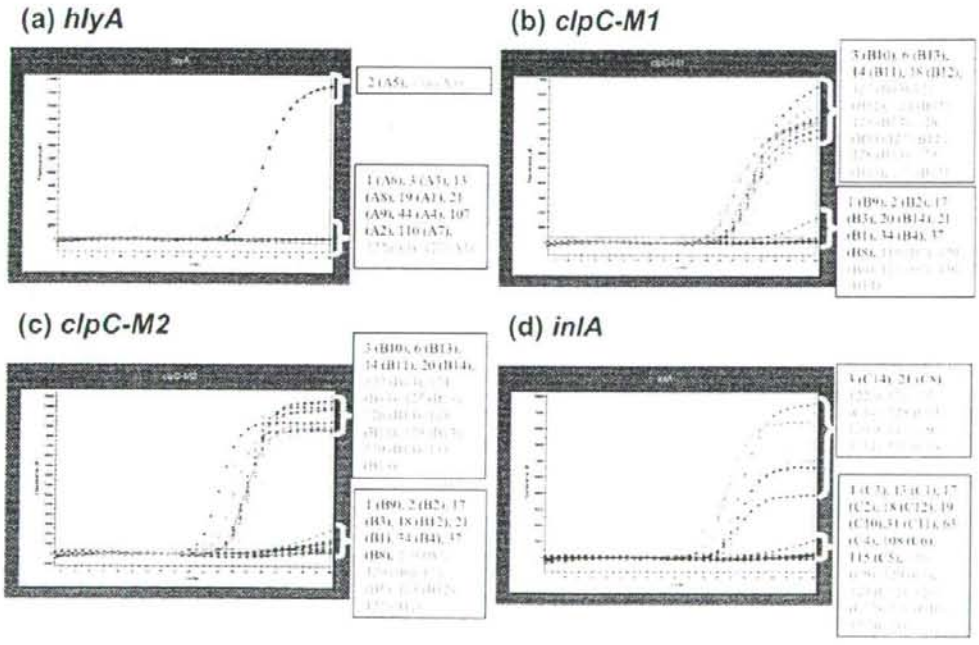

Fig. 1. Real-time plots indicating the specificity of the *hlyA probe* (a), *inlA probe* (b), *clpC-M1 probe* (c), and *clpC-M2 probe* (d) amplification.
Isolate numbers are shown with SNP numbers in parentheses in the boxes on the right of each plot. Numbers in red indicate clinical isolates. The upper box includes isolate numbers corresponding to detected samples. The lower box includes isolate numbers corresponding to undetected samples. Real-time PCR with Cycling Probe Technology was performed as described in Materials and methods.

-30) were detectable. All the clinical isolates detected with this method showed serotype 1/2a (MLST-2, -28, -29, and -30), and the majority of MLST-2, -5, and -6 isolates from food or environment showed serotype 1/2a, with the exception of 3a (No. 23), 3c (No. 25), and UT (Nos. 108 and 109). It seemed that *L. monocytogenes* identified by this cycling probe PCR method are genetically similar to the serotype 1/2a clinical isolates.

