be useful for studying MV-based vaccines in humanized mouse models without the need to sacrifice the mice.

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REFERENCES

Akkina, R. (2013). New generation humanized mice for virus research: comparative aspects and future prospects. *Virology* 435, 14–28. doi: 10.1016/j.virol.2012.10.007

Brandler, S., Marianneau, P., Loth, P., Lacote, S., Combredet, C., Frenkiel, M. P., et al. (2012). Measles vaccine expressing the secreted form of West Nile virus envelope glycoprotein induces protective immunity in squirrel monkeys, a new model of West Nile virus infection. *J. Infect. Dis.* 206, 212–219. doi: 10.1093/infdis/jis328

Despres, P., Combredet, C., Frenkiel, M. P., Lorin, C., Brahic, M., and Tangy, F. (2005). Live measles vaccine expressing the secreted form of the West Nile virus envelope glycoprotein protects against West Nile virus encephalitis. *J. Infect. Dis.* 191, 207–214. doi: 10.1086/426824

Fujino, M., Yoshida, N., Kimura, K., Zhou, J., Motegi, Y., Komase, K., et al. (2007). Development of a new neutralization test for measles virus. *J. Virol. Methods* 142, 15–20. doi: 10.1016/j.jviromet.2007.01.001

Griffin, D. E. (2007). "Measles virus," in *Fields Virology*, eds D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus, 5 Edn (Philadelphia: Lippincott Williams & Wilkins, a Wolters Kluwer Business), 1551–1585.

Hamaia, S., Li, C., and Allain, J. P. (2001). The dynamics of hepatitis C virus binding to platelets and 2 mononuclear cell lines. *Blood* 98, 2293–2300. doi: 10.1182/blood.V98.8.2293

Hashimoto, K., Ono, N., Tatsuo, H., Minagawa, H., Takeda, M., Takeuchi, K., et al. (2002). SLAM (CD150)-independent measles virus entry as revealed by recombinant virus expressing green fluorescent protein. *J. Virol.* 76, 6743–6749. doi: 10.1128/JVI.76.13.6743-6749.2002

Hummel, K. B., Lowe, L., Bellini, W. J., and Rota, P. A. (2006). Development of quantitative gene-specific real-time RT-PCR assays for the detection of measles virus in clinical specimens. *J. Virol. Methods* 132, 166–173. doi: 10.1016/j.jviromet.2005.10.006

Ito, R., Takahashi, T., Katano, I., and Ito, M. (2012). Current advances in humanized mouse models. *Cell. Mol. Immunol.* 9, 208–214. doi: 10.1038/cmi.2012.2

Jiang, Q., Zhang, L., Wang, R., Jeffrey, J., Washburn, M. L., Brouwer, D., et al. (2008). FoxP3+CD4+ regulatory T cells play an important role in acute HIV-1 infection in humanized Rag2−/−gammaC−/− mice in vivo. *Blood* 112, 2858–2868. doi: 10.1182/blood-2008-03-145946

Kato, S. I., Nagata, K., and Takeuchi, K. (2012). Cell tropism and pathogenesis of measles virus in monkeys. *Front. Microbiol.* 3:14. doi: 10.3389/fmicb.2012.00014

Kimberly, W. T., Zheng, J. B., Town, T., Flavell, R. A., and Selkoe, D. J. (2005). Physiological regulation of the beta-amyloid precursor protein signaling domain by c-Jun N-terminal kinase JNK3 during neuronal differentiation. *J. Neurosci.* 25, 5533–5543. doi: 10.1523/JNEUROSCI.4883-04.2005

Koga, R., Ohno, S., Ikegame, S., and Yanagi, Y. (2010). Measles virus-induced immunosuppression in SLAM knock-in mice. *J. Virol.* 84, 5360–5367. doi: 10.1128/JVI.02525-09

Lin, W. H., Kouyos, R. D., Adams, R. J., Grenfell, B. T., and Griffin, D. E. (2012). Prolonged persistence of measles virus RNA is characteristic of primary infection dynamics. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14989–14994. doi: 10.1073/pnas.1211138109

Lorin, C., Mollet, L., Delebecque, F., Combredet, C., Hurtrel, B., Charneau, P., et al. (2004). A single injection of recombinant measles virus vaccines expressing human immunodeficiency virus (HIV) type 1 clade B envelope glycoproteins induces neutralizing antibodies and cellular immune responses to HIV. *J. Virol.* 78, 146–157. doi: 10.1128/JVI.78.1.146-157.2004

Ohno, S., Ono, N., Seki, F., Takeda, M., Kura, S., Tsuzuki, T., et al. (2007). Measles virus infection of SLAM (CD150) knockin mice reproduces tropism and immunosuppression in human infection. *J. Virol.* 81, 1650–1659. doi: 10.1128/JVI.02134-06

Reyes-del Valle, J., Hodge, G., Mcchesney, M. B., and Cattaneo, R. (2009). Protective anti-hepatitis B virus responses in rhesus monkeys primed with a vectored measles virus and boosted with a single dose of hepatitis B surface antigen. *J. Virol.* 83, 9013–9017. doi: 10.1128/JVI.00906-09

Sawada, A., Komase, K., and Nakayama, T. (2011). AIK-C measles vaccine expressing fusion protein of respiratory syncytial virus induces protective antibodies in cotton rats. *Vaccine* 29, 1481–1490. doi: 10.1016/j.vaccine.2010.12.028

Sellin, C. I., and Horvat, B. (2009). Current animal models: transgenic animal models for the study of measles pathogenesis. *Curr. Top. Microbiol. Immunol.* 330, 111–127. doi: 10.1007/978-3-540-70617-5_6

Singh, M., Cattaneo, R., and Billeter, M. A. (1999). A recombinant measles virus expressing hepatitis B virus surface antigen induces humoral immune responses in genetically modified mice. *J. Virol.* 73, 4823–4828.

Stebbings, R., Fevrier, M., Li, B., Lorin, C., Koutsoukos, M., Mee, E., et al. (2012). Immunogenicity of a recombinant measles-HIV-1 clade B candidate vaccine. *PLoS ONE* 7:e50397. doi: 10.1371/journal.pone.0050397

Sun, Z., Denton, P. W., Estes, J. D., Othieno, F. A., Wei, B. L., Wege, A. K., et al. (2007). Intrarectal transmission, systemic infection, and CD4+ T cell depletion in humanized mice infected with HIV-1. *J. Exp. Med.* 204, 705–714. doi: 10.1084/jem.20062411

Tatsuo, H., Ono, N., Tanaka, K., and Yanagi, Y. (2000). SLAM (CDw150) is a cellular receptor for measles virus. *Nature* 406, 893–897. doi: 10.1038/35022579

Terahara, K., Ishige, M., Ikeno, S., Mitsuki, Y. Y., Okada, S., Kobayashi, K., et al. (2013). Expansion of activated memory $CD4^+$ T cells affects infectivity of CCR5-tropic HIV-1 in humanized NOD/SCID/JAK3^{null} mice. *PLoS ONE* 8:e53495. doi: 10.1371/journal.pone.0053495

Traggiai, E., Chicha, L., Mazzucchelli, L., Bronz, L., Piffaretti, J. C., Lanzavecchia, A., et al. (2004). Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science* 304, 104–107. doi: 10.1126/science.1093933

Wege, A. K., Melkus, M. W., Denton, P. W., Estes, J. D., and Garcia, J. V. (2008). Functional and phenotypic characterization of the humanized BLT mouse model. *Curr. Top. Microbiol. Immunol.* 324, 149–165. doi: 10.1007/978-3-540-75647-7_10

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Novel Clinical Features of Recurrent Human Respiratory Syncytial Virus Infections

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Children and elderly individuals are often infected easily and repeatedly with human respiratory syncytial virus (HRSV); however, the features of recurrent infection in the same individual are defined poorly. To clarify the clinical significance of repeated HRSV infections in relation to subgroup epidemiology, this study performed prospective and longitudinal analyses in children with lower respiratory tract infections over 20 consecutive epidemics between 1985 and 2005 at a pediatric outpatient clinic in Kawasaki, Japan. HRSV infections were confirmed by 2 types of reverse-transcription PCR. Samples obtained from patients with repeated infections were subjected to sequence analysis and cloning analysis. A total of 1,312 lower respiratory tract infections observed in 1,010 patients were diagnosed as HRSV infections. Repeated HRSV infections occurred in 208 of the 1,010 patients. Analysis of the patients with repeated infections revealed that children were often infected multiple times even within a single short epidemic. Some patients were re-infected with strains having the same or virtually identical N gene sequences. In patients infected more than 4 times, cloning analysis revealed more frequent dual infections with both subgroups (23.8%). The HRSV-A subgroup caused subsequent homologous infections more frequently than did HRSV-B; furthermore, HRSV-A infections provided no protection from a second homologous infection. In contrast, HRSV-B infections offered significant protection against a second homologous infection. Statistical analysis revealed alleviation of symptoms with a reduced rate of dyspnoeic attacks only in the group re-infected with homologous HRSV-A strains. Thus, this study elucidates new clinical features of recurrent HRSV infection. ***J. Med. Virol.***

KEY WORDS: human respiratory syncytial virus (HRSV); repeated infections; subgroup epidemiology; clinical characteristics

INTRODUCTION

Human respiratory syncytial virus (HRSV), of the family *Paramyxoviridae*, subfamily *Pneumovirinae*, genus *Pneumovirus*, is the leading cause of lower respiratory tract infections in infants and children [Parrott et al., 1973]. All infants experience at least 1 HRSV infection by 2 years of age [Glezen et al., 1986]. Despite the presence of circulatory antibodies against HRSV, recurrent infections in older children and adults occur throughout life, and protective immunity against re-infections is incomplete and brief [Hall et al., 1991]. There are 2 major antigenic subgroups, A (HRSV-A) and B (HRSV-B), and viruses from these subgroups are considered genetically distinct on the basis of sequencing data [Matheson et al., 2006]. Several findings have raised the possibility that antigenic differences between HRSV subgroups may contribute to re-infection, although the results were not conclusive [Mufson et al., 1987]. Both subgroups are subdivided into

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several strains or genotypes according to the attachment glycoprotein (G) gene. More than 3 genotypes in each dominant subgroup usually co-circulate in epidemics, with some strains being replaced each year [Hall et al., 1990; Sullender, 2000; Galiano et al., 2005; Matheson et al., 2006]. The difference between genotypes might be considered to play a role in the establishment of re-infections; however, this requires further investigation [Sullender et al., 1998]. A birth cohort study in Kenya observed that 4 of 12 repeated infections recurred with an identical virus within the same short epidemic [Scott et al., 2005]. Furthermore, in a study with adult volunteers, clinical re-infections occurred repeatedly, even when the subjects were infected with the same strain of HRSV-A within a few months after a natural HRSV-A infection [Hall et al., 1991]. From a study of hospitalized children in Finland, no direct evidence of protection against re-infection with a homologous subgroup was found even within a single season [Waris, 1991]. To clarify the clinical characteristics and significance of HRSV re-infection, a prospective and longitudinal analysis of HRSV infections during 20 consecutive epidemics at the same outpatient clinic was conducted.

