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).

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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

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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

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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[a]				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	B14	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	B14	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	C14	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	B12	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 food-borne 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

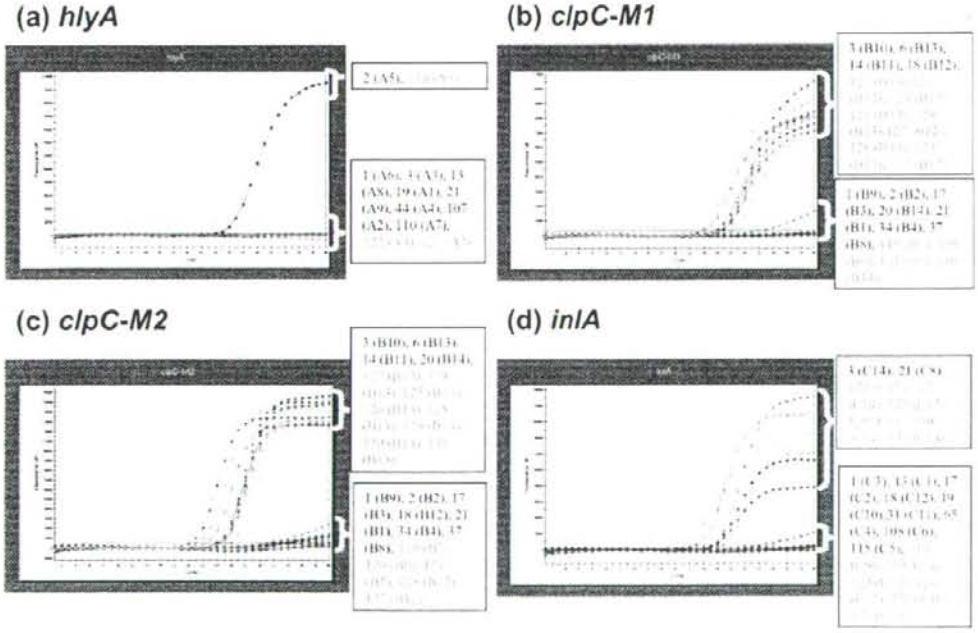

Fig. 1. Real-time plots indicating the specificity of the *hlyA probe* (a), *inlA probe* (b), *clpC-M1 probe* (c), and *clpC-M2 probe* (d) amplification.

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.

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Research Note

Detection of Human Enteric Viruses in Japanese Clams

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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.

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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	−	−	−