In the case of identifying serotype 4b, MLST-4 (Nos. 125, 129, and 131) isolates were selected as representatives, and *clpC-M1*, *clpC-M2*, and *inlA probes* were used. Isolates from food or environment (Nos. 1-3, 6, 14, 17, 18, 20, 21, 34, and 37) and clinical isolates (Nos. 119-131) were selected as representatives of the respective SNP types (Nos. B1-B14) of the *clpC* gene. Isolates from food or environment (Nos. 1, 3, 13, 17-19, 21, 31, 65, 108, and 115) and clinical isolates (Nos. 119-131, except for No. 121) were selected as representatives of the respective SNP types (Nos. C1-C14) of the *inlA* gene. Figure 1b-d shows the results of real-time PCR for detection of specific SNP on the *clpC* or *inlA* genes. By using *clpC-M1* probe, isolates (Nos. 3, 6, 14, 18, 122-129, and 131) of four SNP types (Nos. B10-B13) were identified (Fig. 1b) and these isolates corresponded to MLST-3, -4, -8, -10, -14, -31, -32, -33, -34, or -35. In the case of *clpC-M2 probe*, isolates (Nos. 3, 6, 14, 20, 122, 124-126, and 128-131) of four SNP types (Nos. B10, B11, B13, and B14) were also identified (Fig. 1c) and these isolates corresponded to MLST-3, -4, -8, -12, -31, -32, -33, or -35. In both cases, the isolates with other SNP types were not identified. By using *inlA probe*, isolates (Nos. 3, 21,122, 125, and 128-131) of four SNP types (Nos. C8, C14, C15, and C17) were identified (Fig. 1c) and these isolates corresponded to MLST-3, -4, -12, -13, -31, or -35. By performing real-time PCR using *clpC-M1*, *clpC-M2*, or *inlA probes*, MLST-4 isolates (Nos. 125, 129, and 131) were identified in all three reactions as well as MLST-3 (No. 3), MLST-31 (No. 122: serotype 1/2b), or MLST-35 (No. 128), as predicted by SNP types. According to the results of SNP typing in Table 1, 6 MLST types (MLST-3, -4, -8, -14, -31, and -35) were detectable. The majority of MLST-3, -4, -8, and -14 isolates from food or environment showed serotype 4b, with the exception of 4e (No. 24). Out of the serotype 4b clinical isolates showing MLST-4, -12, -33, -34, and -35, the isolates of two MLST types (-4 and -35) were identified by the cycling probe PCR method. It seemed that the isolates identified by the combined method are genetically similar to parts of the serotype 4b clinical isolates.

Discussion

In order to compare the genetic characteristics between clinical isolates and food-borne or environmental isolates of *L. monocytogenes*, nucleotide sequences of parts of the *hlyA*, *clpC*, *inlA*, and *plcA* genes, which are involved in adhesion and infection of cells, were analyzed. The isolates were categorized into several SNP types or MLST types as shown in Table 1. Many isolates from food or environment were categorized into the same SNP types (characteristic examples; A5, B12-B14, C14) or MLST types (MLST-2, -4, -10, and -12) as clinical isolates, suggesting that these isolates from food or environment may be capable of causing food poisoning. Several researchers have investigated MLST of *L. monocytogenes* and shown that the method is a powerful typing-tool for epidemiology (Revazishvili *et al.*, 2004; Salcedo *et al.*, 2003; Zhang *et al.*, 2004). In particular, MLST using virulence genes seems to be more discriminatory than the standard protocol for epidemiology, PFGE (Revazishvili *et al.*, 2004; Zhang *et al.*, 2004). Thus, like MLST, the SNP typing method based on comparison of the virulence genes of *L. monocytogenes* could become an effective tool for subtyping isolates of *L. monocytogenes*.

Real-time PCR using cycling probes for SNP typing has been performed to detect a point mutation of the *gyrA* gene in quinolone-resistant *Salmonella* (Esaki *et al.*, 2004). By using this method, the mismatch of a single nucleotide between a cycling probe and a target gene has been identified. In the present study, we succeeded in specific identification of isolates (Nos. 2 and 118) grouped into SNP type No. A5 or MLST-2 with serotype 1/2a (Fig. 1a). Furthermore, a combination of reactions using different chimera probes (*clpC-M1*, *clpC-M2*, and *inlA*) successfully led to identification of clinical MLST-4 isolates of serotype 4b (Fig. 1b-d). These results suggest that the isolates from food or environment are predicted to have similar pathogenicity to the clinical isolates. Although we focused on only a few isolates (MLST-2 and -4) as representatives of 1/2a and 4b isolates in this study, by additionally designing other new specific probes, this method could be applied to identification of other highly pathogenic isolates.

Recently, Borucki and Call (2003) developed primer sets for identification of four major serotypes (1/2a, 1/2b, 1/2c, and 4b) using a multiplex PCR method. The method showed high agreement (over 90%) with serotypes of the bacterium, suggesting that modified methods of PCR could become rapid and convenient protocols for application in the food industry and in epidemical research. Our method using real-time PCR combined with Cycling Probe Technology has the potential to become an effective tool for rapid identification of serotype 1/2a and 4b isolates predicted to have similar pathogenicity to the clinical isolates.

To improve reliability of this method in regard to identi-

fication of pathogenicity. collection of more clinical isolates for sequence and pathogenicity analyses are under way.

