TABLE I. RT-PCR and IgG/IgM Responses to CCHFV rNP in the Serum Samples Drawn From Nine "Panel A" Patients With CCHF as Diagnosed by Positive RT-PCR

	Day(s) after onset, RT-PCR, IgM, and IgG ELISA							
	1st samples (collected within day 3)				2nd samples (collected between days 5 and 11)			
Patient ID	Day(s)[a]	RT-PCR[b]	IgM (OD_{405})[c]	IgG (OD_{405})[d]	Day(s)[a]	RT-PCR[b]	IgM (OD_{405})[c]	IgG (OD_{405})[d]
1	2	+	−(0.035)	−(0.071)	8	+	−(0.065)	−(0.059)
2	2	+	−(0.015)	−(0.071)	10	+	+(0.216)	−(0.058)
3	2	+	−(0.021)	−(0.059)	8	−	+(1.433)	−(0.160)
4[e]	1	+	−(0.020)	−(0.031)	5	−	+(2.711)	+(0.972)
5	3	+	(0.000)	(0.007)	11	+	+(1.227)	+(0.235)
6	UK[f]	+	−(0.060)	−(0.181)		ND[g]		
7	UK	+	(0.016)	(0.024)		ND		
8	UK	+	+(2.208)	−(0.023)		ND		
9	UK	+	+(0.860)	−(0.019)		ND		

[a]Day(s) after onset was defined by taking the day on which fever first appeared as day 1.
[b]"+" and "−" indicate positive and negative reactions in RT-PCR, respectively.
[c]"+" and "−" indicate positive and negative reactions in IgM-capture ELISA, respectively. The OD_{405} values at a dilution level of 1:100 are shown.
[d]"+" and "−" indicate positive and negative reactions in IgG ELISA, respectively. The OD_{405} values at a dilution level of 1:400 are shown.
[e]This case was reported in the previous report [Tang et al., 2003].
[f]"UK" indicates unknown.
[g]"ND" indicates not drawn.

set of samples and four in the second set of samples (Table I). Positive IgM responses were demonstrated in four of the five patients (Patients 1–5) from whom 2nd samples were collected, but no IgM responses were demonstrated in any of the five 1st samples from these patients (Table I). The IgM antibody to CCHFV was not detected in Patient 1 within 8 days from onset.

Of the nine patients, in whom the CCHFV genome was amplified, a significant rise in IgG antibody titer was demonstrated in two patients (Patients 4 and 5, Table I). Furthermore, a significant rise in IgG antibody titer was demonstrated in 4 individuals (Patients 10–13, Table II) of the 14 "Panel B" patients from whom a paired serum sample was collected. In summary, IgG responses defined as a positive significant rise in IgG antibody titer were demonstrated in six patients. The CCHFV rNP-based IgM-capture ELISA showed a positive reaction in the sera collected from all of the 6 patients (Table II), while it did not show a positive reaction in the sera collected from the other 10 "Panel B" patients, in whom IgG responses were not demonstrated.

Relationship of IgM, IgG Responses, and Viremia

The 1st and 2nd blood samples were drawn from five of the "Panel A" patients. The relationship between the time of sample collection after onset and antibody responses was evaluated in these patients.

IgM and IgG responses were demonstrated in four (80%) and two (40%) of the 2nd samples collected between day 5 and day 11, respectively, while no antibody responses were demonstrated in the 1st samples collected within 3 days after onset (Table I). On the other hand, all the 1st samples and two of the 2nd samples showed a positive reaction in RT-PCR (Table I).

Antibody Responses and Viremia

The relationship between the IgM responses and viremia was evaluated using the serum samples collected from the "Panel A" patients (Table I). Only 3 of the 11 RT-PCR-positive sera (27%) were positive by the IgM-capture ELISA, while all of the 3 RT-PCR-

TABLE II. IgM Responses to CCHFV rNP in Six Patients With CCHF Diagnosed by Positive IgG Responses Determined by CCHFV rNP-Based IgG ELISA

		OD_{405}s in IgM-capture (1:100), IgG ELISAs (1:100), and antibody titers					
		1st samples (within day 3)		2nd samples (days 8–14)		3rd samples (3–4 weeks from onset)	
Panel	Patient ID	IgM	IgG	IgM	IgG	IgM	IgG
A	4	0.020, <50	0.031, <100	2.711, ≥400	0.972, 1,600	ND[a]	ND
	5	0.000, <50	0.007, <100	1.227, ≥400	0.235, 400	ND	ND
B	10	0.159, <50	0.181, <100	0.359, 200	1.412, >6,400	ND	ND
	11	0.184, <50	0.128, <100	1.077, ≥400	1.408, >6,400	ND	ND
	12	ND	ND	3.129, >400	0.119, <100	2.761, >400	>3.500, >6,400
	13	ND	ND	3.147, ≥400	0.367, 400	2.669, ≥400	1.943, ≥6,400

[a]"ND" indicates not drawn.

negative samples were positive by the IgM-capture ELISA (Table I). In contrast, viral RNA was amplified by RT-PCR in all of the 11 IgM-negative and IgG-negative, in 3 of the 4 IgM-positive and IgG-negative samples, and in neither of the 2 IgM-positive and IgG-positive samples.

IgM and IgG Antibodies to CCHFV Among Residents in CCHF Endemic Area During the Outbreak Season

Of the 61 "Panel C" sera, 5 showed positive reactions by both IgM-capture and IgG ELISAs, 1 showed positive reaction by the IgM-capture ELISA only, 13 showed positive reactions by the IgG ELISA only, and the rest showed negative reactions by both IgM-capture and IgG ELISAs. Clinical manifestations in four of the six IgM-positive patients were available. All four patients had fever, headache, and backache. Two of the four had symptoms of nasal and gingival hemorrhage.

DISCUSSION

Patients with CCHF are usually seen in very remote areas and the number of patients with CCHF is relatively small. Patients with suspected CCHF do not always visit hospitals for treatment in CCHF endemic areas, because of economic difficulties and problems in gaining access to hospitals. Furthermore, the facilities at the hospitals in such remote areas are usually not adequate for virological testing or for storing serum samples. Therefore, virological testing of CCHF, and the collection and storage of blood samples are very difficult. Under such difficult conditions, the serum samples of patients with and without CCHF in one CCHF endemic area in the western part of the Xinjiang Uygur Autonomous Region were collected. The serum samples collected from 132 subjects including 13 CCHF-patients were used in the present study. Therefore, it is considered to be acceptable to draw conclusions regarding the efficacy of the rNP-based ELISA for serological diagnosis of and epidemiological study on CCHF by analyzing the data obtained in this study.

The patients with positive IgG responses, taken as the detection of a significant rise in IgG antibody titer determined by CCHFV rNP-based IgG ELISA, are considered to be CCHF-positive patients, because the IgG ELISA has been confirmed to have high sensitivity and specificity in detecting specific IgG antibodies to CCHFV [Saijo et al., 2002b]. Within this limited number of patients, the efficacy of the CCHFV rNP-based IgM ELISA in diagnosis of CCHF and the relationship between time after onset, antibody responses, and viremia were evaluated.

Six of the nine "Panel A" patients were confirmed to be IgM-positive by CCHFV rNP-based IgM-capture ELISA. The blood samples of the other three "Panel A" patients with IgM-negative result were collected within about 10 days after onset. If the blood had been collected a little later, IgM responses to CCHFV rNP would have been detected. IgM antibodies to CCHFV were detected in all the 6 patients with IgG responses to CCHFV (Table II), while no IgM antibodies were detected in any of the other 10 "Panel B" individuals in whom IgG responses not demonstrated. IgM antibodies to CCHFV were demonstrated in 6 of the 61 serum samples collected from the "Panel C" residents living in a CCHF endemic area during the outbreak season, while none of the Japanese sera showed a positive reaction in the IgM-capture ELISA. These data indicate that the IgM-capture ELISA using His-CCHFV rNP has high sensitivity and specificity in detecting IgM antibodies to CCHFV and that it is useful for the rapid and accurate diagnosis of CCHF. If the sensitivity and specificity are analyzed using the paired sera collected from 14 "Panel B" subjects composed of 4 CCHF-patients and 10 non-CCHF-patients, both the sensitivity and specificity would be considered to be 100%.

Although, further study is needed, the data in the present study suggest that the IgM antibodies to CCHFV become detectable within at least 2 weeks from onset. It was found that viremia was still present at the stage in which positive IgM responses but not IgG responses were observed and that the viremia was already eliminated at the stage in which positive IgG responses were observed. To increase the sensitivity in detecting viremia, nested RT-PCR was performed for serum samples. However, mononuclear phagocytes are one of the main targets of CCHFV infections [Burt et al., 1997]. Therefore, it is possible that the sensitivity of the nested RT-PCR for detection of the CCHFV genome increases when whole blood samples rather than serum samples are used.