MATERIALS AND METHODS

Patients and Clinical Specimens

This study was performed at a private pediatric outpatient clinic in Kawasaki, Japan. A pediatrician in the clinic monitored children with symptoms of lower respiratory tract infection prospectively during the period from December 1985 to August 2005. The diagnosis of lower respiratory tract infection was based on major clinical manifestations such as expiratory wheezing, shortness of breath, hoarseness, barking cough with or without inspiratory stridor, deep or wet chest cough, rhonchi, and rales. Duration of fever $\geq$38°C and the existence of respiratory difficulty (retraction, expiratory wheezing, tachypnoea $\geq$50 breath/min, and/or orthopnoea) on the day of visit or during the illnesses were recorded as clinical features by the pediatrician. Nasopharyngeal secretions or nasal swabs were collected from all patients with lower respiratory tract infections, as described previously [Yui et al., 2003]. If an HRSV infection occurred $\geq$14 days after a previous infection or if the 2 infections were determined to involve different subgroups, the event was defined as a separate infection [Hall et al., 1991]. When a patient was diagnosed with HRSV infection for the first time during a visit to the outpatient clinic, this was determined as the first HRSV infection.

Informed consent for participation in this study was obtained from the parents of all the children. The study protocol was approved by the ethics committee of the Kitasato Institute for Life Sciences, Kitasato University.

Detection of HRSV Antigen

From a total of 1,735 clinical specimens, 1,690 were examined immediately for the presence of HRSV antigens with an enzyme-linked immunosorbent assay (ELISA) kit (Ortho Diagnostics, Raritan, NJ), a TestPack RSV enzyme immunoassay (EIA) kit (Abbott Laboratories, North Chicago, IL) or immunochromatography (IC) using ImmunoCard STAT RSV (Meridian Bioscience, Cincinnati, OH). From 1985 to 1986, an ELISA kit was used for direct antigen detection. Then, EIA was performed until December in 2004, when the manufacturer stopped providing the test kit. The IC test was used subsequently (Fig. 1).

RNA Extraction and RT-PCR

Total RNA was extracted directly from respiratory specimens (nasopharyngeal secretions or nasal swabs) using acid guanidinium thiocyanate phenol–chloroform with minor modifications [Yui et al., 2003], as described previously or using the High Pure Viral RNA Kit (Roche Applied Science, Manheim, Germany) according to the manufacturer's instructions. To amplify HRSV-A and HRSV-B simultaneously, an N gene region conserved between the 2 subgroups was selected (Table I). Early in the study, reverse-transcription PCR (RT-PCR) was performed using primer set 1 (Prs1), as described previously [Yui et al., 2003]. Later in the study, an increased number of samples tested negative by PCR using Prs1, although they were positive by EIA and IC. Because HRSV sequence mutation(s) at the position of Prs1 may cause a failure of PCR amplification, another primer set (Prs2) was synthesized (Table I). First-strand cDNA synthesis was carried out using the CN3 primer (for viral RNA) and CCN6 primer (for mRNA). cDNA was amplified by the first PCR using n-F1(+) and n-B1(−), followed by nested PCR using EcoF3′ (+) and NotB3 (−). When an HRSV infection was suspected clinically or epidemiologically, RT-PCR was conducted irrespective of the rapid antigen detection assay (Fig. 1). All RT-PCR procedures were performed according to the protocol described by Kwok and Higuchi [1989]. Every assay was performed with a negative control.

Restriction Fragment-Length Polymorphism

HRSV subgroups were distinguished using restriction fragment-length polymorphisms (RFLP) of the RT-PCR products, as reported previously [Yui et al., 2003]. In brief, the RT-PCR product of HRSV-A was digested with *Bgl*II and that of HRSV-B was digested with *Hae*III. All PCR products were subjected to RFLP, except the clones obtained from the samples selected for cloning.

Nucleotide Sequencing

All the samples obtained from patients with repeated infections were subjected to sequence analysis.

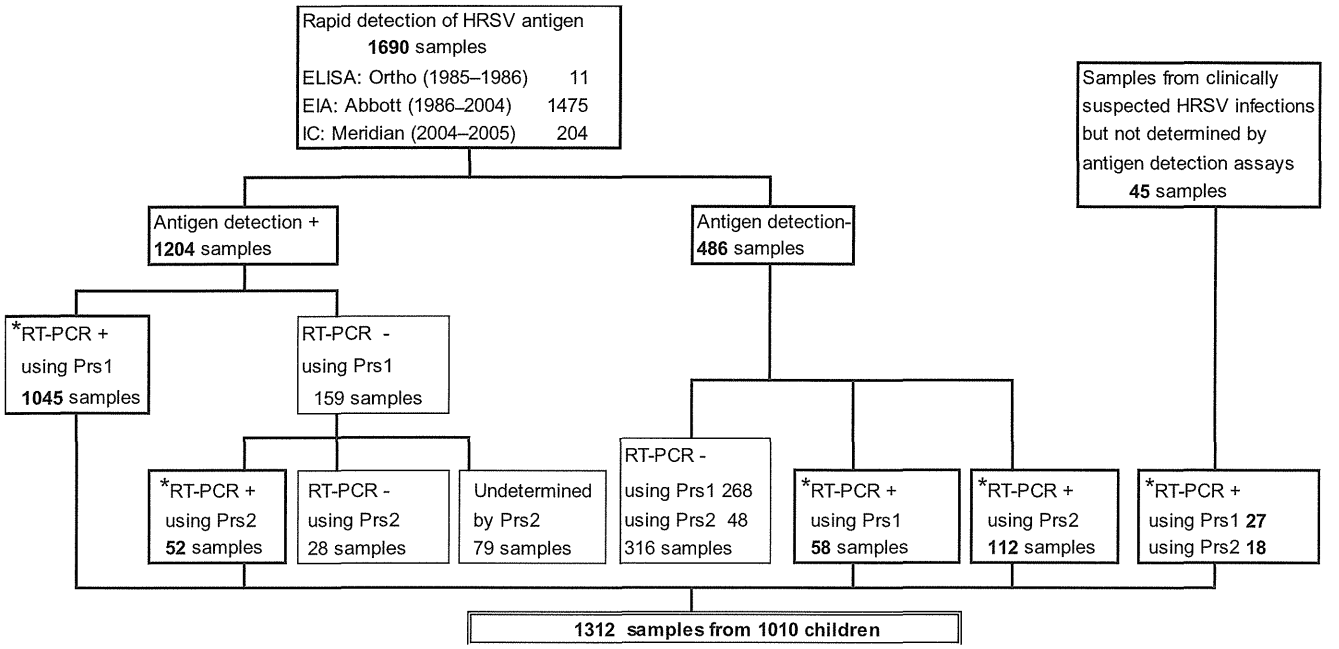

Fig. 1. Study flow diagram. Among the 1,735 clinical specimens, 1,690 were subjected to rapid antigen detection assay and 45 samples from clinically suspected HRSV infections were subjected to RT-PCR. A total of 1,312 specimens obtained from 1,010 children with symptoms of lower respiratory tract infections were confirmed to be infected with HRSV using 2 types of RT-PCR using the primers Prs1 and Prs2. The asterisk represents confirmed HRSV infections. ELISA, enzyme-linked immunosorbent assay; EIA, enzyme immunoassay; IC, immunochromatography; Prs1, RT-PCR primer set 1 shown in Table I; Prs2, RT-PCR primer set 2 shown in Table I.

When a subgroup could not be determined by RFLP due to an atypical cutting pattern, the samples were also subjected to direct sequencing. RT-PCR products were extracted from low-melting-temperature 1% agarose gel and used for sequencing. The nucleotide sequence was determined with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) using an automated 3130/3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). RT-PCR products from samples that were suspected of dual infections of HRSV-A and HRSV-B, based on the RFLP pattern and samples from children infected with HRSV more than 4 times, were inserted into pBluescript II SK (−) (Stratagene, La Jolla, CA); then, nucleotide sequences of more than 20 individual clones from each PT-PCR product were determined

TABLE I. Sequences and Positions of the PCR Primers Used

Primer	Sequence (5′–3′)	Positions
RT-PCR primer set 1 (Prs1)		
C-RSN (+)	GGGTCGACAATTCACTGGGTTAATACCTAT	1274–1295*
RSN-F1 (+)	GCCCCGGGGAGATAGAATCTAGAAAATCCT	1477–1498
RSN-B1 (−)	GCGGAGCTCTTTGGGTTGTTCAATATATGG	1998–2018
RSN-F2 (+)	CCGGTACCGAAATGGGAGAGGTAGCTCC	1516–1535
RSN-B2 (−)	CCGCATGCATAAACCTCAACAACTTGTTCC	1938–1959
RT-PCR primer set 2 (Prs2)		
CN3 (+)	GCTCTTAGCAAAGTCAAGTTGAA	1099–1121*
CCN6 (−)	TCTGTACTCTCCCATTATGCCTA	2087–2109
n-F1 (+)	GAGATAGAATCTAGAAAATCCTACAAAA	1477–1504
n-B1 (−)	TGGGTTGTTCAATATATGGTAGA	1994–2016
EcoF3′ (+)	TGGTGAATTCGCTCCAGAATACAGGCA	1531–1547
NotB3 (−)	AGTTGCGGCCGCATAAACCTCAACAACTTGTTCC	1938–1959
Colony direct PCR primers		
M13m4 (+)	GTTTTCCCAGTCACGAC	580–596**
M13RV (−)	CAGGAAACAGCTATGAC	812–828

The number of nucleotides is based on the genomic location of HRSV-A strain A2* (GenBank accession number M11486) and pBluescript II SK (−)** (GenBank accession number X52330). Underlined letters indicate the linker sequences.

(cloning analysis). A pair of primers, M13m4 (+) and M13RV (−) shown in Table I, was used for colony direct sequencing.

Statistical Analysis

Comparisons between mean values, paired but not normally distributed, were assessed with the Wilcoxon rank sum test. Categorical data were compared by the Chi-square test or Fisher's exact test. All comparisons were conducted at the two-tailed 0.05 level of significance using Stat-View 5.0 software (SAS Institute, Tokyo, Japan).

Nucleotide Sequence Accession Numbers

The N-gene sequences determined in this study have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB722450–AB723492.

RESULTS

Laboratory Diagnosis of HRSV Infection

Among the 1,735 clinical specimens, 1,690 were subjected to the rapid antigen detection assay, of which 1,204 (71.2%) tested positive for the HRSV antigen (Fig. 1). Early in the study, RT-PCR using Prs1 were conducted. Of the 1,557 samples subjected to RT-PCR using Prs1, 1,130 (72.6%) were positive for HRSV. On comparing the results of EIA and IC to those of RT-PCR, 159 samples were negative by RT-PCR using Prs1, although the viral antigen was detected by EIA or IC (Fig. 1). A portion (80/159) of these samples was re-examined by RT-PCR using another set of primers, Prs2. Using Prs2, 52 samples (65.0%) tested positive for HRSV RNA. Further, of the 486 samples that were negative for antigen detection, 58 and 112 samples were detected to be positive by PCR using Prs1 and Prs2, respectively. Consequently, a total of 1,312 lower respiratory tract infections were confirmed as HRSV infections in 1,010 patients, that is, positive by antigen detection and RT-PCR using Prs1 (1,045) or Prs2 (52), negative by antigen detection but positive by PT-PCR using Prs1 (58) or Prs2 (112), and only detected by RT-PCR using Prs1 (27) or Prs2 (18). The patients ranged in age from 5 days to 11 years (median, 18 months), and the duration analyzed was 20 epidemic years. The definition of 1 epidemic year was from September to August of the next year.