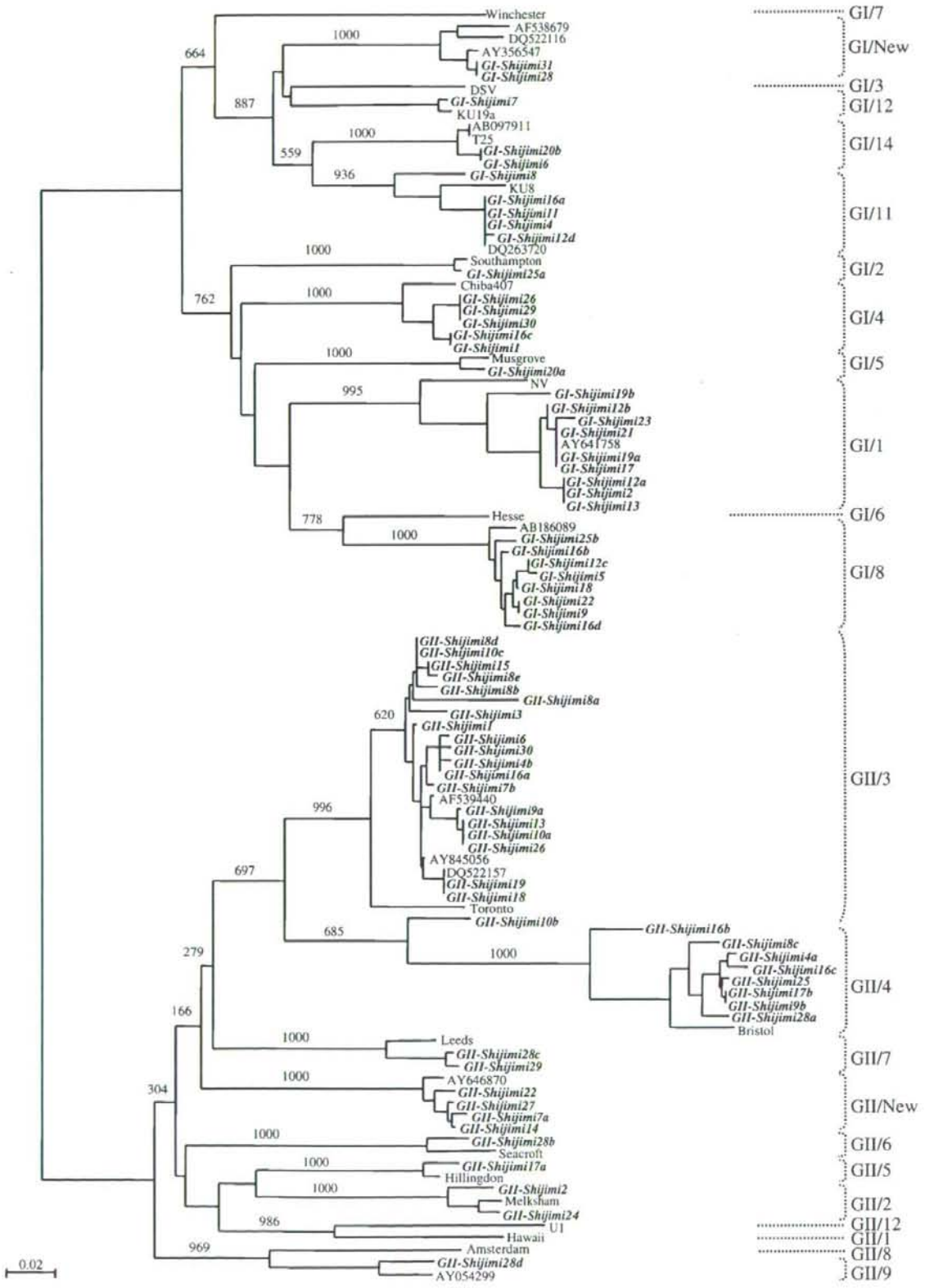
TABLE 1. *Continued*

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-


Winchester
AF538679
DQ522116
AY356547
GI-Shijimi31
GI-Shijimi28
DSV
GI-Shijimi7
KU19a
AB097911
T25
GI-Shijimi20b
GI-Shijimi6
GI-Shijimi8
KU8
GI-Shijimi16a
GI-Shijimi11
GI-Shijimi4
GI-Shijimi12d
DQ263720
Southampton
GI-Shijimi25a
Chiba407
GI-Shijimi26
GI-Shijimi29
GI-Shijimi30
GI-Shijimi16c
GI-Shijimi1
Musgrove
GI-Shijimi20a
NV
GI-Shijimi19b
GI-Shijimi12b
GI-Shijimi23
GI-Shijimi21
AY641758
GI-Shijimi19a
GI-Shijimi17
GI-Shijimi12a
GI-Shijimi2
GI-Shijimi13
Hesse
AB186089
GI-Shijimi25b
GI-Shijimi16b
GI-Shijimi12c
GI-Shijimi5
GI-Shijimi18
GI-Shijimi22
GI-Shijimi9
GI-Shijimi16d
GII-Shijimi8d
GII-Shijimi10c
GII-Shijimi15
GII-Shijimi8e
GII-Shijimi8b
GII-Shijimi8a
GII-Shijimi3
GII-Shijimi1
GII-Shijimi6
GII-Shijimi30
GII-Shijimi4b
GII-Shijimi16a
GII-Shijimi7b
AF539440
GII-Shijimi9a
GII-Shijimi13
GII-Shijimi10a
GII-Shijimi26
AY845056
DQ522157
GII-Shijimi19
GII-Shijimi18
Toronto
GII-Shijimi10b
GII-Shijimi16b
GII-Shijimi8c
GII-Shijimi4a
GII-Shijimi16c
GII-Shijimi25
GII-Shijimi17b
GII-Shijimi9b
GII-Shijimi28a
Bristol
Leeds
GII-Shijimi28c
GII-Shijimi29
AY646870
GII-Shijimi22
GII-Shijimi27
GII-Shijimi7a
GII-Shijimi14
GII-Shijimi28b
Seacroft
GII-Shijimi17a
Hillingdon
GII-Shijimi2
Melksham
GII-Shijimi24
U1
Hawaii
Amsterdam
GII-Shijimi28d
AY054299
664
1000
887
559
936
762
778
620
996
697
685
279
166
304
986
969
0.02
GI/7
GI/New
GI/3
GI/12
GI/14
GI/11
GI/2
GI/4
GI/5
GI/1
GI/6
GI/8
GII/3
GII/4
GII/7
GII/New
GII/6
GII/5
GII/2
GII/12
GII/1
GII/8
GII/9