Acknowledgements We thank Mr. Hsu-Ming Wen for help in preparation of this manuscript.

References

Alberti-Segui, C., Kathryn, K.R. and Higgins, D.E. (2007). Differential function of *Listeria monocytogenes* listeriolysin O and phospholipases C in vacuolar dissolution following cell-to-cell spread. *Cell. Microbiol.*, **9**, 179-195.

Bekkaoui, F., Poisson, I., Crosby, W., Cloney, L. and Duck, P. (1996). Cycling probe technology with RNase H attached to an oligonucleotide. *BioTechniques*, **20**, 240-248.

Best, E.L., Fox, A.J., Frost, J.A. and Bolton, F.J. (2005). Real-time single-nucleotide polymorphism profiling using Taqman technology for rapid recognition of *Campylobacter jejuni* clonal complexes. *J. Medical Microbiol.*, **54**, 919-925.

Borucki, M.K. and Call, D.R. (2003). *Listeria monocytogenes* serotype identification by PCR. *J. Clin. Microbiol.*, **41**, 5537-5540.

Cai, S., Kabuki, D.Y., Kuaye, A.Y., Cargioli, T.G., Chung, M.S., Nielsen, R. and Wiedmann, M. (2002). Rational design of DNA sequence-based strategies fro subtyping *Listeria monocytogenes*. *J. Clin. Microbiol.*, **40**, 3319-3325.

Esaki, H., Noda, K., Otsuki, N., Kojima, A., Asai, T., Tamura, Y. and Takahashi, T. (2004). Rapid detection of quinolone-resistant *Salmonella* by real time SNP genotyping. *J. Microbiol. Methods*, **58**, 131-134.

Gaillard, J.L., Berche, P., Frehel, C., Gouln, E. and Cossart, P. (1991). Entry of L. monocytogenes into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. *Cell*, **65**, 1127-1141.

Gasanov, U., Hughes, D. and Hansbro, P.M. (2005). Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review. *FEMS Microbiol. Rev.*, **29**, 851-875.

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., Berche, P., Bloecker, H., Brandt, P., Chakraborty, T., Charbit, A., Chetouani, F., Couvé, E., de Daruvar, A., Dehoux, P., Domann, E., Domínguez-Bernal, G., Duchaud, E., Durant, L., Dussurget, O., Entian, K.-D., Fsihi, H., García-Del Portillo, F., Garrido, P., Gautier, L., Goebel, W., Gómez-López, N., Hain, T., Hauf, J., Jackson, D., Jones, L.-M., Kaerst, U., Kreft, J., Kuhn, M., Kunst, F., Kurapkat, G., Madueño, E., Maitournam, A., Mata Vicente, J., Ng, E., Nedjari, H., Nordsiek, G., Novella, S., de Pablos, B., Pérez-Diaz, J.-C., Purcell, R., Remmel, B., Rose, M., Schlueter, T., Simoes, N., Tierrez, A., Vázquez-Boland, J.-A., Voss, H., Wehland, J. and Cossart, P. (2001). Comparative genomics of *Listeria* species. *Science*, **294**, 849-852.

Mengaud, J., Vicente, M.-F., Chenevert, J., Pereira, J.M., Geoffroy, C., Gicquel-Sanzey, B., Baquero, F., Perez-Diaz, J.-C. and Cossart, P. (1988). Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. *Infec. Immun.*, **56**, 766-772.

Nair, S., Milohanic, E. and Berche, P. (2000). ClpC ATPase is required for cell adhesion and invasion of *Listeria monocytogenes*. *Infec. Immun.*, **68**, 7061-7068.

Nelson, K.E., Fouts, D.E., Mongodin, E.F., Ravel, J., DeBoy, R.T., Kolonay, J.F., Rasko, D.A., Angiuoli, S.V., Gill, S.R., Paulsen, I.T., Peterson, J., White, O., Nelson, W.C., Nierman, W., Beanan, M.J., Brinkac, L.M., Daugherty, S.C., Dodson, R.J., Durkin, A.S., Madupu, R., Haft, D.H., Selengut, J., Aken, S.V., Khouri, H., Fedorova, N., Forberger, H., Tran, B., Kathariou, S., Wonderling, L.D., Uhlich, G.A., Bayles, D.O., Luchansky, J.B. and Fraser, C.M. (2004). Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. *Nucleic Acids Res.*, **32**, 2386-2395.