Of the 1,312 HRSV infections, 756 (57.6%) and 517 (39.4%) were caused by HRSV-A and HRSV-B, respectively. Through RFLP and cloning analysis, 39 (3.0%) HRSV infections were verified as dual infections with both HRSV-A and HRSV-B. Further, 617 of 756 (81.6%) HRSV-A infections, 487 of 517 (84.7%) HRSV-B infections, and 20 of 39 (51.3%) infections caused by both HRSV-A and HRSV-B were observed in patients aged less than 3 years. These results showed that HRSV-A and HRSV-B co-circulated continuously over 20 epidemic years, and the overall pattern of subgroup prevalence changed every 1 or 2 years (Fig. 2). From 1994 to 2005, 1 year of HRSV-A predominance was followed by 1 or 2 intervening years where the 2 subgroups presented in similar numbers or HRSV-B predominated.

Rate of Repeated HRSV Infection

From the sequencing and cloning analyses, a total of 208 children were noted to have experienced multiple HRSV infections (2–9 times). Consequently,

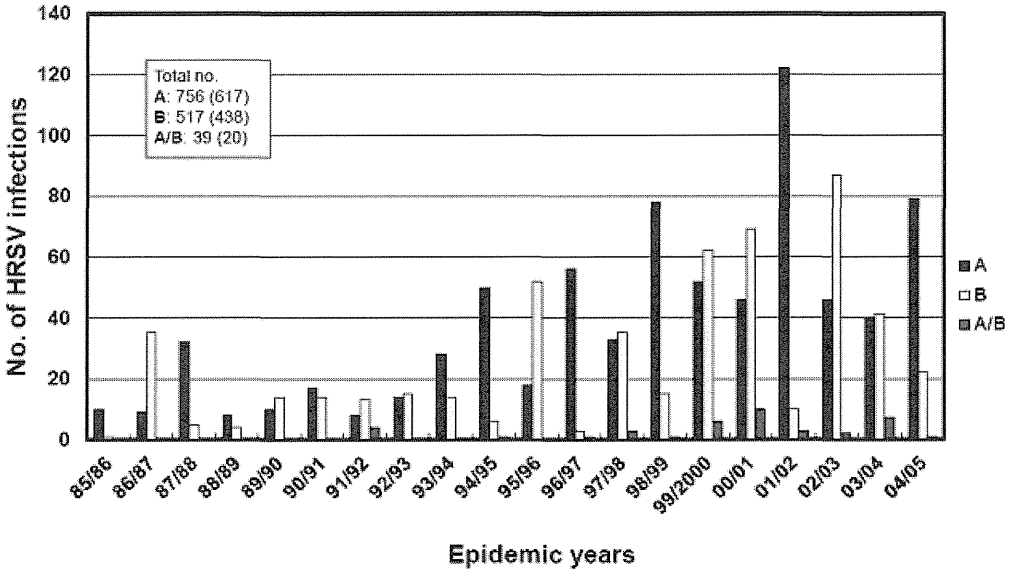

Fig. 2. HRSV subgroup epidemiology. Distribution of HRSV subgroups circulating in Kawasaki, Japan, during 1985–2005. The number in parentheses indicates HRSV infections among patients aged less than 3 years. A, HRSV-A; B, HRSV-B; A/B, dual infection with HRSV-A and HRSV-B.

TABLE II. Patterns of Occurrence Among Children Infected Repeatedly With HRSV

Patterns of occurrence in repeated infections	No. of patients	No. of specimens	HRSV subgroup		
			A	B	A/B
Twice	151	302	186	103	13
Three times	42	126	72	48	6
Four times	6	24	16	4	4
Five times	3	15	8	2	5
Six times	3	18	11	3	4
Eight times	2	16	8	4	4
Nine times	1	9	5	2	2
Total	208	510	306[a]	166[a]	38

[a]Difference between percentages of sample numbers of repeated cases for the total number of HRSV-A and HRSV-B cases is statistically significant ($P = 0.0029$). A/B indicates dual infection with HRSV-A and HRSV-B.

510 infections (510/1,312 = 38.9%) were recorded as multiple infections observed in the 208 children. Among the 510 infections, 306, 166, and 38 infections were caused by HRSV-A, HRSV-B, and both subgroups, respectively (Table II). The rate of repeated HRSV-A infections among the total circulating HRSV-A infections (306/756 = 40.5%) was higher than that of HRSV-B infections (166/517 = 32.1%). The difference in the rate of HRSV-A and HRSV-B infections was statistically significant ($P = 0.0029$). Among the 208 children infected repeatedly with HRSV, 151 and 42 children were re-infected and infected 3 times, respectively. Further, 6, 3, and 3 children were infected 4, 5, and 6 times, respectively. Two children were infected 8 times, and 1 patient was infected 9 times (Table II).

Subgroup Occurrence Among Children Re-Infected With HRSV

During the 20 epidemic years, 151 children experienced HRSV re-infections (302 infections; Table III). The patients had no underlying conditions such as congenital heart disease, immunodeficiencies, neuromuscular disorders, or chronic respiratory disorders, except previously diagnosed asthma (in 10 patients). Five children with low birth weights (<2,500 g) were included in the analysis, as they were not born prematurely, with a gestational age of at least ≥37 weeks. There were 16 (5.3%) cases of hospital admissions (16/302; hospitalization due to the first infection, 12; hospitalization due to a second case of infection, 4) in the group. In the second infection, 60 children were infected with a homologous subgroup, of which 50 and 10 re-infections by a homologous subgroup were caused by HRSV-A and HRSV-B, respectively. Re-infections with a heterologous subgroup were detected in 78 children, of whom 35 and 43 were first infected with HRSV-A and HRSV-B, respectively. Thirteen patients had dual infections with both subgroups in either the first or the second infection. Of the 151 patients, 133 (88.1%) were re-infected after more than 1 season and 18 patients (11.9%) within a single season (Table III). Re-infection with a homologous strain occurred more frequently with HRSV-A than HRSV-B ($P = 0.0065$). During the study period, the overall ratio of circulating HRSV-A/HRSV-B strains in the community was 3:2. Taking circulating HRSV into account, HRSV-A infections provided no protection from a second infection with a homologous strain ($P = 0.91$). However, patients with HRSV-B infections were protected significantly from a second homologous infection

TABLE III. Subgroup Characteristics of HRSV Isolated From Re-Infected Children

Patterns of occurrence	No. of re-infected children	Interval of re-infection				No. of children analyzed for clinical features
		More than 1 separate season		Within a single season		
A–A*	50	43		7		*47*
A–B	35	32		3		*33*
B–B	10	9		1		*9*
B–A	43	39		4		*40*
A/B	13	10	First (5), Second (5)	3	First (2), Second (1)	
Total	151	133		18		*129*

*The first letter designates the subgroup responsible for the first HRSV infection, and the second letter, that for the second infection. A/B indicates that dual infection with both subgroups is included in the first or second infection. The numbers in parentheses indicate dual-infected HRSV samples in the first or second infection. The numbers in italics indicate re-infected children who were enrolled for the analysis of clinical features.

($P = 0.002$). When the comparison was applied to children aged less than 3 years (total number of patients, 91; age range, 1–36 months; median, 15 months), the protection offered by HRSV-B against a second homologous infection was verified ($P = 0.005$). Sequence analysis indicated that 8 children were re-infected with an HRSV-A strain possessing the same nucleotide sequence in the N gene region (nucleotide positions 1548–1937); further, 4 children were infected with an HRSV-A strain and 1 child was infected with an HRSV-B strain possessing a very similar sequence in the N gene region (differing only in a single synonymous nucleotide substitution). The clinical features of 129 children could be traced (Table III).

Subgroup Occurrence Among Children Infected 3 Times

During the study period, 42 children were infected with HRSV 3 times (126 infections; Table IV). Among these, all possible patterns of occurrence were observed. The patients had no underlying conditions except previously diagnosed asthma (in 8 patients) and a low birth weight (in 2 patients). Overall, hospitalization was required in 9 of 126 cases of infections (7.1%; first infection, 6; second infection, 2; third infection, 1). Among the 42 children, 19 (17 + 2) (45.2%) had experienced HRSV infections multiple times (2 or 3 times) within a single epidemic year. The clinical characteristics of 29 children were analyzed (Table IV).

On comparing the first and second infections among patients infected 3 times, the number of patients infected from HRSV-A to HRSV-A (A–A), from HRSV-B to HRSV-B (B–B), from HRSV-A to HRSV-B (A–B), and from HRSV-B to HRSV-A (B–A) were 8, 3, 7, and 11, respectively. Similarly, on comparing the first and third infections, there were 11 (A–A), 2 (B–B), 4 (A–B), and 12 (B–A) children in the various subgroup infection patterns. Likewise, on comparing the second and third infection, 14 (A–A), 1 (B–B), 5 (A–B), and 9 (B–A) children were classified into the various subgroup infection patterns (Table V). Taking circulating HRSV into account, comparisons among the first and second, the first and third, and the second and third homologous infections revealed that HRSV-A or HRSV-B infections conferred no immunity against the same subgroup. However, considering all the patients, independent of the temporal patterns of repetition, HRSV-B infections provided significant protection from a second infection by a homologous strain ($P = 0.003$). Similarly, HRSV-B infections in children aged less than 3 years (total number of patients, 15; age range, 2–36 months; median, 17 months) also provided significant protection against another homologous infection ($P = 0.003$).

Clinical Features Among Children Re-Infected and Infected 3 Times

To assess the alleviation of clinical symptoms in the second or third infection among children re-infected or infected 3 times, the mean number of febrile days and respiratory difficulty were compared between the first and second infections, the first and third, and the second and third infections (Table V).

Among the re-infected patients, a reduction in dyspnoeic attack rates was observed between the first and second HRSV-A infections ($P = 0.004$) (Table V).

TABLE IV. Subgroup Characteristics of HRSV Isolated From Children Infected 3 Times

		Interval of repeated infection 3 times					
Patterns of occurrence	No. of children infected 3 times	More than one separate season		Two infections within a single season		Three infections within a single season	No. of children analyzed for clinical features
A–A–A*	5	2		2		1	*4*
A–A–B	4	1		2	A–A (2)[a]	1	*4*
A–B–A	8	5		3	A–B (2), B–A (1)	0	*7*
A–B–B	1	1		0		0	*0*
B–B–B	2	1		1		0	*1*
B–B–A	3	1		2	B–A (2)	0	*2*
B–A–B	3	2		1	A–B (1)	0	*1*
B–A–A	10	7		3	B–A (3)	0	*10*
A/B	6	3	First (1)[b], Second (2)	3	Second (1), Third (2)	0	
Total	42	23		17		2	*29*

*The first letter designates the subgroup responsible for the first infection; the second letter, that for the second infection; and the third letter, that for the third infection. A/B indicates dual infection in the first, second, or third infection. The numbers in parentheses indicate the sample numbers for each pattern of occurrence in 2 infections within a single season[a], and dual-infected HRSV samples in the first, second, or third infection[b]. The numbers in italics indicate children infected 3 times who were enrolled for the analysis of clinical features.