ucts were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Norovirus, Aichi virus, and HAV nucleotide sequences were prepared as previously described *(11)*.

Sequence analysis. Norovirus, Aichi virus and HAV nucleotide sequences were prepared with the terminator cycle sequence kit (version 3.1, Applied Biosystems, Warrington, UK) and determined with the ABI 3130 sequencer (ABI, Boston, Mass.). In order to determine the norovirus genotypes in the packages with multiple genotypes, we cloned the reverse transcription PCR products into pCR2.1 (Invitrogen), and at least four clones from each sample were sequenced. The genetic diversity of the adenoviruses was not determined in this study. Norovirus nucleotide sequences were aligned with ClustalX, and the distances were calculated by Kimura's two-parameter method. The norovirus nucleotide sequence data determined in this study has been deposited in GenBank under accession no. EF424485 through EF424557.

RESULTS

Thirty-five (61%) of 57 packages were contaminated with one type of virus, 5 (9%) of 57 packages were contaminated with two different types of viruses, 16 (28%) of 57 packages were contaminated with three different types of viruses, and 5 (9%) of 57 packages were contaminated with at least four different types of viruses (Table 1). Astrovirus was not detected in any of the packages.

Noroviruses. Thirty-one (54%) of 57 packages were contaminated with noroviruses (Table 1). Norovirus GI and GII sequences were detected in 24 and 23 packages, respectively (Fig. 1). A total of 24 norovirus GI sequences were detected, and these clustered into nine different GI genotypes (Fig. 1), including one unpublished GI genotype (GI/1, GI/2, GI/4, GI/5, GI/8, GI/11, GI/12, GI/14, and GI/New). A total of 23 norovirus GII sequences were detected, and these clustered into eight different GII genotypes (Fig. 1), including one unpublished GII genotype (GII/2, GII/3, GII/4, GII/5, GII/6, GII/7, GII/9, and GII/New). More than half of the norovirus-positive packages, 20 (65%) of 31, contained two or more norovirus genotypes. Twenty-three (74%) of 31 norovirus-positive packages were co-contaminated with two or more other types of viruses (Table 1).

Aichi virus. We found that 19 (33%) of 57 packages were contaminated with Aichi viruses. The 19 Aichi virus sequences shared over 95% nucleotide homology, suggesting that the same strain contaminated the clams. These 19 sequences closely matched (approximately 95% nucleotide homology) genogroup A sequences found on the database (data not shown). All of the Aichi virus–positive packages were co-contaminated with other viruses (Table 1).

Rotavirus. Fourteen (42%) of 33 packages were contaminated with rotavirus (24 packages were unavailable for screening). Six different rotavirus G types were detected, i.e., G1, G2, G3, G4, G8, and G9. Of the 14 rotavirus-positive packages nine (53%) contained two or more rotavirus G types (Table 1).