The effectiveness of the newly developed CCHFV rNP-based IgM-capture ELISA for the diagnosis of CCHF should be compared with that of the already developed methods such as indirect immunofluorescence assay [Fisher-Hoch et al., 1992; Burt et al., 1998; Papa et al., 2002] and/or IgM-capture ELISA [Saluzzo and Le Guenno, 1987; Gonzalez et al., 1990; Chapman et al., 1991; Burt et al., 1994; Khan et al., 1997; Rodriguez et al., 1997; Schwarz et al., 1997] using authentic CCHFV antigens. However, CCHFV is regarded as a biosafety level-4 (BLS-4) pathogen in Japan, and viral antigen preparation is difficult. Therefore, the CCHFV rNP-based IgM-capture ELISA was not compared with IgM antibody detection systems using authentic viral antigens. The inability to produce authentic viral antigens was overcome by evaluating the CCHFV rNP-based IgM capture ELISA using the sera collected from patients with and without CCHF.

The main antigenic region in the NP of CCHFV (482 amino acid residues) was located on the central portion from amino acid positions 201 to 306 as reported previously [Saijo et al., 2002b]. The amino acid sequence of this region in NP (Chinese strain 8402) has 97%–100% homology to the other Chinese strains and 92%–96% homology to non-Chinese strains. Furthermore, the antibodies to CCHFV in Asian patients with CCHF were detected by the IgG ELISA using the recombinant NP of CCHFV strain IbAr 10200 (GenBank accession no.

U88410, data not shown). These results indicate that the IgM-capture ELISA using the CCHFV rNP of Chinese strain 8402 might be useful in detecting not only antibodies to CCHFV Chinese strains but also antibodies to strains in other regions.

As CCHFV is a BSL-4 pathogen, the preparation of the CCHFV antigen must be performed in a BSL-4 laboratory and this restriction makes the preparation of CCHF antigens difficult in institutes without a BLS-4 laboratory. It was demonstrated that the rNP-based IgM-capture ELISA offers a definite advantage in the diagnosis of and seroepidemiological study on CCHF. In summary, diagnosis by the combination of IgM-capture ELISA and RT-PCR is more sensitive, accurate, and reliable than that by either single method.

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Rapid and Sensitive Detection of Mumps Virus RNA Directly From Clinical Samples by Real-Time PCR

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A rapid, sensitive, and specific assay to detect mumps virus RNA directly from clinical specimens using a real-time PCR assay was developed. The assay was capable of detecting five copies of standard plasmid containing cDNA from the mumps virus F gene. No cross-reactions were observed with other members of *Paramyxoviridae*, or with viruses or bacteria known to be meningitis pathogens. Seventy-three clinical samples consisting of throat swabs collected from patients with parotitis, and cerebrospinal fluid (CSF) collected from patients with aseptic meningitis, were examined with a real-time PCR assay developed by the authors, reverse-transcription nested-PCR (RT-n-PCR), and virus isolation using cell culture. Like the RT-n-PCR assay, the real-time PCR assay could detect mumps virus RNA in approximately 70% of both throat swabs and CSF samples, while, by tissue culture, mumps virus was isolated from only approximately 20% of CSF and 50% of throat swab samples. In addition, the real-time PCR assay could be developed easily into a quantitative assay for clinical specimens containing more than 1,800 copies of mumps virus RNA/ml by using serial dilutions of the standard plasmid. The results suggest that the real-time PCR assay is useful for identification of mumps virus infections, not only in typical cases, but also in suspected cases, which show only symptoms of meningitis or encephalitis. ***J. Med. Virol. 75: 470–474, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: mumps meningitis; TaqMan PCR; quantitative assay

INTRODUCTION

Mumps virus is a member of the family *Paramyxoviridae*, genus *Rubulavirus* [for review, see Rima et al., 1995; Elango et al., 1988], and its genome is a single strand of RNA with negative polarity, approximately 15 kb in length, encoding seven proteins in the following order: 3′-nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN), and large protein (L)-5′. Mumps virus is a common human pathogen that causes childhood parotitis and also, frequently, complications such as meningitis, pancreatitis, and orchitis. The majority of mumps infections are benign; however, the disease causes more serious and rare complications such as encephalitis, cerebellar ataxia [Cohen et al., 1992], and hearing loss [Okamoto et al., 1994]. Only a few methods have hitherto been available for laboratory diagnosis of mumps virus infections, one based on the detection of mumps virus-specific IgM antibodies in serum, and the other, on virus isolation using tissue culture. Only a few commercial kits for serological tests such as enzyme immunoassay are available, other than methods such as hemagglutination inhibition, immunofluorescence, and complement-fixation assay, which are laborious. Moreover, mumps virus infections may occur without the induction of the specific IgM antibodies, particularly in patients vaccinated previously or in reinfection cases [Gut et al., 1995], and false positive results due to cross-reactivity with other paramyxoviruses complicate serological diagnosis [Julkunen, 1984]. On the other hand, the procedure for mumps virus isolation from clinical samples lacks sensitivity and is time-consuming. A few investigators have reported that the rates of isolation from CSF and nasopharyngeal swab (NPS) samples obtained from patients with confirmed mumps virus infections were about 30–40% and 50%, respectively [Kashiwagi et al., 1997; Poggio et al., 2000]. They also described the development of the reverse transcription (RT)-nested PCR (n-PCR) procedures for rapid and sensitive detection of mumps virus RNA directly from clinical samples, and found that the rate of detection of

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the virus could be markedly increased [Kashiwagi et al., 1997; Jin et al., 1999; Poggio et al., 2000].

The purpose of this study is to describe a rapid and sensitive method of detecting mumps virus RNA directly from clinical samples using a real-time PCR assay. The assay does not require nested PCR procedures to obtain sensitivity, restriction fragment length polymorphism (RFLP), or nucleotide sequencing for identification of the amplified products. The real-time PCR assay described below was evaluated by testing clinical samples consisting of throat swab samples and cerebrospinal fluid (CSF) samples. Also, the results are compared with those from virus isolation and the RT-n-PCR assay described previously.

MATERIALS AND METHODS

Clinical Samples and Mumps Virus Stock

The clinical samples used consisted of 46 throat swab samples obtained from 46 patients with parotitis and 27 CSF samples obtained from 27 patients with aseptic meningitis. Of the meningitis patients, 24 had salivary gland swelling, two patients had had mumps vaccination 2 weeks before onset, and one patient did not show salivary gland swelling. Throat swab samples, which were shown by isolation or by PCR to be positive for other viral or bacterial agents, were used to confirm the specificity of the real-time PCR. They include enteroviruses (poliovirus 1–3, coxsackievirus A and B, enterovirus 71, and echoviruses), influenza A virus (AH1N1, AH3N2), influenza B virus, measles virus, human parainfluenza virus 1–3, human respiratory-syncytial virus, *Listeria monocytogenes, Neisseria meningitidis, Haemophilus influenzae, Streptococcus agalactiae*. Between 10^3 and 10^4 copies of DNA of human herpesviruses 1–6 (Advanced Biotechnologies, Inc., Columbia, MD) and *Escherichia coli, Streptococcus pneumoniae, Staphylococcus aureus* (American type culture collection) were also tested. All of these clinical samples were collected from clinics and hospitals in the Saitama Prefecture, Japan, during the years 1997–2003. Throat swab samples were obtained by wiping the throat with cotton swabs and were soaked in 2 ml of 2.5% veal infusion broth (Becton Dickinson Company, Ltd., Franklin Lakes, NJ) solution with antibiotics and bovine serum albumin. These samples were kept at −80°C until used in virus isolation. They were thawed once for virus isolation, then frozen again and kept at −80°C until used in RNA extraction. For the mumps virus stock, SA963/Ja00 strain [Uchida et al., 2003] was propagated in Vero cells, and the virus stock was stored in 0.5-ml aliquots at −80°C. Plaque titration of the virus stock was done using confluent Vero cells in 6-well tissue culture plates.

Virus Isolation

Virus isolation was performed using Vero and BS-C-1 cell cultures. Cell monolayers on a 24-well tissue culture plate with 1 ml minimal essential medium containing 0.001% acetylated trypsin were inoculated with 0.1 ml of the clinical samples. The cell monolayers were incubated at 37°C for 7 days and were examined daily for the appearance of cytopathic effects (CPE). The samples were passaged twice if no CPE were observed. The presence of mumps virus in cultures showing typical CPE was confirmed by a neutralization test using mumps virus antibody (Denka Seiken Co., Ltd., Tokyo, Japan). Samples without CPE after two blind passages were considered negative for virus isolation.

RNA Extraction and cDNA Synthesis

Viral RNA was extracted from 140 μl of a clinical sample using a QIAamp Viral RNA mini kit (QIAGEN Corp., Inc., Hilden, Germany) according to the manufacturer's instructions. RNA was eluted with 60 μl of diethyl pyrocarbonate-treated water. The cDNA was synthesized in 20 μl of a reaction mixture containing 12 μl of eluted RNA, 200 U of SuperScript II RNase H(−) reverse transcriptase (Invitrogen Corp., Carlsbad, CA) and first strand buffer solution supplied with the enzyme, 0.5 mM of each dNTP mix, 5 mM dithiothreitol, 50 pmol of random hexamers (Invitrogen), and 20 U of RNasin ribonuclease inhibitor (Promega Corp., Madison, WI). The mixture was incubated at 42°C for 2 hr, and the enzyme was inactivated at 95°C for 10 min.