TABLE V. Comparison of Clinical Features Among Children With HRSV Re-Infection and Who Were Infected 3 Times

	Re-infection					Repeated infection 3 times				
		Fever		Dyspnoea			Fever		Dyspnoea	
Patterns of occurrence	No. of children	Mean no. of febrile days ± SD	*P*-Value	Rate of dyspnoea	*P*-Value	No. of children	Mean no. of febrile days ± SD	*P*-Value	Rate of dyspnoea	*P*-Value
A–A*	47	F = 2.1 ± 0.3 S = 2.1 ± 0.3	0.95	F = 28/47** S = 14/47	0.004	33 (8, 11, 14)	Fs = 1.1 ± 0.3 St = 1.6 ± 0.3	0.23	Fs = 16/33 St = 20/33	0.45
B–B	9	F = 2.4 ± 0.6 S = 2.6 ± 0.6	0.89	F = 5/9 S = 7/9	0.61	6 (3, 2, 1)	Fs = 1.8 ± 0.9 St = 1.0 ± 0.7	0.58	Fs = 2/6 St = 2/6	>0.99
A–B	33	F = 2.4 ± 0.3 S = 1.9 ± 0.3	0.31	F = 16/33 S = 16/33	>0.99	16 (7, 4, 5)	Fs = 0.7 ± 0.3*** St = 2.1 ± 0.5	0.04	Fs = 7/10 St = 4/10	0.46
B–A	40	F = 2.1 ± 0.3 S = 2.5 ± 0.3	0.26	F = 24/40 S = 21/40	0.5	32 (11, 12, 9)	Fs = 2.6 ± 0.3 St = 1.7 ± 0.3	0.33	Fs = 15/32 St = 15/32	>0.99
Total	129					87 (29 × 3)				

*The first letter designates the subgroup responsible for the first HRSV infection (in the re-infection) or the second infection (in the repeated infection 3 times); and the second letter, that for the second (re-infection) or third infection (repeated infection 3 times). The letter "F" indicates the first HRSV infection, "S" and "s" indicate the second, and "T" and "t" indicate the third infection. The numbers in parentheses indicate the number of patients between the first and second infection, the first and third, and the second and third, respectively. The Wilcoxon rank-sum test was used for comparing the mean duration of fever in each infection, and Fisher's exact test or Chi-square test was used for comparing the rate of dyspnoea at each infection. **, ***The differences are statistically significant.

Other homologous or heterologous subgroup re-infections were not significantly different for these clinical characteristics even on comparing the infections occurring at an interval of more than 1 epidemic or within a single epidemic. Similarly, a reduction in dyspnoeic attack rates was observed between the first and second HRSV-A infections in the younger age groups (≤3 years; $P = 0.016$). In contrast, when the younger age groups (≤3 years) infected with HRSV-B were re-infected with HRSV-A, an increase in the number of febrile days was observed.

Among patients infected repeatedly 3 times, all the patients were summed up according to the temporal patterns of repetition (i.e., the first and second, the first and third, and the second and third infections). The alleviation of clinical symptoms was compared among all the patients with these 4 infection patterns (i.e., A–A, B–B, A–B, and B–A; Table V). No significant alleviation of clinical symptoms, such as the duration of fever or rate of dyspnoea, was observed in any of the 4 patterns of occurrence. On the contrary, the mean duration of fever in the infecting pattern from HRSV-A to HRSV-B (A–B) was longer in the second or third infection ($P = 0.04$). The alleviation of these symptoms was not verified; however, the number of febrile days was increased in children belonging to the younger age groups who were infected with HRSV-B after infection with HRSV-A.

Clinical Course of Children Infected More Than 4 Times

Fifteen children were diagnosed with HRSV infections more than 4 times. Among these 15 children, 6, 3, 3, and 2 patients as well as 1 patient had a history of HRSV infections 4, 5, 6, 8, and 9 times, respectively. Consequently, 82 HRSV infections were observed in these 15 children. The patients had no underlying conditions, including previously diagnosed asthma and a low birth weight. There were five (6.1%) hospital admissions (5/82; hospitalization due to the first infection, 4; hospitalization due to second infection, 1). The patients' ages ranged from 1 month to 11 years (median, 31.5 months).

All the infections, except 2, were subjected to cloning analysis, which indicated that 19 infections (23.8%) were dual infections of HRSV-A and HRSV-B. Various random patterns of occurrence were observed in the repeated infections. All 15 children had experienced HRSV infections multiple times within the same epidemic season, and 4 of the 15 children showed 3–5 separate as well as different subgroup infections within a single season. However, there was no apparent decrease in the duration of fever for each infective episode in these patients. On the other hand, respiratory difficulties tended to recur even after multiple infections in 9 of the 15 children. The remaining children had showed no dyspnoeic attacks from the very first infection.

DISCUSSION

This study was conducted in an ambulatory care setting but not with birth cohort monitoring. Previous studies have demonstrated that over two-thirds of children were infected with HRSV within their first year of life [Glezen et al., 1986]. It was also reported that several neonates were completely asymptomatic although infected with HRSV [Hall et al., 1979]. Therefore, it is likely that a considerable number of the patients in this study had already experienced HRSV infections before their first symptomatic episode. Nevertheless, the data in this study showed that HRSV lower respiratory tract infections occurred repeatedly in the same individual at a rate of 20.6% (208/1,010), which was higher than those reported by 2 previous studies as 9.0% and 7.8% [Zlateva et al., 2007; Yamaguchi et al., 2011]. The higher rate of detection of recurrent HRSV infections in this study as compared to those in previous studies may be attributed partly to the differences in the detection methods used. Two different RT-PCR primer sets (Prs1 and Prs2) and antigen detection assays were used in this study, whereas in the other report, HRSV infections were detected using a single RT-PCR protocol targeting the G gene, which is more variable than the N gene targeted by the present RT-PCR protocols [Yamaguchi et al., 2011]. In fact, the first RT-PCR (Prs1) failed to detect a substantial number of HRSV infections, which were identified by the second RT-PCR (Prs2) and antigen detection assays. The present analysis supports the need to identify HRSV infections using more than 2 diagnostic methods, since the number of infections might otherwise be overlooked. These data thus demonstrated that repeated HRSV infections occur more frequently in Japan than indicated by the previous study.

From 1994 to 2005, a cyclic pattern of subgroup prevalence was observed in the present study. Each HRSV-A predominant year was followed by 1 or 2 epidemic years with relatively equal proportions of the 2 subgroups or HRSV-B predominance. Similar cyclic patterns have been reported in Belgium [Zlateva et al., 2007] and in Rochester, New York [Hall et al., 1990]; however, the patterns differed among the reports. The former study was performed using 2 kinds of RT-PCR procedures and the latter using an immunofluorescent test with monoclonal antibodies. Epidemiological studies around the world have disclosed HRSV-A predominance [Sullender, 2000]. However, the high total HRSV-B detection rate of 44% in Zlateva's study compared with 39% in this study; the 29% HRSV-B detection rate in Hall's study might be due to the methods used and/or the appropriate selection of RT-PCR primers for HRSV-B, since HRSV-B has greater variability in terms of stop codon usage and insertion or deletion than does HRSV-A [Matheson et al., 2006]. Therefore, the different detection rates for HRSV-B using various procedures could influence the results of molecular epidemiology.

In this study, no significant difference was observed in the rate of repeated HRSV-A infections by a homologous subgroup as compared with those by a heterologous subgroup. A birth-cohort study conducted over 2 consecutive years showed that re-infections with the same strain possessing the G gene sequence occurred in infants even within the same epidemic year [Scott et al., 2005]. This observation is thus consistent with the data recorded in the present study. In addition, this study confirmed that the rate of re-infection with HRSV-A was higher than that with HRSV-B, which has also been shown previously [Yamaguchi et al., 2011]. Importantly, HRSV-B re-infection provided protection from a second infection with a homologous subgroup among the re-infected children and among the children infected with HRSV 3 times, even on comparison with children from the younger age groups ($\leq$3 years). This might reflect the 2 distinct lineages of divergent evolution with extensive genetic and serologic differences between HRSV-A and HRSV-B [Matheson et al., 2006]. In addition, this might also explain the cyclic pattern of subgroup prevalence.

Many children who suffered from multiple infections within a short period of time were observed in this study; some of these were infected more than 3 times within the same season, suggesting that some individuals, such as children infected more than 4 times, are highly susceptible to HRSV. Individual host genetic factors are considered important to the immunopathogenesis of HRSV infections [Miyairi and DeVincenzo, 2008; Oshansky et al., 2009]. This influence involves a complex interaction of age-related immunity, previous HRSV experience on priming immune-developmental genetic processes, genes protecting against viral entry in the early phase, and genes modifying later immunopathology. The results in the present study might reflect the differences among individual host genetic factors that determine the susceptibility to HRSV. For highlighting the importance of host genetic factors, patients with multiple HRSV infections appeared to be roughly divisible into 2 groups; those suffering dyspnoeic attacks even after many HRSV infections, and those with no respiratory difficulties from the start. The clinical features of patients presenting with recurrent dyspnoeic attack after HRSV infections could possibly be diagnosed with asthma later in their clinical course.

A previous study showed that the incidences of lower respiratory tract infections, bronchiolitis, and otitis media with effusion were reduced on the third but not second infection of HRSV, and that age at infection influenced the disease severity [Henderson et al., 1979]. The clinical features of repeated HRSV infections, such as the number of febrile days and the presence of dyspnoea, were compared among individuals. In addition, these clinical features were compared among

individuals infected with the different subgroups; this analysis was not performed in the previous studies. Among children infected repeatedly with homologous or heterologous HRSV, a reduction in neither the febrile period nor the rate of respiratory difficulty was observed at the second or third infection, even on comparison with children from the younger age group (≤3 years). The only difference noted was a reduced rate of dyspnoeic attacks among the group re-infected with the same HRSV-A, which might indicate that individuals in this group showed a lower immunopathologic response to HRSV-A than the individuals in the other group. In contrast, among the patients infected 3 times, a longer duration of febrile period was noted in the second infection, with infection from HRSV-A to HRSV-B, as compared with the first infection. Similarly, among the re-infected younger age group, infection from HRSV-B to HRSV-A was associated with a longer duration of febrile period. HRSV can occur together with other respiratory viruses, especially adenoviruses [De Paulis et al., 2011]. Although it is possible that other micro-organisms modulated the clinical manifestations of HRSV infections at least in some patients in this study, it has been previously reported that viral co-infections do not appear to affect the severity of HRSV infections in hospitalized infants [De Paulis et al., 2011].

The cloning procedure was applied mainly to samples obtained from children with multiple HRSV infections; therefore, a high rate (23.8%) of dual infections was identified. The total rate of dual infections was 3.0%, which was still a relatively high rate of incidence as compared to that reported by an earlier study (0.6%) [Zlateva et al., 2007]. It is possible that more dual infections could be detected by applying cloning procedures to all the samples. The sequence of HRSV-B displays greater variability than that of HRSV-A, which could lead to lower detection rates for HRSV-B and affect the discovery of dual infections, if the primers used to detect HRSV-B are not appropriate for the prevalent strains. It would be worthwhile to examine the exact incidence of dual infections, the manner in which individual clinical symptoms are affected by dual infections, and the evolutionary interaction of HRSV-A and HRSV-B inside the human body.

Recently, it was demonstrated that severe fatal lower respiratory tract infections caused by HRSV are characterized by the absence of a pulmonary cytotoxic lymphocyte response, robust viral loads, and an apoptotic crisis, based on an analysis of autopsy specimens from Chilean infants in the absence of mechanical ventilation [Welliver et al., 2007]. This has cast some doubt on the immunopathogenesis of HRSV disease. The results of the present study may provide additional insights into the wide variations in the severity of HRSV disease, which are affected by the age at infection as well as by genetic polymorphisms among races, individual host genetic factors and socioeconomic conditions [Miyairi and DeVincenzo, 2008; Oshansky et al., 2009]. The susceptibility to HRSV and the disease severity or pattern may vary and depend upon individual factors. If certain genetic factors can predict HRSV disease severity, these findings can lead to either a new vaccination strategy that induces the formation of antibodies blocking the CX3C–CX3CR1 interaction of G protein [Zhang et al., 2010] or antiviral compounds derived from plant lectins [Ooi et al., 2010] as an individualized treatment option.