Adenovirus. Seventeen (52%) of 33 packages were contaminated with adenoviruses, using primers designed to detect the two enteric adenoviruses, i.e., Ad40 and Ad41. Fourteen (82%) of 17 adenovirus-positive packages were co-contaminated with other viruses (Table 1).

HAV and HEV. One (2%) of 57 packages was contaminated with HAV. Sequence analysis of the capsid gene indicated that it belonged to subtype IA. HEV was previously detected in 2 of 46 packages *(17)*. An additional 11 packages were screened for HEV; however these were all negative (Table 1).

DISCUSSION

The current study has shown that Japanese clams (*C. japonica*) purchased in supermarkets and fish markets were highly contaminated with human enteric viruses from the natural environment. Similarly, a 3-year study in France found that mussel samples (*Mytilus galloprovincialis*) were highly contaminated with enteric viruses *(15)*. However, an important difference between the study conducted in France and the current study was that the French mussels were collected in areas where sewage was discharged and were prohibited for human consumption, whereas the Japanese clams were sold in supermarkets and fish markets and were considered suitable for human consumption.

Noroviruses are the dominant cause of outbreaks of gastroenteritis worldwide. In this study, the noroviruses were the dominant virus detected the clam packages (found in 54% of the packages). In a comparative study, noroviruses were detected in only approximately 5 to 9% of Japanese oysters (*Crassostrea gigas* or *Crassostrea nippona*) *(20, 21)*. These results suggested that the Japanese clams were more highly contaminated with noroviruses than were the Japanese oysters, or alternatively, it was just a reflection on the different collection sites, i.e., the clams were collected from brackish waters, whereas the oysters were collected from the sea. Alternatively, the different detection rates in clams and oysters were a result of the different sample preparations. Nevertheless, all of the norovirus sequences detected in the clam packages closely matched other sequences detected in patients with gastroenteritis in Japan (using GenBank BLAST searches), suggesting that the contaminated Japanese clams could cause gastroenteritis in humans, although direct evidence is lacking.

Over the past 10 years, the norovirus GII/4 strains have become the dominant cause of outbreaks of gastroenteritis

←

FIGURE 1. *Phylogenetic analysis of norovirus capsid sequences (approximately 300 nucleotides) showing the different genogroups and genotypes. The numbers on each branch indicate the bootstrap values for the genotype. Bootstrap values of 950 or higher were considered statistically significant for the grouping. The scale represents nucleotide substitutions per site. The frequency of each norovirus genotype was 9, 1, 5, 1, 8, 5, 1, 2, 2, 1, 20, 8, 1, 1, 2, 1, and 4 for GI/1, GI/2, GI/4, GI/5, GI/8, GI/11, GI/12, GI/14, GI/New, GII/2, GII/3, GII/4, GII/5, GII/6, GII/7, GII/9, and GII/New, respectively.*

worldwide. In a recent study, we also found that the GII/4 strains were the dominant cause of outbreaks of gastroenteritis in food-catering settings in Japan *(22)*. In the current study, the norovirus GII/3 sequences were detected more frequently than were the norovirus GII/4 sequences, i.e., 20 versus 8 sequences, respectively (Fig. 1). This result may only reflect that the GII/3 strains were more dominant in this area of Japan; however, the norovirus GII/3 strains were the second most dominant cause of gastroenteritis in Japan, Australia, and Vietnam *(2, 8, 22)*, indicating that this genotype is indeed a major cause of gastroenteritis. Noteworthy were two new norovirus genotypes (GI/New and GII/New; Fig. 1) detected in the clam packages, at three different sites, and several months apart. Similar norovirus sequences were recently reported in patients in Thailand, Taiwan, Hong Kong, and from an outbreak on a U.S. navy ship (data not shown), indicating that there may be a widespread distribution of these two newly identified genotypes.