Real-Time PCR and RT-n-PCR

For the real-time PCR assay, primers F1073 (5′-TCTCACCCATAGCAGGGAGTTATAT-3′) and R1151 (5′-GTTAGACTTCGACAGTTTGCAACAA-3′) and a TaqMan probe (5′-AGGCGATTTGTA GCACTGGATG-GAACA-3′) were designed from the published sequence of the mumps virus F gene from the SA956/Ja00 strain (DDBJ accession number AB085219) [Uchida et al., 2003] using Primer Express software version 1.5 (Applied Biosystems, Foster City, CA). The probe was synthesized with a reporter dye, 6-carboxyfluorescein (FAM), and a quencher dye, 6-carboxytetramethylrhodamine (TAMRA), covalently linked to the 5′ and 3′ ends, respectively (Applied Biosystems). The real-time quantitative PCR was carried out in a final volume of 35 μl reaction mixture containing 4 μl of cDNA, 17.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), each primer at a concentration of 300 nM, and the probe at 100 nM. PCR amplification was performed with an ABI PRISM 7700 Sequence Detector (Applied Biosystems) under the following conditions: initial incubation at 50°C for 2 min and 95°C for 10 min, and then 45 cycles of amplification with denaturation at 95°C for 15 sec and annealing and extension at 59°C for 1 min. Amplification data were collected and analyzed with Sequence Detector software version 1.7 (Applied Biosystems). In each operation, a recombinant plasmid containing one copy of the mumps virus F gene was used as a standard plasmid. To construct the standard plasmids, RNA from the mumps virus strain SA956/Ja00 was transcribed to cDNA, then the cDNA was amplified with primers F8 and F9, described previously [Uchida et al., 2003]. The amplified product

was cloned using the PCR cloning kit (QIAGEN) according to the manufacturer's instructions. The concentration of the purified plasmid DNA was determined with a spectrophotometer at 260 nm and the corresponding number of copies was calculated.

The RT-n-PCR assay was carried out using primer sets SH1 and SH2R for the first amplification and SH3 and SH4R for the nested amplification as described previously [Jin et al., 1999]. The first amplification was carried out in 50 μl of reaction mixture containing 4 μl of cDNA, 10 pmol of each primer (SH1 and SH2R), 1.25 U of Taq DNA polymerase (Promega), 5 μl of 10× buffer supplied with the enzyme, 2 mM $MgCl_2$, and each dNTP at 0.2 mM. The amplification was performed under the following conditions: after denaturation for 5 min at 95°C, 35 cycles of 1 min at 95°C, 1.5 min at 50°C, 2 min at 72°C, and a final extension for 15 min at 72°C were performed. Nested amplification was performed using 2 μl of the 1st amplified product under the same conditions as the 1st amplification. The amplified products were electrophoresed through 2% agarose gels, visualized by staining with ethidium bromide, and confirmed by direct sequencing with an ABI 310 sequencer as described previously [Uchida et al., 2001].

RESULTS

Sensitivity and Specificity of the Real-Time PCR Assay

The sensitivity of the real-time PCR assay was evaluated by using serial dilutions of the standard plasmids. Five replicates of the plasmid dilutions containing 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 copies and 1 copy were tested. One copy of the plasmid was detected once (20% sensitivity) in five operations, while 10 copies of the plasmid were detected with 100% sensitivity with a threshold cycle (Ct value; the cycle at which a significant increment of a fluorescent signal is first detected) ranging from 35.0 to 36.0. Inverse linear correlation was observed between the Ct values and the logarithm of the plasmid copy number from 10^6 to 10^1 (Fig. 1). The coefficient of variation (CV; the ratio between the standard deviation and the mean of repeated measurements) of the Ct values was from 0.9% (for 10^5 copies) to 1.1% (for 10 copies). Five copies of the standard plasmid were also tested in the same way and were detected with 100% sensitivity in five replicates with Ct values ranging from 36.1 to 39.0. The average and CV of the Ct values were 37.6% and 2.8%, respectively.

Tenfold serial dilutions of mumps virus stock (SA963/Ja00 strain) were also examined by the real-time PCR assay and RT-n-PCR. The viral RNA was extracted from 140 μl of each dilution of the virus stock. In three operations, real-time PCR twice detected viral RNA in 10^6-fold dilution (0.5 PFU/ml) of the virus stock, and RT-n-PCR detected once. The virus stock was examined after additional freezing and thawing, and the real-time PCR and the RT-n-PCR detected viral RNA in a 10^6-fold dilution of the virus stock as it had before, but the virus titer of the stock was decreased from 1/2 to 1/10 of the previous PFU/ml (data not shown).

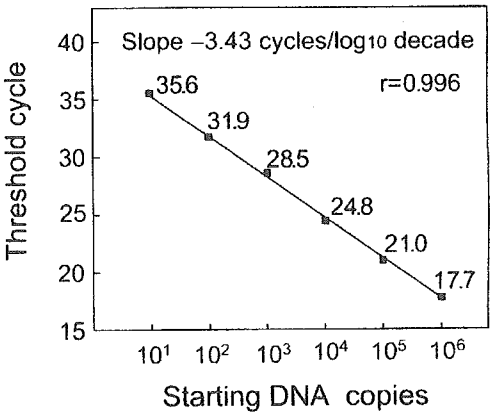

Fig. 1. Standard curve for mumps virus cDNA quantification. Tenfold serial dilutions ranging from 10^6 to 10 copies of the standard plasmid were tested in five replicates, and the mean Ct values were plotted against the copy number. The correlation coefficient (CC) was consistently greater than 0.995.

The specificity of the real-time PCR was evaluated by analyzing bacterial and viral agents, which frequently cause meningitis or encephalitis. No cross-reactions were observed with any of the viruses or bacteria described above (data not shown).

Detection of Mumps Virus in Clinical Samples

A total of 73 clinical samples (46 throat swab samples and 27 CSF samples) were tested using tissue culture, RT-n-PCR and real-time PCR assays. Mumps virus was isolated from 24 throat swabs (52.2%) and 6 CSF (22.2%) samples by culture, while mumps virus RNA was detected in 33 throat swab samples (71.7%) and 19 (70.4%) of the CSF samples by RT-n-PCR.

For the real-time PCR assay, the standard plasmids containing 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 per reaction tube were used to draw a standard curve in each operation, while five copies of the plasmid were used for the detection limit, because the plasmid could be detected with 100% sensitivity. Twenty of 27 (74.1%) CSF and 33 of 46 (71.7%) throat swab samples showed positive results in the real-time PCR assay. All throat swab and CSF samples found by culture or by RT-n-RCR to be positive for mumps virus were also positive with real-time PCR (Fig. 2). The 20 CSF and 33 throat swab samples positive for the virus according to real-time PCR had Ct values ranging from 28.8 to 37.0 and from 23.5 to 36.2, respectively. Of the positive samples, 7 CSF and 2 throat swab samples had larger Ct values than that of 10 copies of the plasmid, and were thus considered not to suitable for quantitative assay. In the samples with Ct values smaller than those of 10 copies of the plasmid, the estimated RNA copy number contained in 1 ml of the original samples ranged from 1,800 to 132,000 copies for CSF samples, and from 3,600 to 4,063,000 copies for throat swab samples.

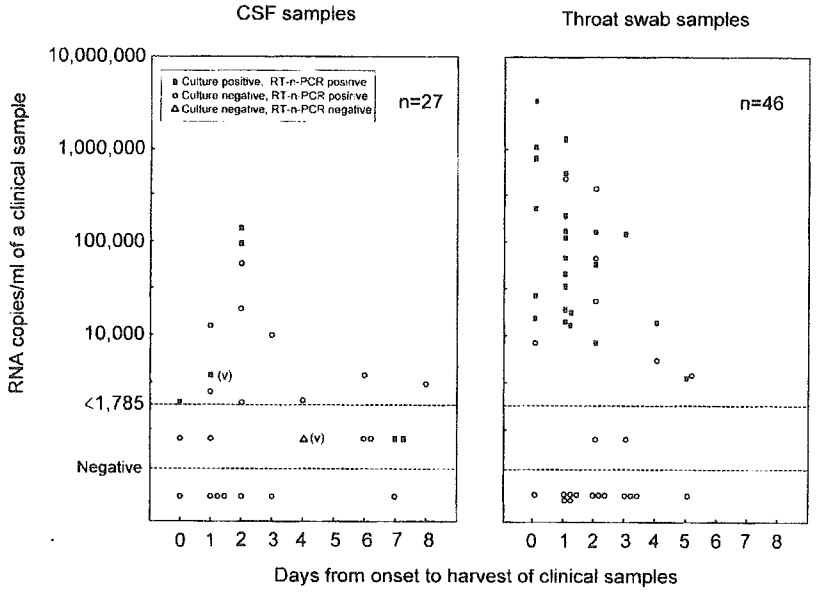

Fig. 2. Estimated mumps virus RNA copy number in clinical samples. The lower limit of quantitative assay was 1,785 copies RNA/ml of a clinical sample. (v) indicates a sample collected from a patient after mumps virus vaccination.