Okutani, A., Okada, Y., Yamamoto, S. and Igimi, S. (2004). Overview of *Listeria monocytogenes* contamination in Japan. *Int. J. Food Microbiol.*, **93**, 131-140.

Pharmaceutical Society of Japan. (2000). "Methods of Analysis in Health Science", 95-97 (in Japanese).

Revazishvili, T., Kotetishvili, M., Stine, O.C., Kreger, A.S., Morris, J.G. Jr. and Sulakvelidze, A. (2004). Comparative analysis of multilocus sequence typing and pulsed-field gel electrophoresis for characterizing *Listeria monocytogenes* strains isolated from environmental and clinical sources. *J. Clin. Microbiol.*, **42**, 276-285.

Rouquette, C., Ripio, M.-T., Pellegrini, E., Bolla, J.-M., Tascon, R.I., Vázquez-Boland, J.A. and Berche, P. (1996). Identification of a ClpC ATPase required for stress tolerance and *in vivo* survival of *Listeria monocytogenes*. *Mol. Microbiol.*, **21**, 977-987.

Salcedo, C., Arreaza, L., Alcalá, B., de la Fuente, L. and Vázquez, J.A. (2003). Development of a multilocus sequence typing method for analysis of *Listeria monocytogenes* clones. *J. Clin. Microbiol.*, **41**, 757-762.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.*, **22**, 4673-4680.

Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J. and Kreft, J. (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.*, **14**, 584-640.

Zhang, W., Jayarao, B.M. and Knabel, S.J. (2004). Multi-virulence-locus sequence typing of *Listeria monocytogenes*. *Appl. Environ. Microbiol.*, **70**, 913-920.

Journal of Food Protection, Vol. 71, No. 8, 2008, Pages 1689–1695

Research Note

Detection of Human Enteric Viruses in Japanese Clams

GRANT S. HANSMAN,[1]* TOMOICHIRO OKA,[1] TIAN-CHENG LI,[1] OSAMU NISHIO,[2] MAMORU NODA,[3] AND NAOKAZU TAKEDA[1]

[1]*Department of Virology II and* [2]*Infectious Diseases Surveillance Center, National Institute of Infectious Diseases, Musashi-murayama, Tokyo 208-0011, Japan; and* [3]*National Institute of Health Sciences, Kami-Yoga 1-18-1, Setagaya-ku, Tokyo, Japan*

MS 08-010: Received 6 January 2008/Accepted 9 March 2008

ABSTRACT

A total of 57 clam packages that were collected from supermarkets and fish markets from 11 different sites in western Japan between 8 December 2005 and 6 September 2006 were examined for human enteric viruses (i.e., norovirus, Aichi virus, rotavirus, adenovirus, hepatitis A virus, and astrovirus), using PCR and reverse transcription PCR. Sixty-one percent of the packages were contaminated with one type of virus, 9% had two different types of viruses, 28% had three different types of viruses, and 9% had at least four different types of viruses. Thirty-one (54%) of 57 packages were contaminated with noroviruses. Norovirus genogroup I and genogroup II sequences were detected in 24 and 23 packages, respectively, and these sequences belonged to nine genogroup I and eight genogroup II genotypes. Aichi viruses were found in 19 (33%) of 57 packages, and these belonged to genogroup A. Rotaviruses (group A) were detected in 14 (42%) of 33 of packages and 9 of 14 rotavirus-positive packages contained two or more rotavirus genogroup types. Adenoviruses (Ad40 and Ad41) were detected in 17 (52%) of 33 packages. One of the 57 (2%) packages was positive with hepatitis A virus (subtype IA). Astrovirus was not detected in any of the packages. This is the first study to detect such a high level of contamination in Japanese clams. These results represent an important finding because the Japanese clams were considered suitable for human consumption. Further studies are needed to determine the health risks associated with eating these highly contaminated clams.