We found that more than half (65%) of the norovirus-positive packages contained two or more norovirus genotypes (Table 1 and Fig. 1). Multiple norovirus genotypes have also been found in oyster-associated outbreaks of gastroenteritis *(10)*, and in a recent study, we found multiple norovirus genotypes in outbreaks of gastroenteritis at various food-catering settings throughout Japan *(22)*. These findings indicate that like oyster-associated outbreaks, clam-associated outbreaks may also be caused by multiple norovirus genotypes, although further studies are needed.

The Aichi virus was found in 33% of the clam packages, and all of these packages were co-contaminated with other viruses. The Aichi virus sequences detected in the packages closely matched other Aichi virus sequences (genogroup A) that were detected in patient stool specimens from oyster-associated gastroenteritis *(26)*. To the best of our knowledge, these results have shown for the first time that the Aichi virus can also accumulated in these Japanese clams. The importance of Aichi virus in human gastroenteritis is still poorly understood, and very few studies have reported Aichi virus infections since its first discovery in 1989 *(25)*. One recent study detected Aichi virus in only 3% (28 of 912) of stool specimens from infants with sporadic cases of gastroenteritis (collected in Japan, Bangladesh, Thailand, and Vietnam), which were negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus *(23)*. Further studies are clearly needed in order to determine the importance of this virus in humans.

Rotavirus was detected in 14 of 33 available clam packages. A similar study in France found rotavirus in 52% of mussel samples and 27% of oyster samples *(15)*. Rotavirus infections usually causes sporadic cases of gastroenteritis in children in the winter season, but our results suggest that rotavirus may persist longer in the environment, at least up to June (Table 1). A great genetic diversity of rotavirus G types was detected in the packages, and we also found that 9 of 17 rotavirus-positive packages contained two or more rotavirus G types. Likewise, a study in Egypt and Spain also found a great genetic diversity of rotavirus G types as well as unusual genotypes in sewage samples *(24)*.

Enteric adenoviruses (Ad40 and Ad41) were detected in 17 of 33 available clam packages. Adenovirus infections in the western part of Japan were reported to be low, with one study reporting adenovirus serotype 41 in only approximately 3% of stool specimens from infants with sporadic cases of gastroenteritis *(6)*. The high detection rate of adenoviruses in these packages may indicate that adenovirus prevalence is variable, although further studies are needed.

One (2%) of 57 packages was contaminated with HAV, and sequence analysis indicated that it belonged to subtype IA. The low detection rate of HAV was also observed in an oyster study that found only 2 of 112 samples positive in Japan *(12)*. The low detection rate of HAV in the clams and oysters was not unusual, because the prevalence of HAV infections is low in Japan, although this may be increasing *(13)*. More surveillance is clearly needed in order to locate other contaminated areas and help control the spread of HAV contamination.

Astroviruses were not detected in any of the Japanese clam packages. This result is surprising because astroviruses were detected in more than half (61%) of African clam samples *(5)*, 50% of French mussel samples *(15)*, and 17% of French oyster samples *(15)*. This result suggested that the astrovirus may not concentrate to detectable levels in certain species of shellfish or the level of contamination differs in each place, which was similarly observed in two other studies *(3, 21)*.

In conclusion, this study has shown that the Japanese clams were highly contaminated with many types of human enteric viruses capable of causing gastroenteritis and/or acute viral hepatitis. At present, the Enforcement Regulation of Food Sanitation Law mainly focuses on bacterial contamination in Japan *(21)*. Clearly, regulations and standards need to be revised in order to address this problem of viral contamination in the Japanese clams. The health risks associated with eating contaminated oysters have been well documented, but further studies are clearly needed in order to determine the health risks associated with eating these contaminated Japanese clams.