DISCUSSION

The general advantages of a real-time PCR assay are rapidity, high sensitivity, low risk of contamination, and possibility of quantitative assay. In this study, the experiments using the standard plasmid and other viral and bacterial agents demonstrated that the real-time PCR assay was (i) sensitive, detecting five copies of mumps virus cDNA, and (ii) specific, showing no cross-reaction with other agents. On the other hand, the examination using clinical samples showed that the real-time PCR assay detected mumps virus in approximately 70% of both CSF and throat swab samples, similar to RT-n-PCR, but mumps virus was isolated from about 20% of CSF and 50% of throat swab samples. This suggests that the real-time PCR assay is capable of sensitive and rapid laboratory diagnosis of mumps virus infections. The primers and the probe designated for the real-time PCR assay are located in the F gene, a highly conserved region among various mumps virus strains [Tecle et al., 2000]. Some strains registered in GenBank have only two or three nucleotide differences in the sequences used for the primers and the probe. It indicates that the primers and the probe are eligible for general use for mumps virus detection.

Among the well-known factors that influence the sensitivity of virus isolation are the number of infectious virus particles in the original sample, the presence of anti-mumps virus antibody in the original sample, increases of defective particles, and loss of virus viability through sample handling, such as freeze-thawing of samples. It is considered that these factors do not much affect the real-time PCR assay.

By making a standard curve with a known number of the standard plasmids, the real-time PCR assay makes it possible to estimate the number of copies of mumps virus RNA in the original sample. For example, a Ct value of 35.0–36.0 is equivalent to 10 copies of the standard plasmid in a reaction tube and corresponds to approximately 1,800 copies of mumps virus RNA per ml of the original sample, assuming 100% efficiency of the reverse transcription step. The real-time PCR could detect 5 copies of standard plasmid; however, the presence of more than 10 copies of the cDNA per reaction tube was suitable for quantitative assay, as described in various studies [Gault et al., 2001; Zhao et al., 2002; Kageyama et al., 2003], because the CV calculated from Ct values of 5 copies of the standard plasmid showed a larger variation than that of more than 10 copies of the plasmid. There were a few samples in this study, in particular CSF samples that showed a significant increase of fluorescence signals repeatedly and Ct values from 36.0 to 37.0, larger than the Ct values for 10 copies of the plasmids. Therefore, to set a detection limit, five copies of the plasmid, which yielded Ct values of 36–39 were used, and the results corresponded well to the results of the RT-n-PCR. One sample was positive on real-time PCR but negative on RT-n-PCR. The results of the assay using the virus stock suggest that the real-time PCR assay is slightly more sensitive than RT-n-PCR. This may cause the discordant results in some samples containing only a small number of mumps virus RNA copies.

It may be interesting to consider the factors that yield differences in the number of copies of mumps virus RNA in each sample. These may be the method of

obtaining throat swab samples, the period from onset to harvesting of samples, and differences of the severity in clinical symptoms in each patient. In this study, the CSF and throat swab samples were harvested from 0 to 8 days and from 0 to 5 days after onset, respectively. In a limited number of the samples examined, the viral RNA concentration in the CSF samples seemed to be higher when they were harvested 2 days after onset. On the other hand, the viral RNA concentration in throat swab samples had a wide range from 3.6×10^3/ml to 4.0×10^6/ml, and the difference of the viral RNA concentrations of samples due to the period from onset to harvest was not clear. Though these samples were obtained from different patients, little information on the severity of their clinical symptoms was obtained. More clinical samples will be required to reveal the correlation between the concentration of mumps virus RNA in the samples and features of the samples.

Some important aspects of mumps virus infections have been described, but information is still limited. These include the importance of mumps virus as a causative agent of central nervous system infections such as meningitis and encephalitis [Poggio et al., 2000] and other complications. The real-time PCR assay described here is rapid and sensitive with a low risk of contamination, and is capable of development into a quantitative assay. It will be useful for identification of not only suspected cases of mumps, but also of atypical and more significant cases including encephalitis and cerebellar ataxia, and in future investigations of the mumps virus infections.

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Discrimination of *Listeria monocytogenes* contaminated commercial Japanese meats

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Abstract

Discrimination was attempted on 14 *Listeria monocytogenes* strains isolated from commercially available Japanese pork and chicken. Examination of the isolates was performed by restriction fragment length polymorphism (RFLP) analysis of the chromosomal DNA and amplified products and comparison of the nucleotide sequences of the amplified products. A polymorphism region containing the repeated sequences in the *iap* gene was amplified by the polymerase chain reaction (PCR). The genetic analyses could discriminate the 14 isolates in combination with traditional serotyping, and some strains isolated from different meats were confirmed to have a genetically close relationship. Genetic analyses used in the present study would be useful for the elucidation of the pathogen tracks from contaminated sources to humans and of the ecological niche in the food environment.

Keywords: *Listeria monocytogenes*; Meats; RFLP; Sequencing

1. Introduction

Listeriosis, a zoonosis caused by *Listeria monocytogenes* (Colburn et al., 1990; Boerlin and Piffaretti, 1991; Lawrence and Gilmour, 1995; Autio et al., 2000; Hoffman et al., 2003), has been recognized as a food-borne disease since the 1980s (Schlech et al., 1983; Fleming et al., 1985; Linnan et al., 1988). Since food-borne outbreaks of listeriosis have gradually increased in Europe and USA, the food-borne listeriosis was required decrease in number to half by 2010, and if possible before 2005 (FSIS: Food Safety and Inspection Service, US Department of Agriculture,

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Washington, 2001). The infection is generally opportunistic but shows severe septicemia, meningitis and encephalitis in the high-risk groups such as immunocompromised persons, pregnant women and newborns (Vazquez-Boland et al., 2001).

Although an outbreak has not been reported, and listeriosis is still now recognized as a sporadic infection in Japan, elucidation of the infection and/or the contamination source of listeriosis are required for the assessment of the contamination from the view point of good public health. The authors previously reported that the genetic diversity of the *iap* gene in EGD strain encoding an extracellular protein p60 (Kuhn and Goebel, 1989; Kohler et al., 1990) might be useful for the discrimination among the *L. monocytogenes* isolates from foods and patients by PCR restriction fragment length polymorphism (RFLP) analysis (Saito et al., 1998) and classification of the determined nucleotide sequences (Saito et al., 2000).

In the present study, discrimination of the *L. monocytogenes* strains isolated from commercial meats was first investigated by RFLP analysis of both the chromosomal DNA and amplified products of the *iap* gene, and then multiple sequence alignments of the products were performed. These results were compared among the isolates from the commercial meats as the basis of possible food-borne pathogens.

2. Materials and methods

The methods are briefly described here as the details have presented in previous reports (Saito et al., 1998, 2000; Ueda et al., 2002).

2.1. Isolation, identification and serotyping of L. monocytogenes from commercial meat

Fourteen strains used in the present study were selected within the strains isolated from a total of 84 samples of pork and chicken purchased (June 1996 to June 1998) from 17 shops in the Kanto area of Japan (Tokyo and other 5 prefectures). Six strains (11P1, 63P1, 75P1, 76P1, 76P2 and 78P1) were isolated from pork whereas the other strains (6C1, 9C1, 17C1, 23C1, 28C1, 72C1, 79C1 and 80C1) were isolated from chicken. UVM broth (MERCK, Tokyo, Japan), Palcam (MERCK) and Oxford (Nihon BD, Tokyo, Japan) agars were used for the preculture broth and the selection of *Listeria* spp., respectively. Identification was performed by the usual method, and then the serovar of the isolates was determined by the Sahumy method (Ueda et al., 2002). The 14 strains contained 3 strains of serovar 1/2a, 6 strains of 1/2b, 4 strains of 1/2c and one strain of 4b.

2.2. RFLP analysis of chromosomal DNA extracted from L. monocytogenes isolates

The chromosomal DNA was extracted and purified from the isolated strains as previously described (Saito et al., 1998). The DNA was digested with restriction enzymes of *Ban* III, *Pst* I and *Xba* I, according to the manufacturer's instructions (Toyobo, Tokyo, Japan). The reactants were separated on 0.8% agarose gel followed by staining with ethidium bromide (EtBr). DNA fragments on the gel were visualized using an UV transilluminator, and a photograph was taken of each gel.