Gastroenteritis is one of the leading causes of death by an infectious disease *(19)*, with more than 700 million cases of acute diarrheal disease occurring annually. The main viral agents that cause gastroenteritis are norovirus, rotavirus, sapovirus, astrovirus, and enteric adenoviruses. These viruses have been detected in environmental samples (e.g., lakes and sewage) as well as in foods such as oysters, clams, sandwiches, and raspberries. Other important viral agents that can accumulate in oysters and clams are hepatitis A virus (HAV) and hepatitis E virus (HEV) *(4, 17)*. The impact of viral contamination in the environment is evident in Japan, where outbreaks of norovirus oyster–associated gastroenteritis increases in winter, and this coincides with the oyster-harvesting season in winter *(21)*. The detection methods for these viruses in environmental samples and clinical specimens have greatly improved over the past 10 years and have provided a better understanding and distribution of these viruses.

The purpose of this study was to detect norovirus, Aichi virus, rotavirus, adenovirus, HAV, and astrovirus in clam packages sold at supermarkets and fish markets, which were destined for human consumption in Japan, using PCR and reverse transcription PCR, and then describe the genetic diversity of the positive noroviruses.

* Author for correspondence. Tel: +81-42-561-0771; Fax: +81-42-561-4729; E-mail: ghansman@nih.go.jp.

MATERALS AND METHODS

Clam samples. A total of 57 clam (*Corbicula japonica*) packages (30 to 60 clams per package) were collected from supermarkets or fish markets (nonexport) from 11 different geographically distinct sites in western Japan between 8 December 2005 and 6 September 2006. The clam packages were screened for norovirus, Aichi virus, rotavirus, adenovirus, HAV, and astrovirus using PCR and reverse transcription PCR. These 57 packages were previously screened for sapovirus *(9)*, and 46 of 57 packages were screened for HEV *(17)*.

Viral extraction. The clams were shucked, the digestive diverticulum removed by dissection on the day of harvest *(16)*, and then weighed and homogenized in nine times their weight of phosphate-buffered saline without magnesium or calcium. One gram of digestive diverticulum (10 to 15 clams per package) was homogenized with an Omni mixer (Omni International, Marietta, Ga.) in 10 ml of phosphate-buffered saline (pH 7.2). After centrifugation at 10,000 × *g* for 30 min at 4°C, the supernatant was layered onto 1 ml of 30% sucrose solution and ultracentrifuged at 154,000 × *g* for 3 h at 4°C. The pellet was resuspended in 140 μl of distilled water and stored at −80°C until use.

DNA and RNA extraction and reverse transcription. Viral DNA (for detection of adenovirus) was extracted from resuspended pellet, using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Viral RNA (for detection of norovirus, Aichi virus, rotavirus, and HAV) was extracted from the resuspended pellet, using QIAmp Viral RNA Mini Kit (Qiagen,). For reverse transcription, the RNA solution was treated with 2 U of RNase-free DNase I (Takara, Tokyo, Japan) for 30 min at 37°C, and was