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REFERENCES

De Paulis M, Gilio AE, Ferraro AA, Ferronato AE, do Sacramento PR, Botosso VF, de Oliveira DB, Marinheiro JC, Hársi CM, Durigon EL, Vieira SE. 2011. Severity of viral coinfection in hospitalized infants with respiratory syncytial virus infection. J Pediatr (Rio J) 87:307–313.

Galiano MC, Palomo C, Videla CM, Arbiza J, Melero JA, Carballal G. 2005. Genetic and antigenic variability of human respiratory syncytial virus (groups A and B) isolated over seven consecutive seasons in Argentina (1995–2001). J Clin Microbiol 43:2266–2273.

Glezen WP, Taber LH, Frank AL, Kasel JA. 1986. Risk of primary infection and reinfection with respiratory syncytial virus. Am J Dis Child 140:543–546.

Hall CB, Kopelman AE, Douglas RG Jr, Geiman JM, Meagher MP. 1979. Neonatal respiratory syncytial virus infection. N Engl J Med 300:393–396.

Hall CB, Walsh EE, Schnabel KC, Long CE, McConnochie KM, Hildreth SW, Anderson LJ. 1990. Occurrence of groups A and B of respiratory syncytial virus over 15 years: Associated epidemiologic and clinical characteristics in hospitalized and ambulatory children. J Infect Dis 162:1283–1290.

Hall CB, Walsh EE, Long CE, Schnabel KC. 1991. Immunity to and frequency of reinfection with respiratory syncytial virus. J Infect Dis 163:693–698.

Henderson FD, Collier AM, Clyde WA Jr, Denny FW. 1979. Respiratory syncytial-virus infections, reinfections and immunity: A prospective, longitudinal study in young children. N Engl J Med 300:530–534.

Kwok S, Higuchi R. 1989. Avoiding false positives with PCR. Nature 339:237–238.

Matheson JW, Rich FJ, Cohet C, Grimwood K, Huang QS, Penny D, Hendy MD, Kirman JR. 2006. Distinct patterns of evolution between respiratory syncytial virus subgroups A and B from New Zealand isolates collected over thirty-seven years. J Med Virol 78:1354–1364.

Miyairi I, DeVincenzo JP. 2008. Human genetic factors and respiratory syncytial virus disease severity. Clin Microbiol Rev 21:686–703.

Mufson MA, Belshe RB, Orvell C, Norrby E. 1987. Subgroup characteristics of respiratory syncytial virus strains recovered from children with two consecutive infections. J Clin Microbiol 25:1535–1539.

Ooi LS, Ho WS, Ngai KL, Tian L, Chan PK, Sun SS, Ooi VE. 2010. Narcissus Tazetta Lectin shows strong inhibitory effects against respiratory syncytial virus, influenza A (H1N1, H3N2, H5N1) and B viruses. J Biosci 35:95–103.

Oshansky CM, Zhang W, Moore E, Tripp RA. 2009. The host response and molecular pathogenesis associated with respiratory syncytial virus infection. Future Microbiol 4:279–297.

Parrott RH, Kim HW, Arrobio JO, Hodes DS, Murphy BR, Brandt CR, Camargo E, Chanock RM. 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. Am J Epidemiol 98:289–300.

Scott PD, Ochola R, Ngama M, Okiro EA, James Nokes D, Medley GF, Cane PA. 2005. Molecular analysis of respiratory syncytial virus reinfections in infants from Coastal Kenya. J Infect Dis 193:59–67.

Sullender WM, Mufson MA, Prince GA, Anderson LJ, Wertz GW. 1998. Antigenic and genetic diversity among the attachment proteins of group A respiratory syncytial viruses that have caused repeat infection in children. J Infect Dis 178:925–932.

Sullender WM. 2000. Respiratory syncytial virus genetic and antigenic diversity. Clin Microbiol Rev 13:1–15.

Waris M. 1991. Pattern of respiratory syncytial virus epidemics in Finland: Two-year cycles with alternating prevalence of groups A and B. J Infect Dis 163:464–469.

Welliver TP, Garofalo RP, Hosakote Y, Hintz KH, Avendano L, Sanchez K, Velozo L, Jafri H, Chavez-Bueno S, Ogra PL, McKinney L, Reed JL, Welliver RC Sr. 2007. Severe human lower respiratory tract illness caused by respiratory syncytial virus and influenza virus is characterized by the absence of pulmonary cytotoxic lymphocyte responses. J Infect Dis 195:1126–1136.

Yamaguchi M, Sano Y, Dapat IC, Saito R, Suzuki Y, Kumaki A, Shobugawa Y, Dapat C, Uchiyama M, Suzuki H. 2011. High frequency of repeated infections due to emerging genotypes of human respiratory syncytial viruses among children during eight successive epidemic seasons in Japan. J Clin Microbiol 49:1034–1040.

Yui I, Hoshi A, Shigeta Y, Takami T, Nakayama T. 2003. Detection of human respiratory syncytial virus sequences in peripheral blood mononuclear cells. J Med Virol 70:481–489.

Zhang W, Choi Y, Haynes LM, Harcourt JL, Anderson LJ, Jones LP, Tripp RA. 2010. Vaccination to induce antibodies blocking the CX3C–CX3CR1 interaction of respiratory syncytial virus G protein reduces pulmonary inflammation and virus replication in mice. J Virol 84:1148–1157.

Zlateva KT, Vijgen L, Dekeersmaeker N, Naranjo C, Van Ranst M. 2007. Subgroup prevalence and genotype circulation patterns of human respiratory syncytial virus in Belgium during ten successive epidemic seasons. J Clin Microbiol 45:3022–3030.

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ORIGINAL ARTICLE

Mumps Hoshino and Torii vaccine strains were distinguished from circulating wild strains

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Abstract Aseptic meningitis and acute parotitis have been observed after mumps vaccination. Mumps outbreaks have been reported in Japan because of low vaccine coverage, and molecular differentiation is required to determine whether these cases are vaccine associated. RT-nested PCR was performed in the small hydrophobic gene region, and viruses were differentiated by restriction fragment length polymorphism assay. A total of 584 nucleotides were amplified. The PCR product of the Hoshino strain was cut into two fragments (313 and 271 nucleotides) by *Mfe*I; that of the Torii strain was digested with *Eco*T22I, resulting in 332- and 252-nucleotide fragments. Both strains were genotype B and had an *Xba*I site, resulting in two fragments: 299 and 285 nucleotides. Current circulating wild types were cut only by *Xba*I or *Mfe*I. However, the *Mfe*I site of the wild types was different from that of the Hoshino strain, resulting in 451- and 133-nucleotide fragments. Using three restriction enzymes, two mumps vaccine strains were distinguished from wild types, and this separation was applied to the identification of vaccine-related adverse events.

Keywords Mumps Hoshino strain · Mumps Torii strain · Molecular differentiation · Wild circulating genotypes

Introduction

In Japan, the MMR vaccine was introduced in 1989 but discontinued in 1993 because of an unexpectedly high incidence of aseptic meningitis caused by components of the mumps vaccine [1, 2]. The mechanisms responsible for the high incidence of aseptic meningitis with the MMR vaccine have not been elucidated in comparison to monovalent mumps vaccines used since 1993. Nagai et al. [3] investigated the incidence of aseptic meningitis after vaccination and identified 10 cases among 21,465 vaccine recipients. Moreover, 13 patients with aseptic meningitis were reported among 1,051 cases of naturally acquired mumps confirmed by viral isolation together with genome detection. The incidence of aseptic meningitis after vaccination was 1/27 of that observed for natural infections. However, in the post marketing study, the incidence of aseptic meningitis was approximately 0.01 % (1 case in 10,000 recipients) and that of acute parotitis, 2–3 %.

The mumps virus strains were divided into 12 genotypes based upon the sequence diversity of the small hydrophobic (SH) genome region [4, 5]. Parental strains of the Hoshino and Torii vaccine strains, isolated in the 1960s, are genotype B [6, 7]. Circulating wild-type strains were all genotype B in the 1970s and earlier and were genotypes J and B in the 1980s to 1990s. Genotype G appeared in the 2000s. Genotypes D, I, and L have been isolated sporadically [8–10], and recently genotype G was globally the major circulating genotype [11]. Large outbreaks have been observed every 3–5 years because of the low vaccine coverage, 30 % to 40 %. The mumps vaccine is voluntary (its cost is not covered by the government), and a guardian's decision usually depends on information on mumps outbreaks. Some recipients were immunized during the incubation period of natural infection, making it difficult to determine whether the mumps illness was caused by a natural infection or the vaccine.

In previous reports, the Hoshino vaccine strain was distinguished from circulating wild strains using the

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reverse transcription-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) in the hemagglutinin-neuraminidase (HN) gene with *Sca*I and *Afl*II [12]. A simpler method was also reported through digestion with *Sca*I after DNA amplification by reverse transcription loop-mediated isothermal amplification (RT-LAMP) [13]. These methods are applied after immunization with the Hoshino vaccine. Now, two vaccine strains of the Torii and Hoshino are used, but no method of differentiation has been developed for the Torii strain. In this report, 584 nucleotides were amplified in the SH gene, and the two vaccine strains were distinguished from circulating wild types by unique restriction enzyme sites.

Materials and methods

Mumps virus and clinical samples

The Hoshino (Kitasato Institute, Tokyo, Japan) and Torii (Takeda Pharmaceutical, Osaka, Japan) vaccine strains were recovered from marketed vaccines. MuVi/Tokyo.JPN/77 (genotype B), MuVi/Akita.JPN/93-AK (genotype I), MuVi/Tokyo.JPN/94-H (genotype J), MuVi/Tokyo.JPN/94-0K (genotype B), and MuVi/Tokyo.JPN/01-III-10 (genotype L) were used as wild-type representatives for genotypes B, J, and L, which have already been reported [8–10]. A total of 47 clinical samples were examined: 20 cases of aseptic meningitis after immunization with the Torii strain, 25 cases after immunization with the Hoshino strain, and 2 cases of orchitis after immunization with the Hoshino strain. Two wild-type strains (MuVi/Tokyo.JPN/10-K and MuVi/Tokyo.JPN/10-F) were isolated and identified as genotype G. Cerebrospinal fluid (CSF) samples from the patients with aseptic meningitis and two salivary swab samples or nasopharyngeal swab (NPS) from the patients with orchitis were used.

RNA extraction

Total RNA was extracted from 200 μl CSF and salivary swabs or NPS using a magnetic bead RNA purification kit (MagExtractor-viral RNA; Toyobo, Osaka, Japan) and the RNA pellet was suspended in 30 μl distilled water.

RT-PCR and RLFP

RNA was transcribed to cDNA with a random hexamer using a PrimeScript RT reagent Kit (TaKaRa Bio, Japan) and amplified using Ex *Taq* DNA polymerase (TaKaRa Bio). The first PCR was done using MP F 921+ (5′TCTAT AATTCAATTGCCAGA) and MP HN241− (5′TGTCTGC AATTGAAGACAAC) and the nested PCR, using MpF0+ 5′GTCGATGATCTCATCAGGTAC) and Mp HN1− (5′C CAATATTCGGAAGCAGGTTCGGA), amplifying 584 nucleotides including the primer sequences from the genome positions 6139 to 6722 [10]. PCR products underwent electrophoresis after digestion with *Eco*T22I, *Mfe*I, and *Xba*I (New England BioLabs Japan).

Sequence analysis

PCR products were excised from low-melting gel electrophoresis and purified. DNA sequences were determined by the dye terminator method using an Applied Biosystems 3130 (Life Technologies Japan).