2.3. Polymorphism analysis of PCR products

A part of the *iap* gene, which contains the repeated variable nucleotide sequences, was amplified by the polymerase chain reaction (PCR) using a pair of primers, SI3A (5′-ACTGGTTTCGTTAACGGTAAA-3′) and SI3B (5′-AGAACCTTGATTAGCATTCGT-3′) (Saito et al., 1998). The amplified product, which is 687bp in length for the fragment of the SV1/2a EGD (EGD) strain, was digested with the restriction enzymes of *Alu* I, *Alw*N I and *Fnu*4H I. The reactants were separated on 4% NuSieve GTG agarose gels (FMC Bio products, Rockland, ME) followed by staining with EtBr. DNA fragments on the gel were visualized using an UV transilluminator, and a photograph was taken of each gel.

2.4. Determination of the nucleotide sequence

A part of the amplified products was used to determine the nucleotide sequences using dye-primers of SI4AD (5′-AATACGGTGTTTCTGTTCAAG-3′) and SI4BD (5′-TTTAGTGTAACCAG- AGCAATC-3′) as previously described (Saito et al., 2000). A comparative analysis was carried out using the nucleotide sequences determined in this study and

that of the EGD strain as reported by Kohler et al. (GenBank accession number X52268).

3. Results

3.1. RFLP analysis of chromosomal DNA and PCR products from L. monocytogenes isolates

The chromosomal DNA extracted from the 14 isolates was digested with the restriction enzymes of *Xba* I, *Ban* III and *Pst* I and the RFLP analysis was attempted by agarose gel electrophoresis. The digested patterns by *Xba* I are shown in Fig. 1. The digested patterns appeared to be identical among each group of the 3 strains of serovar 1/2b (79C1, 6C1, 17C1) isolated from chickens and the 3 strains of serovar 1/2c (76P1, 75P1, 78P1) from pork (lanes 6 to 8 and 11 to 13, respectively), though other fingerprints showed different patterns from each other. The chicken-derived isolates, 79C1, 6C1 and 17C1, were purchased from different 3 stores but the pork-derived isolates, 76P1, 75P1 and 78P1, were purchased from the same store. The same fingerprint patterns among the 3 chicken isolates or pork isolates were also obtained by restriction enzyme digestion with *Ban* III and *Pst* I (data not shown).

RFLP analysis was also performed for the amplified products from a part of the *iap*. Fig. 2 shows the cleavage patterns of the 14 isolates obtained from digestion with *Alw*N I and the restriction map of the amplified product from the EGD strain. Three bands were observed on the agarose gels. The middle band, termed the B fragment in Fig. 2, showed variability in its size among the isolates, though the upper and lower bands, the A and C fragments, respectively, showed the same size among all the isolates. The length polymorphism of the B fragment showed that the isolates used in this study were classified into at least 5 types. The variability of the B fragment was

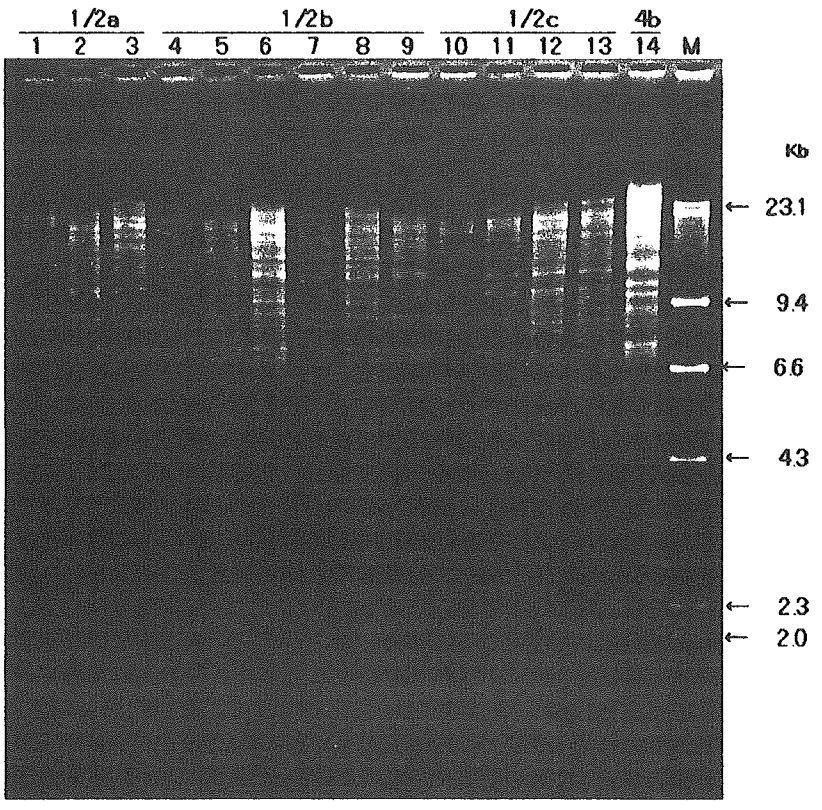

Fig. 1. Fingerprinting pattern of the chromosomal DNA digested by a restriction enzyme, *Xba* I. Lane number and the sample: (1) 11P1; (2) 76P2; (3) 80C1; (4) 9C1; (5) 72C1; (6) 79C1; (7) 6C1; 8, 17C1; (9) 63P1; (10) 23C1; (11) 76P1; (12) 75P1; (13) 78P1; (14) 28C1; M—size marker (λ-*Hin*d III digest).

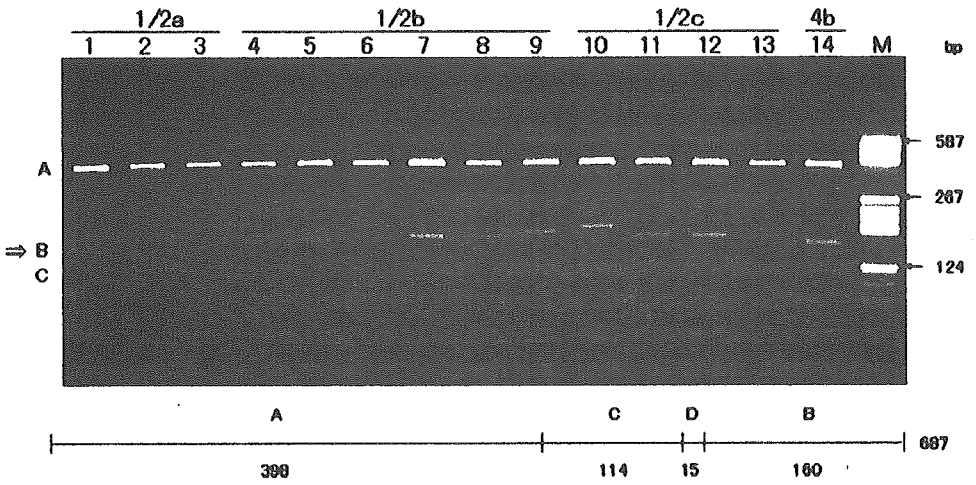

Fig. 2. RFLP analysis of the amplified products of the *iap* gene. The products were digested with *Alw*N I. The cleavage map of the EGD strain by the same restriction enzyme is shown under the photograph. Lane number and the sample: (1) 11P1; (2) 76P2; (3) 80C1; (4) 9C1; (5) 72C1; (6) 79C1; (7) 6C1; (8) 17C1; (9) 63P1; (10) 23C1; (11) 76P1; (12) 75P1; (13) 78P1; (14) 28C1; M—size marker (λ-*Hin*d III digest).

not likely to depend on the serovar of the isolate (see arrow in the photo). However, the B fragments of 3 strains from chickens (lanes 6 to 8) and 3 strains from pork (lanes 11 to 13) showed the same size among each group as shown by the RFLP analysis of the chromosomal DNA. RFLP analysis with *Fnu*4H I and *Alu* I also classified each chicken isolate and pork isolate into the same type.

3.2. Comparison of nucleotide sequences of PCR products from L. monocytogenes isolates

The nucleotide sequence was determined for the PCR products containing the B fragment showing genetic polymorphism by RFLP analysis. Multiple sequence alignments were carried out with nucleotide sequences determined in the present study and that of the EGD strain as previously described (Saito et al., 2000) (Fig. 3). A sequence variation containing nucleotide substitutions, insertions, deletions and number of repeated sequences was found among the isolates and EGD strain. The sequence variation is summarized in Fig. 4.

Nucleotide substitutions between the isolates from meats and EGD strain, as shown in the figure, were found in a total of 33 nucleotide positions. The isolates investigated in the present study were divided into 2 groups by the frequency of the nucleotide substitutions; one of the groups has substitutions of less than 10 positions (7 isolates) and the others were of more than 20 positions (7 isolates), though no isolates were observed which had a number of substitutions between 10 and 20 positions. Almost all substitutions of 7 higher diversity isolates to the EGD strain were located out of the repeated sequence in the variable region of the *iap* gene. No nucleotide substitutions were found among 3 isolates, 76P1, 75P1 and 78P1 from pork that were closely related to each other by RFLP analysis of both the chromosomal DNA and PCR products. Three isolates, 79C1, 6C1 and 17C1, from chicken had the same substitutions in 23 positions, and the chicken-derived 9C1 also had the same 23 substitutions. The identical nucleotide substitutions were also found in isolates without a common historical background, as far as we know, such as between pork-derived 11P1 and chicken-derived 80C1 and between 2 chicken-derived strains, 72C1 and 28C1.