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短　報

パンに含まれるノロウイルスの回収法の検討

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Key words: norovirus, bread

序　文

二枚貝以外の食品が原因であると推定されるノロウイルス（NoV）による食中毒事件が急増しているが，これらの食品からのウイルス検出報告は少ない．特に学校給食における食中毒事件では，疫学的に原因食がパンと推定される事例が少なからずあるものの，パン自体からNoVを検出した事例は稀である．この原因の一つとして，パンからNoVを効率的に回収する方法が確立されていないことが挙げられる．実際，パンからウイルスを回収する場合，他の食品の場合と比べウイルスの回収率が極めて低いことが指摘されている[1)]．本研究では，学校給食で提供される機会が最も多いコッペパンをαアミラーゼで消化することで，付着しているNoVを効率良く回収する実験系を検討し，若干の知見を得たので以下に報告する．

材料および方法

NoV（genogroup II）は，患者便10%乳剤からショ糖密度勾配法にて精製し，PBS（−）に浮遊させた．精製して得た9.4×10^5コピー/100μLおよび同液から希釈して得た理論上9.4×10^4コピー/100μL，9.4×10^3コピー/100μLの濃度のgenogroup IIのNoV浮遊液をコッペパン12.5gの表面に100μL塗布し，30分間室温に放置後さらに4℃で一夜放置した．パン表面を3g採取し，PBS（−）で10%乳剤を作成し，αアミラーゼ（Wako）溶液を0mg/mL，5mg/mL，10mg/mLおよび30mg/mLの濃度になるよう添加し，室温で30分間振とう後4℃で一晩静置した（Fig. 1）．αアミラーゼで処理した乳剤を9,100×gで4℃20分間遠心し，野田らの方法[2)]により，NaClを最終濃度1M，ポリエチレングリコール6000を最終濃度12%となるよう上清に加え，4℃で一晩静置し，9,100×gで4℃20分間遠心した．沈渣を140μLのdoubly distilled waterに再懸濁し，QIAamp viral RNA mini kit（QIAGEN）を用いてRNAを抽出し，1単位のDNase I（Takara）による37℃30分間の処理後，ランダムヘキサマー（Promega）を用いてSuperScriptII（Invitrogen）によりcDNAを作成し，COG2F，COG2Rプライマーおよび RING2-TPTaqMan プローブを用いたKageyamaらによる方法[3)]によるリアルタイムPCR法でウイルスゲノムの定量を行った．厚生労働省通知平成15年11月5日食安監発第1105001号に基づき，実測値で10コピー（希釈率を補正した換算値で400コピーに相当）を検出限界とした．対照として，25cm²のサランラップに塗布したNoVのPBS（−）による回収試験も行った（Fig. 1）．また，αアミラーゼ処理によるウイルスの抽出・回収以外の方法として，ダクロン綿棒を用いたパン表面のふき取りによるウイルスの回