Results

Sequence analysis and restriction enzyme sites

The Hoshino and Torii strains were sequenced; alignments at the restriction enzyme sites are depicted in Fig. 1. The *Eco*T22I site (genome position 6386–6391) was unique to

	*Eco*T22 I	*Xba* I	*Mfe* I	*Mfe* I
	6386 6391	6437 6442	6451 6456	6589 6594
Torii [B]	ATGCAT	TCTAGA	CAGTTG	CAACTG
Hoshino [B]	-----G	------	--A---	T-----
JPN/77 [B]	-----G	------	---C--	----C-
JPN/93AK [I]	-----G	------	-----A	---T--
JPN/94H [J]	-----G	------	--T--A	----C-
JPN/94OK [B]	-----G	------	---C--	----C-
JPN/01-III-10 [L]	-----A	------	-----T	------
JPN/10K [G]	-----G	-----G	-----A	---T--
JPN/10F [G]	-----G	-----G	-----A	---T--

Fig. 1 Sequence alignment of the Torii and Hoshino vaccine strains and representative wild strains. MuVi/Tokyo.JPN/77 (genotype B), MuVi/Akita.JPN/93-AK (genotype I), MuVi/Tokyo.JPN/94-H (genotype J), MuVi/Tokyo.JPN/94-0K (genotype B), and MuVi/Tokyo.JPN/01-III-10 (genotype L) were used. MuVi/Tokyo.JPN/10-K and MuVi/Tokyo.JPN/10-F (genotype G) are isolated in this study. Nucleotide changes are depicted in comparison with the Torii strain, and restriction enzyme sequences are highlighted in *grey*

Table 1 DNA sizes of restriction fragments after treatment with *Eco*T22I, *Mfe*I, and *Xba*I

Mumps strains	*Eco*T22I	*Mfe*I	*Xba*I
Hoshino genotype B	–	+ (313/271)	+ (299/285)
Torii genotype B	+ (332/252)	–	+ (299/285)
Wild genotypes B, J, L	–	–	+ (299/285)
Wild genotype I	–	+ (451/133)	+ (299/285)
Wild genotype G	–	+ (451/133)	–

the Torii strain and *Mfe*I site (6451–6456) to the Hoshino strain. Genotypes B, I, J, and L had an *Xba*I (6437–6442) site, and old genotype I and the currently circulating genotype G had an *Mfe*I site (6589–6594) newly introduced by nucleotide change, not at position 6451–6456 of the Hoshino strain. Based on the results of the sequence analysis, RFLP and predicted fragment lengths are shown in Table 1. The PCR product of the Hoshino strain was cut into two fragments (313 and 271) by *Mfe*I and that of the Torii strain into two fragments (332 and 252) by *Eco*T22I. These two strains were also cut by *Xba*I into two fragments (299 and 285). RFLP of the circulating wild type had mainly two patterns: genotypes B, J, and L were cut by *Xba*I and genotype G by *Mfe*I but differently from the Hoshino strain.

The results of RFLP are shown in Fig. 2. The PCR product of the Hoshino vaccine strain was cut by *Mfe*I and *Xba*I, and that of the Torii strain by *Eco*T22I and *Xba*I. As for the RFLP of wild type, the PCR product of MuVi/Akita.JPN/93-AK (genotype I) was cut by both *Mfe*I and *Xba*I with different fragment sizes from the Hoshino strain. MuVi/Tokyo.JPN/94-OK (genotype B) was cut by *Xba*I, and the same RFLP pattern was noted for MuVi/Tokyo.JPN/94-H (genotype J) and MuVi/Tokyo.JPN/01-III-10 (genotype L). PCR products of MuVi/Tokyo.JPN/10-K and/10-F (genotype G) were cut by *Mfe*I. They showed different patterns from the vaccine strains, as predicted from the sequencing results.

Differentiation of vaccine strains from wild types

A total of 47 clinical samples were obtained: 20 cases of aseptic meningitis after immunization with the Torii strain, 25 cases after immunization with the Hoshino strain, and 2 cases of orchitis after immunization with the Hoshino strain. The results of RT-PCR and RFLP are shown in Table 2. RT-PCR was negative for two CSF samples from the recipients of the Torii strain, and among 18 RT-PCR positives, 16 were identified as the Torii vaccine strain. Among 25 CSF samples obtained from the recipients of the Hoshino strain, 3 were negative by RT-PCR, and 20 were considered positive for the vaccine strain. Two from each

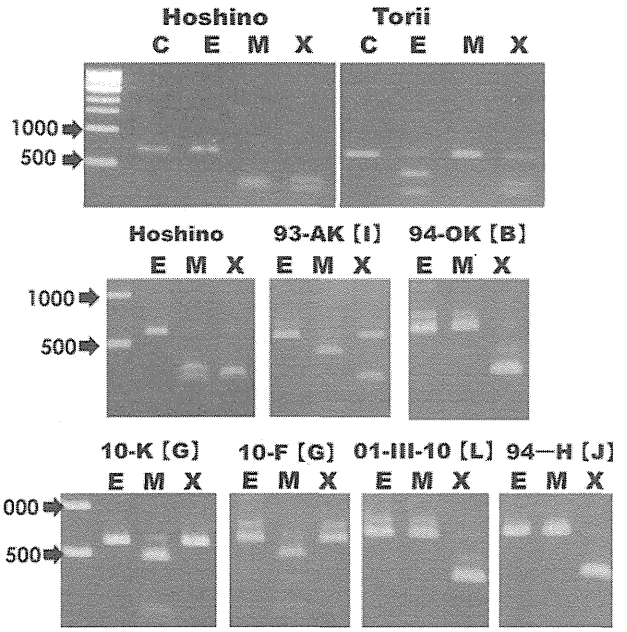

Fig. 2 Restriction fragment length polymorphism (RFLP) of the Hoshino and Torii vaccine strains and circulating wild strains. C, control; E, treatment with *Eco*T22I; M, treatment with *Mfe*I; X, treatment with *Xba*I. 93-AK, MuVi/Akita.JPN/93-AK [genotype I]; 94-H, MuVi/Tokyo.JPN/94-H [genotype J]; 94-OK, MuVi/Tokyo.JPN/94-0K [genotype B]; 01-III-10, MuVi/Tokyo.JPN/01-III-10 [genotype L]; 10-K, MuVi/Tokyo.JPN/10-K [genotype G]; 10-F, MuVi/Tokyo.JPN/10-F [genotype G]

were identified as wild strains. In 2 cases of orchitis after vaccination with the Hoshino strain, RT-PCR was positive in 1 case, identified as the wild type. Five of 45 patients with suspected adverse events were identified as having a concurrent wild-type genotype G.

Some strains identified as causing adverse events were sequenced; the phylogenetic analysis is shown in Fig. 3. Cases 1 and 2 were patients with aseptic meningitis after immunization with the Hoshino strain and cases A and B after that with the Torii strain. The sequencing results showed they were identical to the respective vaccine strains.

Table 2 Results of differentiation of mumps virus genome for clinical samples obtained from patients with aseptic meningitis and orchitis

	PCR negative	PCR positive	
		Vaccine strain	Wild strain
Aseptic meningitis after vaccination with			
Torii ($n = 20$)	2	16	2
Hoshino ($n = 25$)	3	20	2
Orchitis after vaccination with			
Hoshino ($n = 2$)	1		1

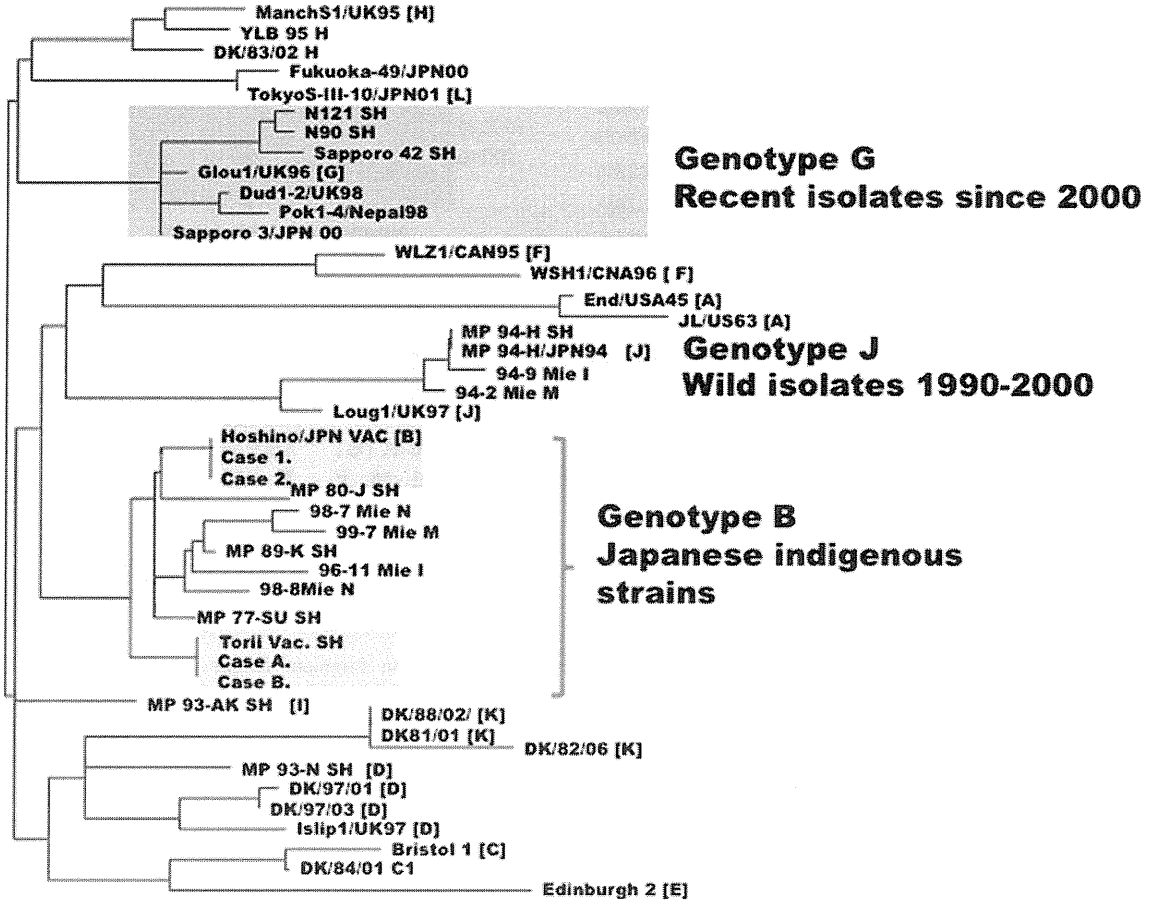

Fig. 3 Phylogenetic analysis of vaccine-associated cases in the small hydrophobic (SH) genome region. Cases 1 and 2 were patients with aseptic meningitis after immunization with the Hoshino strain and cases A and B after immunization with the Torii strain