Nucleotide insertions and deletions were found in some strains. The highly divergent group had the insertion of 3 nucleotides (AAT) after nucleotide position 1282 of the EGD strain and the deletion of 3 nucleotides at positions 1307 to 1309 (GCA), which are located upstream of the repeated sequence. However, the deletion of 3 nucleotides was found in the sequences of the 63P1 and 23C1 isolates in the repeated sequence. No nucleotide insertions and deletions were found among the nucleotide sequences of the pork-derived 76P1, 75P1 and 78P1, and among those of the chicken-derived 79C1, 6C1 and 17C1 as shown by the genetic analysis described above.

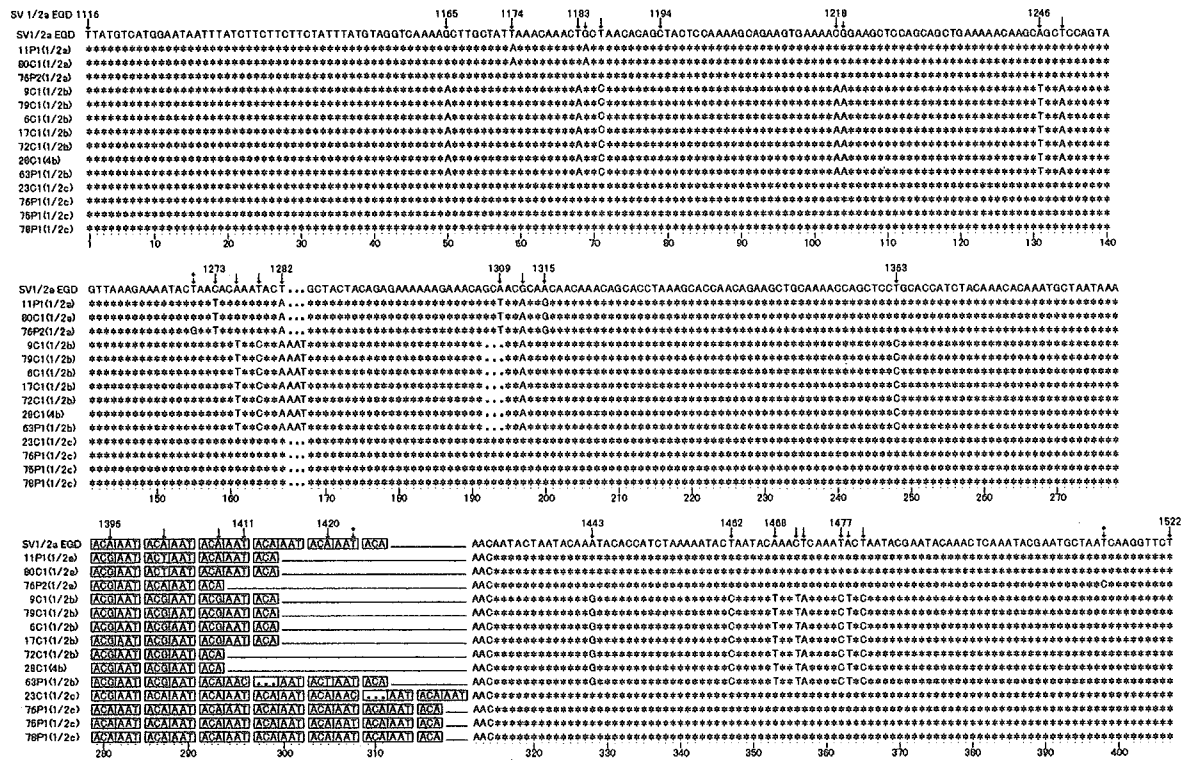

Fig. 3. The nucleotide sequences for *iap* gene in *L. monocytogenes* isolates. Nucleotides identical to those of the SV1/2a EGD strain and gaps are indicated as asterisks and bars, respectively. Nucleotide numbers above the nucleotide sequences refered the report by Kohler et al. Boxes of the nucleotide positions from 1394 to 1426 show the repeat sequence structures in the *iap* gene.

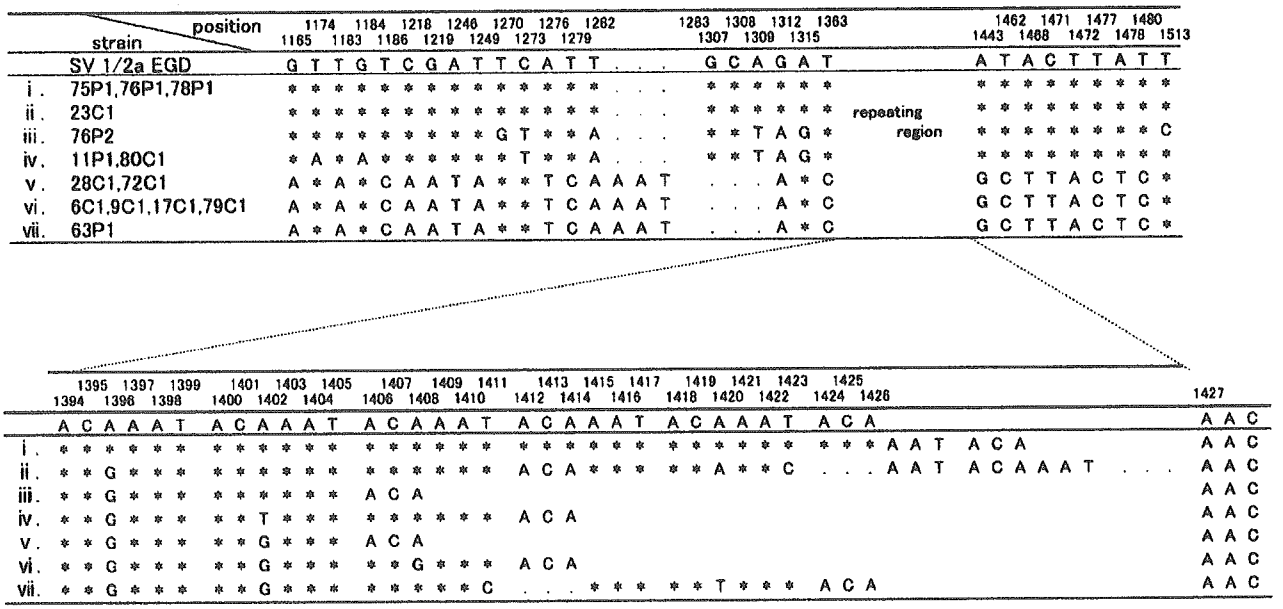

Fig. 4. Sequence variation, nucleotide substitutions, insertion and deletion, and then repeat structure in the variable region of *iap* gene determined in the present study. Asterisks show the identical nucleotides to those of the SV1/2a EGD strain and dots of the nucleotide positions are nucleotide deletions. The shadow region of the nucleotide position from 1396 to 1423 is the repeat sequence structures in the *iap* gene.

The repeated sequences, which consist of a unit of 6 nucleotides of ACAAAT and end-sequence of ACA located on positions 1394 to 1426 in the EGD strain, were compared to the isolates derived from meats. Some isolates were found to contain the unit of ACGAAT and/or ACTAAT. The sequences also showed the difference in repeating number of units from 1 to 6 cycles but no 3 cycles among the isolates. The repeating numbers were 2 and 5 in the chicken-derived 76P1, 75P1 and 78P1, and in the pork-derived 79C1, 6C1 and 17C1, respectively.

Table 1
Discrimination of 14 *L. monocytogenes* isolates from commercial Japanese meats

Strain		Serovar	Date	Meat	Store	Nucleotide substitution	Deletion	Insertion	Repeat structure	Cleavage pattern
1	11P1	1/2a	'96.06.24	Pork	Aa	9	0	0	2	
2	76P2	1/2a	'98.02.10	Pork	Ab	8	0	0	1	
3	80C1	1/2a	'98.02.13	Chicken	Ac	9	0	0	2	
4	9C1	1/2b	'96.06.17	Chicken	Aa	23	1	1	2	
5	72C1	1/2b	'98.02.09	Chicken	Ac	22	1	1	1	
6	79C1	1/2b	'98.02.12	Chicken	Ac	23	1	1	2	○
7	6C1	1/2b	'96.06.16	Chicken	Bd	23	1	1	2	○
8	17C1	1/2b	'96.06.22	Chicken	Ce	23	1	1	2	○
9	63P1	1/2b	'97.05.15	Pork	Df	24	2	1	4	
10	23C1	1/2c	'96.06.24	Chicken	Aa	2	1	0	6	
11	76P1	1/2c	'98.02.10	Pork	Ab	0	0	0	5	△
12	75P1	1/2c	'98.02.10	Pork	Ab	0	0	0	5	△
13	78P1	1/2c	'98.02.11	Pork	Ab	0	0	0	5	△
14	28C1	4b	'97.06.20	Chicken	Ec	22	1	1	1	

Store shows 5 different stores of A to E from which the meats were purchased, and 6 different meat plants of a to f which were labeled on the package of each meat. ○ and △ in the cleavage pattern; the same fingerprint patterns were obtained for each symbol after digestion by restriction enzymes.