Fig. 1　Scheme of NoV recovery from bread

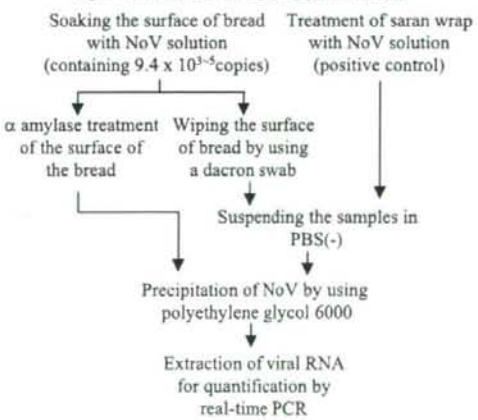

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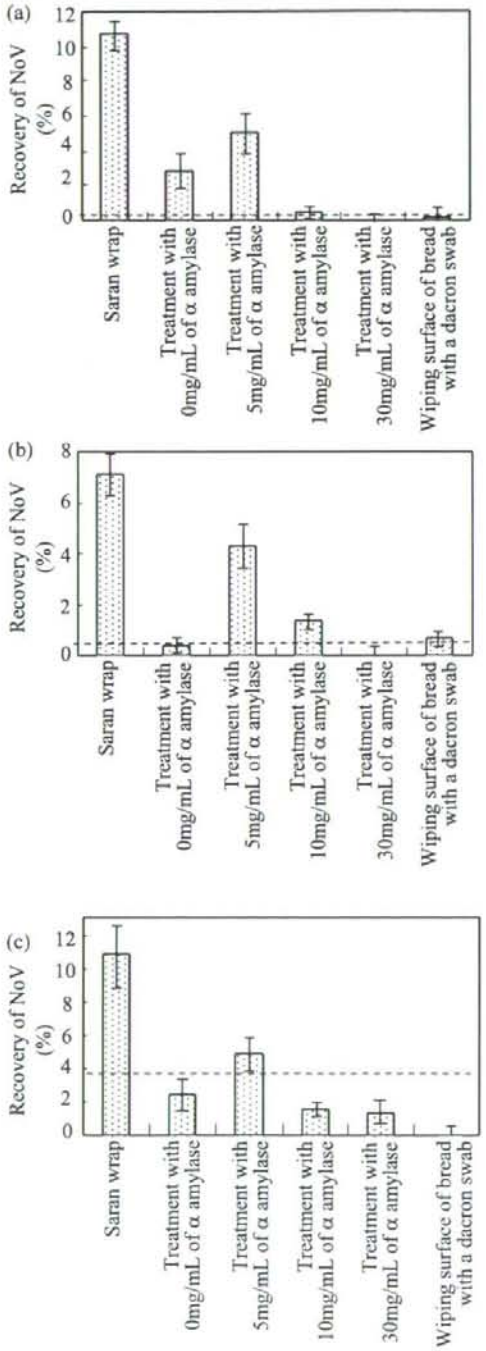
Fig. 2 Efficiency of NoV recovery (n=6)
Efficiency of NoV recovery from bread soaked with NoV solution containing (a) 9.4×10^5 copies, (b) 9.4×10^4 copies, and (c) 9.4×10^3 copies of NoV genome is shown with standard error. Dotted line : limit of detection.



収を試みた (Fig. 1).

成 績

NoV 回収試験の成績を Fig. 2に示した. サランラップからの回収率は 10% 前後であった. パンへの NoV の添加量にかかわらず, αアミラーゼ処理は 5mg/mL の濃度の時に回収率が 5% 前後と最大になり, ダクロン綿棒を用いた回収法よりも高い回収率を得た.

考 察

今回の実験においては, パンを処理する際のαアミラーゼの至適濃度は 5mg/mL であり, それ以上の濃度で添加した場合には NoV の回収率が低下した. 高濃度のアミラーゼで処理を行ったサンプルでは, 遠心操作によって上清と沈殿を完全に分離できなかった. そのため, 回収した上清中に混入した沈殿物由来成分が, その後の NoV 回収行程を阻害した可能性がある.

森らはネコカリシウイルスを用いたパンからの添加回収試験で, 従来法で 0.1% 以下, 界面活性剤を用いた方法で 0.5% の回収率であったと報告している[1]. 一方, 今回の実験では, αアミラーゼ処理をパンに行うことにより, 5% 前後の NoV 回収が可能になった. 多くの NoV 食中毒患者からは, 便 1g 当たりゲノム数に換算して, 10^8 から 10^9 コピーに相当する多量のウイルスが排泄されると考えられている[4]. また, NoV の感染力は強く, 100 粒子以下のウイルス量で感染が成立すると考えられている[5]. このため, NoV は極めて微量の便の食品汚染でも食中毒を発生しうることを我々は指摘している[4]. 理論上, 本法を用いることで, 仮に 1g 中 10^8 コピーの NoV ゲノムを含む便が食品取扱者の手からパンに 0.1mg 付着した場合でも (パン全体に 10^4 粒子の NoV が付着した場合), NoV の検出が可能であると考えられる. しかし, 回収率が 5% 程度であるので, さらなる回収法の改善が必要であると思われる.

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A Study of Recovery of Norovirus from Bread

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