TABLE 1. *Details of the clam samples*

Package no.	Site	Mo/day/yr	No. of norovirus genotypes	Norovirus strain name (genogroup/genotype)	Aichi virus	Rotavirus: G types	Adeno-virus	HAV	HEV (from *(17)*)	Sapovirus (from *(9)*)
1	A	12/08/05	2	GI-Shijimi1 (GI/4), GII-Shijimi1 (GII/3)	Aic-1	G1/G4	−	−	−	−
2	D	12/10/05	−		−	−	+	−	−	−
3	A	12/22/05	2	GI-Shijimi2 (GI/1), GII-Shijimi2 (GII/2)	Aic-2	G9	+	−	−	−
4	D	12/17/05	−		−	G8	+	−	−	−
5	K	01/10/06	1	GII-Shijimi3 (GII/3)	Aic-3	NTa	NT	−	−	−
6	A	01/14/06	3	GI-Shijimi4 (GI/11), GII-Shijimi4a (GII/4), GII-Shijimi4b (GII/3)	Aic-4	G4	+	−	−	−
7	J	01/16/06	1	GI-Shijimi5 (GI/8)	Aic-5	NT	NT	−	−	−
8	A	01/20/06	2	GI-Shijimi6 (GI/14), GII-Shijimi6 (GII/3)	−	NT	NT	−	−	−
9	B	01/22/06	3	GI-Shijimi7 (GI/12), GII-Shijimi7a (GII/New), GII-Shijimi7b (GII/3)	Aic-6	G?	+	−	−	−
10	B	01/22/06	−		−	−	+	−	−	−
11	C	01/21/06	3	GI-Shijimi8 (GI/11), GII-Shijimi8a (GII/3), GII-Shijimi8b (GII/3), GII-Shijimi8c (GII/4), GII-Shijimi8d (GII/3)	−	−	−	−	−	−
12	D	01/24/06	3	GI-Shijimi9 (GI/8), GII-Shijimi9a (GII/3), GII-Shijimi9b (GII/4)	Aic-7	G?	+	−	−	−
13	C	01/26/06	3	GII-Shijimi10a (GII/3), GII-Shijimi10b (GII/3), GII-Shijimi10c (GII/3)	Aic-8	−	+	−	−	SaV-3
14	D	02/07/06	1	GI-Shijimi11 (GI/11)	Aic-18	NT	NT	−	+	−
15	B	02/05/06	4	GI-Shijimi12a (GI/1), GI-Shijimi12b (GI/1), GI-Shijimi12c (GI/8), GI-Shijimi12d (GI/11)	Aic-9	−	+	−	−	−
16	D	02/19/06	2	GI-Shijimi13 (GI/1), GII-Shijimi13 (GII/3)	Aic-10	G1/G8/G9	+	−	−	−
17	B	02/17/06	1	GII-Shijimi14 (GII/New)	−	G4	+	−	−	−
18	C	02/25/06	1	GII-Shijimi15 (GII/3)	Aic-11	G2/G4	−	−	−	−
19	D	03/01/06	7	GI-Shijimi16a (GI/11), GI-Shijimi16b (GI/8), GI-Shijimi16c (GI/4), GI-Shijimi16d (GI/8), GII-Shijimi16a (GII/3), GII-Shijimi16b (GII/4), GII-Shijimi16c (GII/4)	Aic-12	NT	NT	−	+	−
20	B	03/02/06	3	GI-Shijimi17 (GI/1), GII-Shijimi17a (GII/5), GII-Shijimi17b (GII/4)	Aic-19	−	+	−	−	−
21	C	03/10/06	2	GI-Shijimi18 (GI/8), GII-Shijimi18 (GII/3)	−	−	+	−	−	SaV-1
22	B	03/14/06	3	GI-Shijimi19a (GI/1), GI-Shijimi19b (GI/1), GII-Shijimi19 (GII/3)	Aic-13	−	+	−	−	−
23	A	03/14/06	−		−	NT	NT	−	−	−
24	E	03/13/06	−		−	NT	NT	−	−	−
25	E	03/14/06	−		−	NT	NT	−	−	−
26	B	03/15/06	2	GI-Shijimi20a (GI/5), GI-Shijimi20b (GI/14)	Aic-14	−	+	−	−	−
27	D	03/17/06	1	GI-Shijimi21 (GI/1)	−	G1/G2/G8	+	−	−	−
28	F	03/18/06	2	GI-Shijimi22 (GI/8), GII-Shijimi22 (GII/New)	−	G1/G2/G3/G8/G9	+	−	−	−
29	E	03/18/06	−		−	NT	NT	−	−	−
30	E	03/18/06	−		−	NT	NT	−	−	−