Discussion

The mumps virus is classified into 12 distinct genotypes, with genotype B indigenous to Japan [4, 5, 8]. Genotype J was a dominant circulating strain with some genotype B strains in 1990–2000, and genotype G appeared in 2000–2012 with sporadic outbreaks of genotype L [8–10]. Mumps has been circulating in Japan because of a low immunization rate, approximately 30–40 %, and mump outbreaks have also been reported in the EU and USA where high immunization coverage was achieved with two doses of MMR [14–16]. The outbreaks were caused by the accumulation of susceptible individuals with an insufficient two-dose MMR vaccination in childhood. Several vaccine strains have been developed, and the Jeryl Lynn strain, belonging to genotype A, has been widely used as a component of MMR. Neutralization test (NT) antibody titers in sera obtained after vaccination with Jeryl Lynn were lower against genotype G than those against the vaccine strain, but they completely neutralized the other genotypes [17, 18]. In contrast, the antigenicity of genotype A of the vaccine strain was quite different from the recent circulating wild types and considered one of the reasons for the recirculation of the mumps virus [19]. There would be some problem with immunogenicity and persistence of immunity after immunization with the Jeryl Lynn strain. In Japan, two vaccine strains, Hoshino and Torii, are used and they belong to genotype B. In our previous report, there was no antigenic difference among circulating wild types [10]. Immunogenicity paralleled the incidence of adverse reactions. The incidence of aseptic meningitis after immunization with the Jeryl Lynn strain was reported to be 1 case in 100,000, and that of Torii or Hoshi was higher. The mump vaccine is still a voluntary one and so the cost is not covered by regional governments. Thus, guardians consider a mumps vaccination only when an outbreak is coming according to surveillance data. Some recipients were vaccinated by chance during the incubation period, and infection with the wild type became mixed into the vaccine-adverse events [12, 13]. In this report, 4 cases were identified as wild types among 45 cases with aseptic meningitis and 1 of the 2 cases of orchitis after immunization. From the results of surveillance reports, mumps outbreaks were observed in moderate grade, and

approximately 10 % of the vaccine-associated cases were infected with the wild type around the immunization day. Most adverse events developed 2–3 weeks after vaccination, but wild-type-related illness developed a few days earlier. There was no difference in clinical symptoms and clinical laboratory findings between vaccine-related adverse events and wild-type-related illness [3]. Five samples in aseptic meningitis and 1 in orchitis showed negative for mumps RT-PCR. Enterovirus RT-PCR for the mump PCR-negative samples showed negative for 5 mumps PCR-negative clinical samples [20]; these were considered to be low virus doses or in inappropriate stocking or transporting conditions.

Vaccine safety is a major concern and depends on postmarketing surveillance. Postmarketing surveillance from 1994 to 2010 is summarized, adding new data to the previous report [21], compared with the incidence of natural infections, in Table 3. The incidence of aseptic meningitis was <1–15 % among mumps infections with different incidences [22], and enhanced surveillance data showed 2.9 % of mumps patients were hospitalized, 6.1 % had orchitis, 0.3 % had meningitis, and 0.25 % had pancreatitis in England in 2002–2006 [23]. For the other complications, permanent deafness was considered to occur in approximately 1 per 20,000 cases, but it would actually be higher, 1 per 1,000 cases [24]. The results of postmarketing studies are shown from 1994 to 2010. A total of 3.5 million doses of the Hoshino vaccine were shipped, and acute parotitis was observed in 2–3 % of recipients. Among them, 117 nasopharyngeal swabs were examined and 89 were positive for RT-PCR: 64 were identified as the Hoshino vaccine strain and 25 were wild type. Among CNS complications, 5 cases of encephalopathy, 223 cases of aseptic meningitis, and 3 cases of acute disseminated encephalomyelitis (ADEM) were reported. Two cases were identified as enterovirus infections by RT-PCR [20]. When 85 CSF samples were examined in 223 cases of aseptic meningitis, 58 were considered vaccine-associated illnesses among 66 PCR positives. In this study period from 2008 to 2012, approximately 10 % of the patients suspected of having a vaccine-associated illness were identified as having wild-type infections during the mump outbreaks. Therefore, a simple differentiation method would contribute to further understanding of the safety of mumps vaccines.

Table 3 Complications of mumps and vaccine adverse events after vaccination with the Hoshino strain reported from 1994 to 2010

Complications	Natural infection	Vaccination (3.5 million)
Acute parotitis	70 %	2–3 %[a]
CNS complications		
Encephalopathy	1/5,000–6,000	5 (1: enterovirus)
Aseptic meningitis	1–2 %	223[b]
ADEM		3 (1: enterovirus)
Deafness	1/15,000 (1/1,000)	4
Orchitis	25 % in adolescents	15[c]
Oophoritis	5 %	
Pancreatitis	4 %	2
Other		1: ITP
		1: allergic purpura

Incidence of complications during natural infection refers to Ref. [22]

CNS, central nervous system; ADEM, acute disseminated encephalomyelitis; ITP, idiopathic thrombocytopenic purpura

[a] Of 117 nasopharyngeal swab (NPS) samples examined from patients with acute parotitis after vaccination with the Hoshino strain, PCR was positive in 89; 64 were identified as the vaccine strain and 25 as the wild type

[b] Of 85 CSF samples examined, 66 were PCR positive; 58 were identified as the vaccine strain and 8 as the wild type

[c] Three NPS samples were examined; one was the vaccine strain and two were the wild type

Conflict of interest The author has a conflict of interest. T.N. has received a research fund for the development of a new concept of live recombinant vaccines (20 million yen a year) from Daiichi-Sankyo Pharmaceutical.

References

1. Kimura M, Kuno-Sakai H, Yamazaki S. Adverse events associated with MMR vaccines in Japan. Acta Paediatr Jpn. 1996;38:205–11.
2. Ueda K, Miyazaki C, Hidaka Y, Okada K, Kusuhara K, Kadoya R. Aseptic meningitis caused by measles–mumps–rubella vaccine in Japan. Lancet. 1995;346:701–2.
3. Nagai T, Okafuji T, Miyazaki C, Ito Y, Kamada M, Kumagai T, et al. A comparative study of the incidence of aseptic meningitis in symptomatic natural mumps patients and monovalent mumps vaccine recipients in Japan. Vaccine. 2007;25:2742–7.
4. Jin L, Rima B, Brown D, Örvell C, Técle T, Afzal M, et al. Proposal for genetic characterization of wild-type mumps strains: preliminary standardisation of the nomenclature. Arch Virol. 2005;150:1903–9.
5. WHO. Mumps virus nomenclature update: 2012. A report based on the WHO mumps nomenclature update meeting, Geneva, 22 September 2011. Wkly Epidemiol Rec. 2012;87:217–24.
6. Sasaki K, Higashihara M, Inoue K, Igarashi Y. Studies on the development of a live attenuated mumps virus vaccine. Kitasato Arch Exp Med. 1976;49:43–52.
7. Hoshino M, Nishimitsu M, Ichimori Y, Oka Y, Kouno R, Yamashita K, et al. Development of live attenuated mumps Torii vaccine strain: development and biological characteristics. Clin Virol. 1981;9:323–30. (in Japanese).
8. Kashiwagi Y, Takami T, Mori T, Nakayama T. Sequence analysis of F, SH, and HN genes among mumps virus strains in Japan. Arch Virol. 1999;144:593–9.
9. Takahashi M, Nakayama T, Kashiwagi Y, Takami T, Sonoda S, Yamanaka T, et al. Single genotype of measles virus is dominant whereas several genotypes of mumps virus are co-circulating. J Med Virol. 1996;62:278–85.
10. Inou Y, Nakayama T, Yoshida N, Uejima H, Yuri K, Kamada M, et al. Molecular epidemiology of mumps virus in Japan and proposal of two new genotypes. J Med Virol. 2004;73:97–104.

11. Otto W, Mankertz A, Santibanez S, Saygilli H, Wenzel J, Jilg E, et al. Ongoing outbreak of mumps affecting adolescents and young adults in Bavaria, Germany, August to October 2010. Eur Surveill 2010;15(50):19748
12. Kashiwagi Y, Kawashima H, Takekuma K, Hoshika A, Mori T, Nakayama T. Detection of mumps virus genome directly from clinical samples and a simple method for genetic differentiation of the Hoshino vaccine strain from wild strains of mumps virus. J Med Virol. 1997;52:195–9.
13. Yoshida N, Fujino M, Ota Y, Notomi T, Nakayama T. Simple differentiation of mumps Hoshino vaccine strain from wild strains by reverse transcription loop-mediated isothermal amplification (RT-LAMP). Vaccine. 2007;25:1281–6.
14. CDC. Mumps epidemic—United Kingdom, 2004–2005. MMWR. 2006;55:173–5.
15. CDC. Mumps outbreak at a summer camp: New York, 2005. MMWR. 2006;55:175–7.
16. Barskey AE, Glasser J, LeBaron CW. Mumps resurgences in the United States: a historical perspective on unexpected elements. Vaccine. 2009;27:6186–95.
17. Rubin SA, Audet SA, Sullivan B, Carbone KM, Bellini WJ, Rota PA, et al. Antibody induced by immunization with Jeryl Lynn mumps vaccine strain effectively neutralizes a heterologous wild-type mumps virus associated with a large outbreak. J Infect Dis. 2008;198:508–15.
18. Rubin SA, Link MA, Sauder CJ, Zhang C, Ngo L, Rima BK, et al. Mumps outbreaks in vaccinated populations: no evidence of immune escape. J Virol. 2012;86:615–20.
19. Atrasheuskaya AV, Blatun EM, Kulak MV, Atrasheuskaya A, Karpov IA, Rubin S. Investigation of mumps vaccine failures in Minsk, Belarus, 2001–2003. Vaccine. 2007;25:4651–8.
20. Takami T, Kawashima H, Takei Y, Miyajima T, Mori T, Nakayama T, et al. Usefulness of nested PCR and sequence analysis in a nosocomial outbreak of neonatal enterovirus infection. J Clin Virol. 1998;11:67–75.
21. Nakayama T, Onoda K. Vaccine adverse events reported in post-marketing study of the Kitasato Institute from 1994–2004. Vaccine. 2007;25:570–6.
22. Plotkin SA, Rubin SA. Mumps vaccine. In: Plotkin SA, Orenstein W, Offit P, editors. Vaccines. 5th ed. Philadelphia: Saunders Elsevier; 2008. p. 435–65.
23. Yung CF, Andrews N, Bukasa A, Brown KE, Ramsay M. Mumps complications and effects of mumps vaccination, England and Wales, 2002–2006. Emerg Infect Dis. 2011;17:661–7.
24. Hashimoto H, Fujioka M, Kinumaki H, Kinki Ambulatory Pediatrics Study Group. An office-based prospective study of deafness in mumps. Pediatr Infect Dis J. 2009;28:173–5.

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NOTE

Simple method for differentiating measles vaccine from wild-type strains using loop-mediated isothermal amplification

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ABSTRACT

Because of increasing measles vaccine coverage, the proportion of patients with modified measles has been increasing. Such patients have low-grade fever with very mild eruptions similar to vaccine-related adverse events. Differentiation between these two pathogenic conditions is required to improve the quality of laboratory-based measles surveillance. In this study, vaccine-specific and wild-type specific primer sets were designed for loop-mediated isothermal amplification in the N gene, and vaccine strains, C1, D3, D4, D5, D8, D9, G3 and H1 wild strains were examined. Three vaccine strains were efficiently amplified using a vaccine-specific primer set with an approximately 10-times higher sensitivity than wild-type primer. Modified measles was differentiated from vaccine-associated cases by this system, but limitations were encountered with the other genotypes.

Key words LAMP, measles vaccine, modified measles.