4. Discussion

The authors suggested that the epidemiology for the *L. monocytogenes* infection would become more available by molecular biological analysis combined with the traditional discrimination by serotyping used in previous studies (Saito et al., 1998, 2000). In the present study, the molecular biological analysis as well as serotyping was used to determine the prevalence of *L. monocytogenes* in meats purchased from shops in the Kanto area of Japan to evaluate the usefulness of the analysis for the elucidation of tracking in a pathogens in the food environment. Table 1 summarizes the serovars, some information of meats and the genetic characteristics obtained from alignments of the nucleotide sequences of PCR products for the *iap* gene and from the RFLP analysis of chromosomal DNA and PCR products.

Some strains of serovar 1/2a of 11P1 and 80C1, or 1/2b of 9C1 and 79C1 were observed to have a distinct fingerprinting pattern by RFLP analysis of the chromosomal DNA, though the amplified products showed identical genetic characteristics by the alignments of the nucleotide sequences and RFLP analysis between the isolates of the same serovar. Thus, the exact identification for *L. monocytogenes* is suggested to be essential not only genetic analysis of the restricted region, such as the PCR product, but also that at the whole genome level.

Three isolates of serovar 1/2b (79C1, 6C1, 17C1) and 3 strains of serovar 1/2c (76P1, 75P1, 78P1) were confirmed to have the same characteristics by analysis of the whole genome fingerprinting as well as that of the PCR products. The identity of the serovars and the gene characteristics suggest a close relation among the isolates in each serovar. The closely related strains of serovar 1/2c were isolated from pork purchased from the same store on almost the same date. The geographical and historical information suggests that the meats were contaminated with *L. monocytogenes* from a common source, though the contamination source remains unclear. Other closely related strains of serovar 1/2b, however, were isolated from chickens purchased from 3 geographically distinct shops on different dates and years. This information suggests the chickens to be contaminated from different sources of the organism. These show the possibility that the contamination of chickens by the isolates has already spread to the poultry industries in some areas of Japan. The 76P1 and 76P2 strains, which were simultaneously isolated from the same pork, were different serovars of 1/2c and 1/2a, respectively, and showed distinct gene characteristics by RFLP analysis and comparative assay of the nucleotide sequences. These results show that multiple types of *L. monocytogenes* produced simultaneous contamination in some infection sources.

In summary, genetic analyses used in the present study discriminated the *L. monocytogenes* isolates derived from various meats that were purchased from geographically distinct shops over a 2-year period. The analyses were then useful for the confirmation of some isolates that have a genetically close relation. Further investigation using the molecular biological method combined with serotyping would be needed to determine in detail the *L. monocytogenes* contamination in meats and to clarify the infectious route from contaminated sources to humans.

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Thallium Contamination in Wild Ducks in Japan

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ABSTRACT: Although thallium (Tl) is toxic to both humans and animals, there is little information on contamination in wildlife. In this study, Tl contents in wild ducks in Japan were determined. Contents of Tl in kidney and liver ranged from 0.42 to 119.61 and 0.10 to 33.94 μg/g dry weight, respectively. Significant correlations between Tl contents in kidney and liver were observed for all dabbling ducks except mallard (*Anas platyrhynchos*); similar correlations were not observed in diving ducks. Variation in Tl content was observed between sampling locations with the highest mean Tl content in the Eurasian wigeon (*Anas penelope*) collected in Ibaraki Prefecture.

Key words: Duck, thallium, wild bird.

Thallium (Tl) exists naturally in the environment (Mulkey and Oehme, 1993; Asami, 2001), and background levels of <0.00001 mg/l have been reported for fresh water and seawater (Mason, 1966). Concentrations in nonpolluted soil of 0.10–0.56 mg/kg dry weight (wt.) (Asami et al., 1996), 0.07–0.91 mg/kg dry wt. (Hoffer et al., 1990), and 0.292–1.172 mg/kg dry wt. (Qi et al., 1992) have been reported. In plants, concentrations do not generally exceed 0.05 mg/kg dry wt. (Wierzbicka et al., 2004) and, in animals, Tl levels are normally less than 1 ppb and 10 ppb in blood and tissues, respectively (Mulkey and Oehme, 1993). In humans, Tl has been detected in both kidney and liver, but concentrations were less than 4.05 and 1.42 ng/g wet wt., respectively (Weinig and Zink, 1967).

Although rodenticides and insecticides containing Tl have been regulated in many countries since the 1960s and 1970s (Asami, 2001), coal-burning power plants, certain cement plants, and mining and smelting operations can represent sources for this element (Pielow, 1979; Prinz et al., 1979; Sabbioni et al., 1984; Ewers, 1988). Nriagu and Pacyna (1988) estimated that the total emission of Tl from coal combustion and cement production ranged from 3,320 to $6{,}950\times10^3$ kg/yr. This metal and its compounds are also used in various industrial products, such as the production of sulfuric acid, dye and pigments, semiconductors, and superconducting ceramics (Mulkey and Oehme, 1993; Asami, 2001). Thallium has been suggested as a priority elemental pollutant with regard to human health (Keith and Telliard, 1979), and a significant positive correlation between the Tl content of moss and the incidence of circulatory disease in humans has been reported (Heim et al., 2002).

With the exception of reports of acute poisoning due to rodenticides and insecticides (Cromartie et al., 1975; Clausen and Karlog, 1977), there is little information on Tl contamination in wildlife. In this study, we determined levels of Tl in several species of wild ducks collected in various locations in Japan.

Samples were collected from 58 dabbling ducks representing five species and 15 diving ducks representing three species (Fig. 1). Dabbling ducks included spotbill ducks (*Anas poecilorhyncha*, $n=19$), mallards (*Anas platyrhynchos*, $n=7$), common teal (*Anas crecca*, $n=6$), northern pintails (*Anas acuta*, $n=11$) and Eurasian wigeon (*Anas penelope*, $n=15$). Diving ducks included greater scaup (*Aythya marila*, $n=6$), tufted ducks (*Aythya fuligula*, $n=6$) and pochards (*Aythya ferina*, $n=3$). Ducks were captured between 1993 and 1995 as a part of unrelated projects being conducted by the Japanese Ministry of the Environment. Other birds were supplied

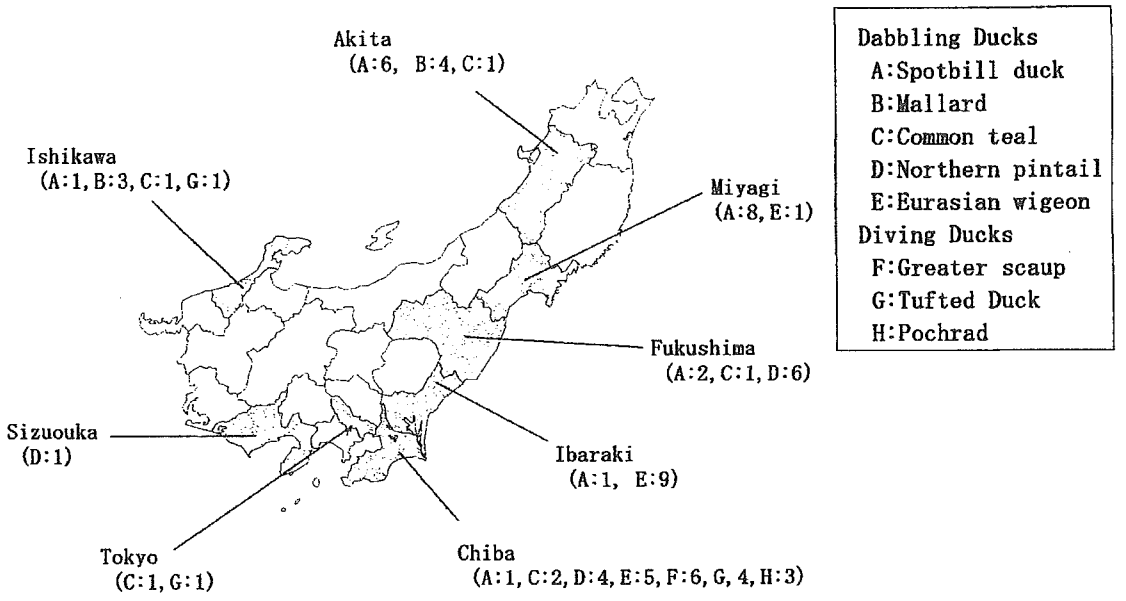

FIGURE 1. Sampling locations for wild ducks.

through the Gyotoku Bird Observatory in Chiba Prefecture.