TABLE 1. *Continued*

Package no.	Site	Mo/day/yr	No. of norovirus genotypes	Norovirus strain name (genogroup/genotype)	Aichi virus	Rotavirus: G types	Adeno-virus	HAV	HEV (from *(17)*)	Sapovirus (from *(9)*)
31	G	03/18/06	1	GI-Shijimi23 (GI/1)	–	NT	NT	–	–	–
32	H	03/18/06	–		–	NT	NT	–	–	–
33	D	03/30/06	1	GII-Shijimi24 (GII/2)	Aic-15	G1/G8	–	–	–	–
34	B	04/07/06	3	GI-Shijimi25a (GI/2), GI-Shijimi25b (GI/8), GII-Shijimi25 (GII/4)	–	–	–	–	–	–
35	D	04/13/06	2	GI-Shijimi26 (GI/4), GII-Shijimi26 (GII/3)	Aic-16	G1/G2/G8	–	–	–	SaV-2
36	B	04/26/06	1	GII-Shijimi27 (GII/New)	–	G2/G4	–	–	–	–
37	A	05/16/06	5	GI-Shijimi28 (GI/New), GII-Shijimi28a (GII/4), GII-Shijimi28b (GII/6), GII-Shijimi28c (GII/7), GII-Shijimi28d (GII/9)	Aic-17	–	–	–	–	SaV-4
38	D	05/13/06	2	GI-Shijimi29 (GI/4), GII-Shijimi29 (GII/7)	–	NT	NT	1A	–	–
39	D	05/27/06	–		–	G8/G9	–	–	–	–
40	A	05/29/06	2	GI-Shijimi30 (GI/4), GII-Shijimi30 (GII/3)	–	–	–	–	–	–
41	I	06/14/06	–		–	–	–	–	–	–
42	D	06/16/06	–		–	G3	–	–	–	–
43	A	06/16/06	–		–	–	–	–	–	–
44	D	06/23/06	–		–	G?	–	–	–	–
45	B	07/05/06	–		–	–	–	–	–	–
46	D	07/06/06	–		–	–	–	–	–	–
47	A	07/13/06	–		–	NT	NT	–	–[b]	–
48	D	07/21/06	–		–	NT	NT	–	–[b]	–
49	B	07/21/06	–		–	NT	NT	–	–[b]	–
50	A	07/27/06	–		–	NT	NT	–	–[b]	–
51	A	08/06/06	–		–	NT	NT	–	–[b]	–
52	D	08/07/06	–		–	NT	NT	–	–[b]	–
53	B	08/10/06	–		–	NT	NT	–	–[b]	–
54	D	08/23/06	–		–	NT	NT	–	–[b]	–
55	I	09/04/06	–		–	NT	NT	–	–[b]	–
56	B	09/04/06	1	GI-Shijimi31 (GI/New)	–	NT	NT	–	–[b]	–
57	D	09/06/06	–		–	NT	NT	–	–[b]	–
Total				31/57	19/57	17/33	17/33	1/57	2/46	4/57

[a] NT, not tested.
[b] Tested in this study.

followed by the inactivation of the enzyme at 75°C for 5 min. Reverse transcription was performed with 15 μl of RNA solution and 15 μl of reverse transcription mixture that contained 1 mM dNTP mixture, 10 mM dithiothreitol, 0.75 μg of random hexamers (Takara), 33 U of RNase inhibitor (Takara), 300 U of reverse transcriptase Superscript II (Invitrogen, San Diego, Calif.), and 4.5 μl of Superscript II buffer (Invitrogen).

PCR. For the norovirus PCR, the primers were designed to amplify the 5′ end of the capsid gene *(10, 14)*. For norovirus genogroup I (GI), COG1F and G1SKR primers were used for the first PCR, and then G1SKF and G1SKR primers were used for the nested PCR. For norovirus genogroup II (GII), COG2F and G2SKR were used for the first PCR, and then G2SKF and G2SKR primers were used for the nested PCR. For the Aichi virus, C94b and 264K primers were used, and these were designed to amplify the 3C–D junction (protease-polymerase) *(26)*. For the rotavirus (group A), primers were designed to amplify the major outer capsid glycoprotein VP7, and the rotavirus type was determined by PCR size *(7)*. For the adenovirus, primers were designed to detect the E1B region of enteric adenoviruses, i.e., Ad40 and Ad41, and determined by PCR size *(1)*. For HAV, we used a set of nested in-house primers designed to amplify the capsid gene. For the first HAV PCR, we used sense HAV+2799 primer (5′-ATTCAGAT TAGACTGCCTTGGTA-3′) and antisense HAV-3273 primer (5′-CCAAGAAACCTTCATTATTTCATG-3′). For HAV nested PCR, we used sense HAV+2907 primer (5′-GCAAATTACAAT CATTCTGATGA-3′) and antisense HAV-3162 primer (5′-CTTC YTGAGCATACTTKARTCTTG-3′). The HAV PCR conditions were the same as those for the norovirus *(14)*. For the astrovirus, PreCAP1 and 12GR primers were used to amplify the first PCR product, and then Mon244 and 82b primers were used for nested PCR, which were designed to amply the 5′ end of the capsid gene *(18)*. Two types of positive controls and a virus-free negative control per five assays for norovirus PCR were used. All PCR prod-