Although the number of measles-related deaths decreased from 873,000 in 1999 to 164,000 in 2008, the goal set to reduce measles deaths by 2010 by 90% of those prior to 2000 was not achieved (1). Global single dose measles vaccination coverage increased from 72% in 2000 to 82% in 2007, when the two-dose immunization strategy was recommended for countries with high coverage (>95%) with the single dose measles vaccine. Most countries (88%) now implement the two-dose strategy (1). However, since late 2009, measles transmission has increased and outbreaks have become widespread in the European Union region because of failure to vaccinate susceptible populations (2). The World Health Assembly updated the goal of measles elimination to 95% reduction of 2000 figures for measles mortality by 2015 (3). In 2007–2008 in Japan, measles outbreaks with different characteristics occurred, namely most patients were young adults or adolescents attending high schools and universities in the early stages of the outbreak (4, 5). Because a relatively large proportion of those with adult measles had previously received single dose measles vaccination, they developed only mild grade fever and rash rather than typical measles symptoms (5). The patients with modified measles exhibited symptoms similar to the adverse events associated with vaccines, 10–20% of vaccine recipients developing low-grade fever (6). The diagnoses of modified measles were confirmed by virus isolation, detection of virus genome, or the serological responses of significant increase in IgG antibodies and/or presence of IgM antibodies (7).

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List of Abbreviations: BIP, backward inner primer; FIP, forward inner primer; LAMP, loop-mediated isothermal amplification; RT-LAMP, reverse transcription loop-mediated isothermal amplification; vac-specific, vaccine-specific; wt-specific, wild type-specific.

However, using IgM positivity to diagnose modified measles in patients who have previously been vaccinated is not reliable. Virus isolation is not always successful because of issues like timing of sample collection, transport, low virus load, and short period of virus excretion in modified measles. The measles virus genome can be directly detected in clinical materials, and sequencing of the PCR products for molecular genotyping, targeting the N gene, would differentiate between adverse effects of vaccines and modified measles. However, this technique is time consuming and clinical samples do not contain sufficient amounts of viral RNA to perform sequencing analysis.

Circulating wild-type genotypes of measles virus are classified into 24 subclades. Of these, B1, C1, D1, E and F are considered inactive (8). In Japan, three measles vaccine strains have been used; the AIK-C and Schwarz FF8 strains, which have been further attenuated from the Edmonston strain, and CAM from the domestic wild-type, genotype A (9). It is critical to develop a simple method with high sensitivity and specificity for making an accurate diagnosis. Recent advances in molecular technology have improved the sensitivity and simplicity of genome amplification. A rapid diagnostic procedure for the detection of the measles genome using RT-LAMP has been reported (10, 11). In this study, that technique was modified for the discrimination of measles vaccine strain from wild circulating strains.

In Japan, C1, D3, D5 Palau-type, H1, D5 Bangkok-type and D9 have circulated since 1984 (5, 12). The following representative strains were used in this study: MVi/Tokyo.JPN/84-K [C1], MVi/Tokyo.JPN/37.99(Y) [D3], MVi/Tokyo.JPN/21.00 (O) [D5 Palau-type], MVi/Tokyo.JPN/20.00(S) [H1], MVi/Tokyo.JPN/17.07 [D5 Bangkok-type] and MVi/Aichi.JPN/44.06 [D9]. In 2011, D4, D8, D9 and G3 strains were imported from outside the country (National Institute of Infectious Diseases, Infectious Disease Surveillance Center; http://idsc.nih.go.jp/disease/measles/2011). MVi/Chiba C.JPN/08.11 [G3], MVi/Hiroshima.JPN/09.11 [D8] and MVi/Osaka C.JPN/09.11 [D4] were also investigated. During the measles outbreak in 2007–2008, 18 clinical samples were obtained from measles patients and those with non-typical measles characterized by mild febrile illness and eruptions (5). Of these, 13 stored samples were available for this study. Five vaccine-associated cases that occurred after 2008 were also investigated.

Total RNA was extracted from 200 μL of culture medium or clinical samples using a magnetic beads RNA purification kit (TOYOBO, Osaka, Japan) and re-suspended in 30 μL of distilled water. cDNA was synthesized from 5 μL of RNA, using a One Step PrimeScript RT-PCR Kit (TaKaRa Bio, Otsu, Japan) with poly T primer and random hexamer. Five microliters of cDNA was used for amplification of the measles genome by LAMP. The LAMP method was characterized by auto-cycling DNA synthesis using *Bst* DNA polymerase with strand displacement (New England BioLabs, Ipswich, MA, USA) and a specially designed set of primers, as is shown in Figure 1. Six LAMP primers were synthesized, recognizing eight different positions: of these F3, B3, F Loop, and BIP were the same as has previously been reported (11). In the present study, vaccine- and wild-type-specific FIPs and B Loop primers were used: vac-specific FIP (FIP-*Vac*: 5′-**T**TGTCCTCAGTAGTATG-CATTGCAGGTATCACTGCCGAGGATG), wt-specific FIP (FIP-*Wt*: 5′-**C**TGTCCTCAGTAGTATGCATTG-CAGGTATCACTGCCGAGGATG), vac-specific B loop (B Loop-*VAC*: 5′-TGAGAATGAGCTACC**GA**) and wt-specific B loop (B Loop-*Wt*: 5′-TGAGAATGAGCTAC-C**AG**) (Fig. 1). The bold letters at the 5′ end of the FIP and the 3′ end of the B loop are specific for the vaccine and wild-type sequences. The reaction mixture and procedure have previously been reported; differentiation between vaccine and wild-type strain was based on which primer set reached the threshold of LAMP amplification faster (11).

The N gene open reading frame of AIK-C and MVi/Tokyo.JPN/37.99(Y) [D3] were amplified and inserted into the multi-cloning sites of pBluescript SK II to construct pAIK-N and pWt D3-N. Serial dilutions of pAIK-N and pWt D3-N were subjected to vac-specific and wt-type specific LAMP; the results are shown in Figure 2. The detection limit of the measles AIK-C vaccine genome was 1–10 copies by vac-specific LAMP but 10–100 copies by wt-specific LAMP, indicating the latter is less efficient. Wild-type pWt D3-N was detected with a detection limit of 10–100 copies by wt-specific LAMP, more than 10 min faster than by vac-specific LAMP. This detection limit is similar to 30–100 copies achieved by the original RT-LAMP (11). The measles vaccine strain genomes from Schwarz FF-8 and CAM (10^4 TCID 50/mL) were amplified over 10 min faster using vac-specific LAMP than with wt-specific LAMP. The time differences between vac-specific and wt-specific LAMP in amplification increased with further dilutions.

Wild-type measles genotypes C1, D3, D4, D5 Palau, D5 Bangkok, D8, D9, G3 and H1 were also investigated. These isolates contained approximately 10^4 TCID 50/mL and serial 10-fold dilutions of cDNA were subjected to vac- and wt-specific LAMP. The results for the recent isolates D4, D5, D8, D9 and G3 are shown in Figure 3. The measles genome was efficiently amplified by wt-specific LAMP, more than 10 min earlier than by vac-specific LAMP in D4, D5 Palau, D5 Bangkok, D8 and D9 genotypes. As for the G3 isolate, 1:100 dilution of G3 was

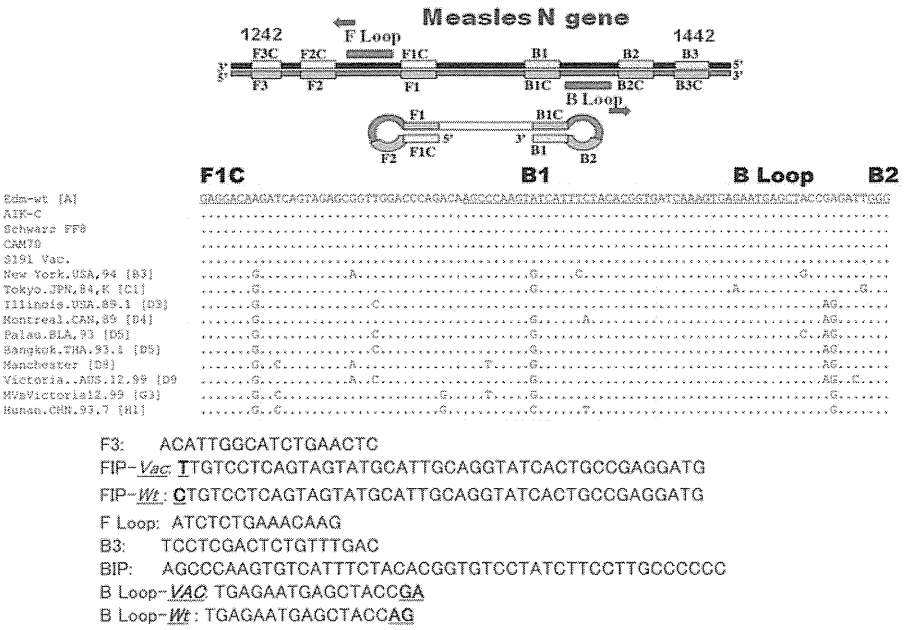

Fig. 1. Primer design for vaccine-specific and wild-type specific LAMP primers. The upper panel shows the location of LAMP primers schematically. The middle panel shows sequence alignments of vaccine and wild strains between F1C and B2 primer positions. Different nucleotides are depicted in comparison with the Edmonston and vaccine strains (AIK-C, Schwarz FF8, CAM70 and S191). The underlined sequence is the original measles LAMP primers. The lower panel shows the vac-specific and wt-specific LAMP primer sequences. The first bold letter at the 5′ end of FIP-*Vac* and FIP-*Wt* and the two bold letters at the 3′ end of B Loop-*Vac* and B Loop-*Wt* are specific to the indicated sequences.

amplified by wt-LAMP 3 min faster than by vac-LAMP and approximately 10 min faster for 1:1000 dilutions. The amplification profile of H1 was similar to that of G3. However, amplification times for C1 by vac-specific and wt-specific LAMP were within 1–2 min (data not shown).

Clinical samples obtained from the patients with low-grade fever and rash were examined; the results are shown in Figure 2. One had developed a febrile illness 10 days after immunization and the other during a measles outbreak, as previously reported (5). In the patient suspected of having an adverse event of vaccine, the measles genome was amplified only by the vac-specific LAMP, whereas the wild-type genome was amplified more efficiently by the wt-specific LAMP in the patient with modified measles during a measles outbreak. These two cases were confirmed by sequence analysis. Of the 13 stored samples from the 2007–2008 outbreak, all were efficiently amplified by wt-specific LAMP, as they had been by the original RT-LAMP. Of the five vaccine-associated cases after 2008, four were identified as vaccine strain. The remaining case was negative by both LAMP and RT-PCR. Conventional nested PCR and real-time PCR were also negative. This apparent discrepancy may have been caused by incorrect sample taking or transporting conditions.

After the World Health Organization in the Western Pacific Region set the goal of eliminating measles by 2012, a two dose schedule with a catch-up campaign for the forthcoming 5 years was implemented in Japan in 2008 (4). Thereafter, the number of reported cases of measles decreased from 11,015 in 2008 to 457 in 2010. Of 457 cases in 2010, approximately two-thirds were laboratory-confirmed, making the estimated incidence 3.58 per million. Some imported cases with D4, D8, D9 and H1 genotypes were reported (13). After the tsunami disaster in 2011, D4, D8, D9 and G3 strains were imported from outside the country (National Institute of Infectious Diseases, Infectious Disease Surveillance Center; http://idsc.nih.go.jp/disease/measles/2011pdf).

In the control phase, identification of the index case is difficult, especially for modified measles, because cross-reactivity with IgM measles antibody makes it hard to differentiate measles infection from rubella, parvovirus B19 infection and other virus infections (14). The proportion of modified measles cases with low-grade febrile illness and mild rash, similar to vaccine-associated illness is increasing. Restriction fragment length polymorphism was developed to differentiate between vaccine-associated adverse events and wild measles infection (15). The measles genome is amplified by RT-nested PCR, PCR products are electrophoresed after