Samples (approximately 200 mg) of kidney and liver from each duck were dried, weighed, and digested. Thallium contents were determined using an inductively coupled plasma atomic emission spectrometer (ICP-AES, Spectro A.I., Germany) as described in the previous reports (Mochizuki et al., 1999, 2002a, b, c). The detection limit for Tl was 0.007 ppm, and for statistical analyses, nondetectable values were estimated to contain half of the detection limit. Results are presented as mean±standard error (SE). Peason's correlation coefficients were calculated in Excel® (Microsoft Corporation, Redmond, Washington, USA).

Thallium contents in kidney and liver for each species are shown in Table 1. Mean contents of Tl in kidney were greater than 4 μg/g dry wt. in all dabbling spe-

TABLE 1. Thallium contents (μ g/g dry wt.) in the kidney and liver of each species and the correlation between thallium contents of kidney and that of liver. The correlation with an asterisk shows significant correlation (**$P<0.01$, *$P<0.05$). n=number of samples.

		Kidney		Liver		
Species	n	Mean±SE	Range	Mean±SE	Range	Correlation
Dabbling ducks						
Spotbill duck	19	7.15±1.48	(0.91–23.73)	4.57±1.32	(0.60–26.62)	0.601**
Mallard	7	8.11±1.96	(2.65–15.76)	4.50±1.42	(1.09–11.86)	0.692
Teal	6	10.68±6.18	(2.12–40.33)	5.32±2.91	(0.37–19.12)	0.881**
Pintail	11	4.00±1.35	(1.01–15.70)	6.52±3.21	(0.12–33.94)	0.692*
Wigeon	15	20.38±8.08	(0.42–119.61)	4.27±1.14	(0.48–14.72)	0.848**
Total	58	10.46±2.34	(0.42–119.61)	4.93±0.85	(0.12–33.94)	0.411**
Diving ducks						
Scaup	6	2.10±0.32	(1.05–2.98)	2.71±1.15	(0.43–7.85)	0.214
Tufted duck	6	3.04±0.62	(1.02–6.60)	5.27±3.83	(0.10–24.33)	0.810
Pochard	3	2.13±0.33	(1.63–2.75)	2.09±0.85	(0.42–3.21)	−0.019
Total	15	2.48±0.36	(1.02–6.60)	3.61±1.56	(0.10–24.23)	0.745**

TABLE 2. Thallium contents (μg/g dry wt.) in the kidney and liver of each prefecture. *n*=number of samples.

	Dabbling ducks			Diving ducks		
Prefecture	*n*	Kidney	Liver	*n*	Kidney	Liver
Akita	11	8.43±2.05	5.73±2.32			
Chiba	12	3.32±0.78	2.83±0.70	13	2.28±0.22	2.04±0.59
Fukushima	9	4.08±1.56	6.93±3.94			
Ibaraki	10	28.25±11.44	5.26±1.55			
Ishikawa	5	14.73±6.74	6.96±3.13	1	6.60	24.23
Miyagi	9	7.80±2.15	3.99±0.74			
Sizuoka	1	8.71	1.93			
Tokyo	1	2.18	1.22	1	1.02	3.32

cies, while those in diving ducks were less than 3 μg/g dry wt. for all species. The total mean value for dabbling duck species (10.5 μg/g dry wt.) was approximately four times higher than the mean for diving duck (2.5 μg/g dry wt.). Although Tl levels in liver also were higher in species of dabbling ducks, mean values were less than 6.5 μg/g dry wt. in all species, and these differences were not statistically significant.

The Tl levels in ducks in this study were lower than those reported from birds of prey (63 ppm wet wt.) that died due to acute poisoning by Tl (Cromartie et al., 1975), and wood mice (kidney; 44.05, liver; 11.34 μg/g dry mass) and magpies (maximum value of kidney; 45 μg/g dry wt.) captured in a polluted area next to a zinc mine (Dmowski et al., 1998). Thallium contents in kidney and liver exceeding 0.5 ppm wet wt. (approximately 2 μg/g dry wt.) are believed to be indicative of poisoning (Clausen and Karlog, 1977). Mean contents of Tl observed for all species of ducks in this study (Table 1) exceeded this proposed threshold. There was no indication, however, that any of the ducks sampled in this study were physically affected.

Mochizuki et al. (1999, 2002a, c) reported spatial variation in contents of cadmium, molybdenum, and vanadium in wild birds, suggesting that birds may represent effective indicators of environmental contamination. This same relationship may exist with Tl contents in ducks, which approximated 10 μg/g dry wt. in all prefectures except Chiba Prefecture (Table 2). In Japan, high Tl contents have been reported in plants (28.9 mg/kg dry wt.) and bottom sediment (79.9 mg/kg dry wt.) associated with the Hosokura mines in Miyagi Prefecture (Asami, 2001). Background concentrations of 4.81 mg/kg dry wt. in sediments collected 20 km from this point source have also been reported. A high Tl content (maximum, 4.13 mg/kg dry wt.) has also been reported for one area near the copper mine in Ibaraki Prefecture and the maximum/background ratio of 10.7 was reported from soil near the mine. Although the authors could not find similar reports in other prefectures, the prefectures of Akita, Fukushima, and Ishikawa have active and idle silver, copper, lead, and zinc mines (Hata, 1997; Asami, 2001) that have been suggested as possible sources of Tl (Ewers, 1988). There are no such mines in the Chiba Prefecture, which may explain the low levels of Tl observed in ducks sampled from this location (Table 2).

With cadmium (Mochizuki et al., 2002a), vanadium (Mochizuki et al., 1999), and lead (Gerhardsson et al., 1995), a correlation between the contents observed in kidney and liver has been reported in wildlife species and humans. Similar correlations for Tl were obtained from all dabbling species except for the mallard and all diving species in the present study (Table 1). Sample sizes for individual species by location were too low for a meaningful comparison of kidney and liver Tl con-

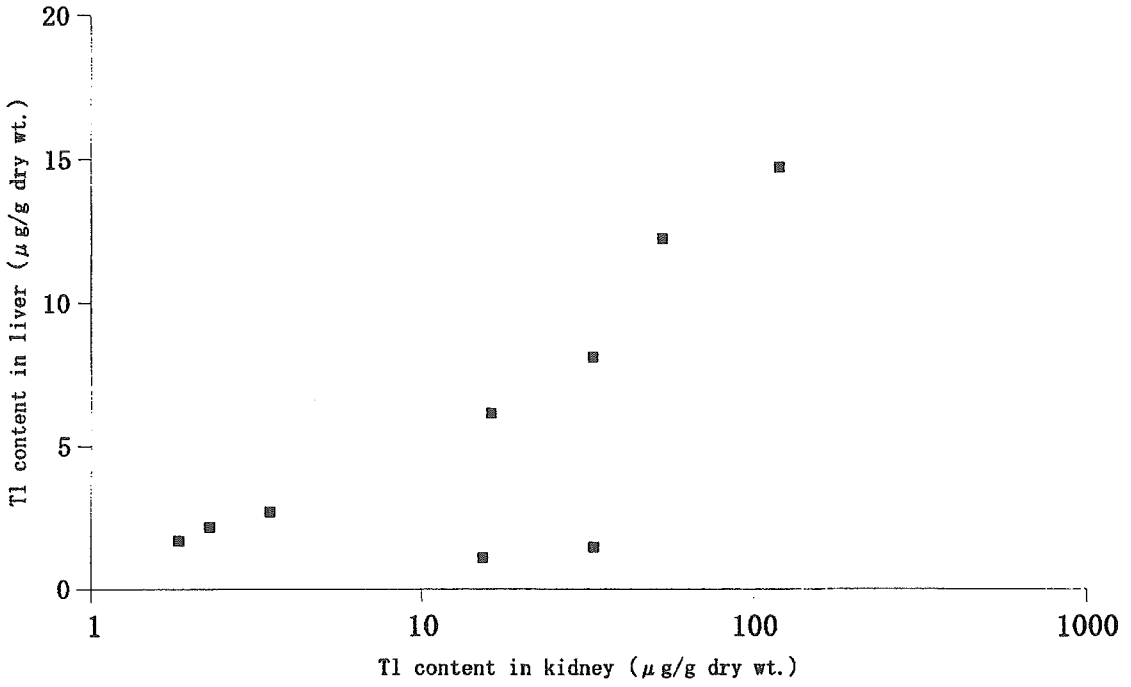

FIGURE 2. Relation between the Tl contents (μg/g dry wt.) in kidney and liver of Eurasian wigeon sampled from Ibaraki Prefecture.

tents. However, a significant correlation was obtained with the results from Eurasian wigeon (n=9) from Ibaraki Prefecture (R=0.856, P<0.01) (Fig. 2). Results of this study suggest that elevated contents of Tl are common in ducks in Japan but the potential clinical significance of these Tl levels is not understood. Additional monitoring of waterfowl may provide a means to better understand potential sources of Tl to wildlife populations.

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