isolates from North America to representative genotypes [Peret et al., 1998; Frabasile et al., 2003].

Recent studies also demonstrated a worldwide circulation of very distinct RSV genotypes and their temporal rather than geographical clustering [Garcia et al., 1994; Cane and Pringle, 1995; Choi and Lee, 2000]. However, there is less information about the distribution pattern of RSV epidemic strains in the multiple communities over extended periods of time. Such studies will provide information about the actual circulation pattern of distinct RSV genotypes. In this study, the genetic relationship was examined of RSV strains isolated in two communities during approximately 20 seasons in Japan. We also compared Japanese strains with reference strains from Western and African countries belonging to RSV genotypes defined previously.

## MATERIALS AND METHODS

### Virus Isolates

The RSV strains examined in this study were isolated from children in the urban areas of Sapporo and Tokyo. The children were 1 month–3 years old and all developed lower respiratory tract infections. RSV was isolated in HEp-2 cell culture. RSV identification and subgrouping were performed by ELISA with subgroup specific monoclonal antibodies [Tsutsumi et al., 1989; Nagai et al., 1993] or by subgroup-distinctive RT-PCR [Sullender et al., 1993] as described previously. Several isolates from each epidemic season were examined which included 53 group A isolates: 32 in Sapporo (1980–2002) and 17 in Tokyo (1982–1999); and 56 group B isolates: 41 in Sapporo (1980–2001) and 15 in Tokyo (1985–1999). Only a limited number of Tokyo strains were obtained for testing, because fewer strains were stocked in each season and re-isolation of the virus from frozen materials sometimes failed. Only four strains in a single season (1999) were obtained from Osaka. Sapporo RSV group A and B isolates were described in part previously [Kamasaki et al., 2001; Seki et al., 2001].

### RNA Extraction and cDNA Synthesis

RNA extraction and cDNA synthesis were described previously [Seki et al., 2001]. Briefly, the frozen stock viruses were cultured in HEp-2 cells in 24-well semimicroplates and the total RNA was extracted by adding 0.8 ml of RNAzolB (Biotex Laboratories, Houston, TX) to each well when an extensive cytopathic effect was observed. The following procedure was carried out in accordance with the instructions concerning RNAzolB. The RNA pellet was dissolved in DNAse-free, RNAse-free water. For cDNA synthesis, 100 ng of the total RNA was used for RT-PCR with a random hexamer.

### PCR

The oligonucleotides used for PCR amplification were based on the published sequences of the G and F protein gene of the A2 (group A) and CH18537 (group B) strains [Johnson et al., 1987; Johnson and Collins, 1988]. Primers were set to cover the second variable region of the G gene. The 3′ oligonucleotide for both group A and B was as follows: GTTATGACACTGGTATACCAACC (nt 164–184 of the F protein gene) [Sullender et al., 1993]. The two 5′ oligonucleotide conferring subgroup specificity were as follows: (CCCGGGCATGC) GATCCCAAACCTCAAACCAC (nt 655–674 of the G gene of A2) and (CCCGGG)AAACCAACCATCAAACCCACA (nt 598–618 of the G gene of CH18537). These primers were designed for the present study. PCR was carried out in a total volume of 50 μl containing 1 μM each primer, 250 μM each dNTP (Takara, Kyoto, Japan), 10 mM Tris (pH 9.0), 500 mM KCl, 1% Triton X-100, 1.5 mM $MgCl_2$, and 50–100 ng of cDNA sample. After being denatured at 94°C for 5 min and cooled to 80°C, the mixture was seeded with 2 U of thermostable Taq polymerase (Promega, Madison, WI). Thirty-three cycles of amplification were carried out with a DNA thermal cycler (Perkin-Elmer, Norwalk, CT). Each cycle consisted of warming at 95°C for 40 sec, at 55°C for 1 min, and at 72°C for 1 min. Finally, the preparations were incubated at 72°C for 10 min.

### Nucleotide Sequencing and Computer Analysis

PCR products were sequenced directly by the autosequencer ABI PRISM 310 Genetic Analyzer (PE-ABI, Foster City, CA). The sequence of 194 nucleotides (nt 724–917) for group A and 252 nucleotides (nt 664–915) for group B at the C-terminal third of the G gene was selected from all isolates. Phylogenetic analysis was done by the neighbor-joining method with MEGA3 beta version 3.

The 10 subgroup A and 9 subgroup B sequences assigned previously to specific genotypes [Venter et al., 2001; Frabasile et al., 2003] were analyzed together. These consist of NY108 (GA1), NY20 (GA6) [Peret et al., 2000], Mon/3/88 (GA2), Mon/4/90 (GA3), Mon/8/92 (GA5), Mad/5/92 (GA7) [Garcia et al., 1994], CH09 (GA4) [Peret et al., 1998], Moz/12/99 (Cluster A-1), Moz/201/99 (Cluster A-2) [Roca et al., 2001], and SA99V1239 (SAA1) [Venter et al., 2001] for subgroup A; B1 (GB1) [Karron et al., 1997], CH93-9b (GB2) [Peret et al., 1998], NY97 (GB3), NY01 (GB4) [Peret et al., 2000], Moz/202/99 (Cluster B-1), Moz/44/99 (Cluster B-2) [Roca et al., 2001], SA0025 (SAB1), SA99V800 (SAB2), and SA98V192 (SAB3) [Venter et al., 2001] for subgroup B. Prototype strains (Long for group A and 18537 for group B) were also included.

When more than several isolates from the plural cities make one cluster and this cluster does not contain any isolates which sequences have been assigned previously to specific genotypes, these isolates were claimed to form new genotypes.

## RESULTS

The RSV isolates with unique sequences for each epidemic season in each community were included in the phylogenetic analysis. As a result, among 53 group A and 56 group B sequences, 49 (30 in Sapporo, 17 in

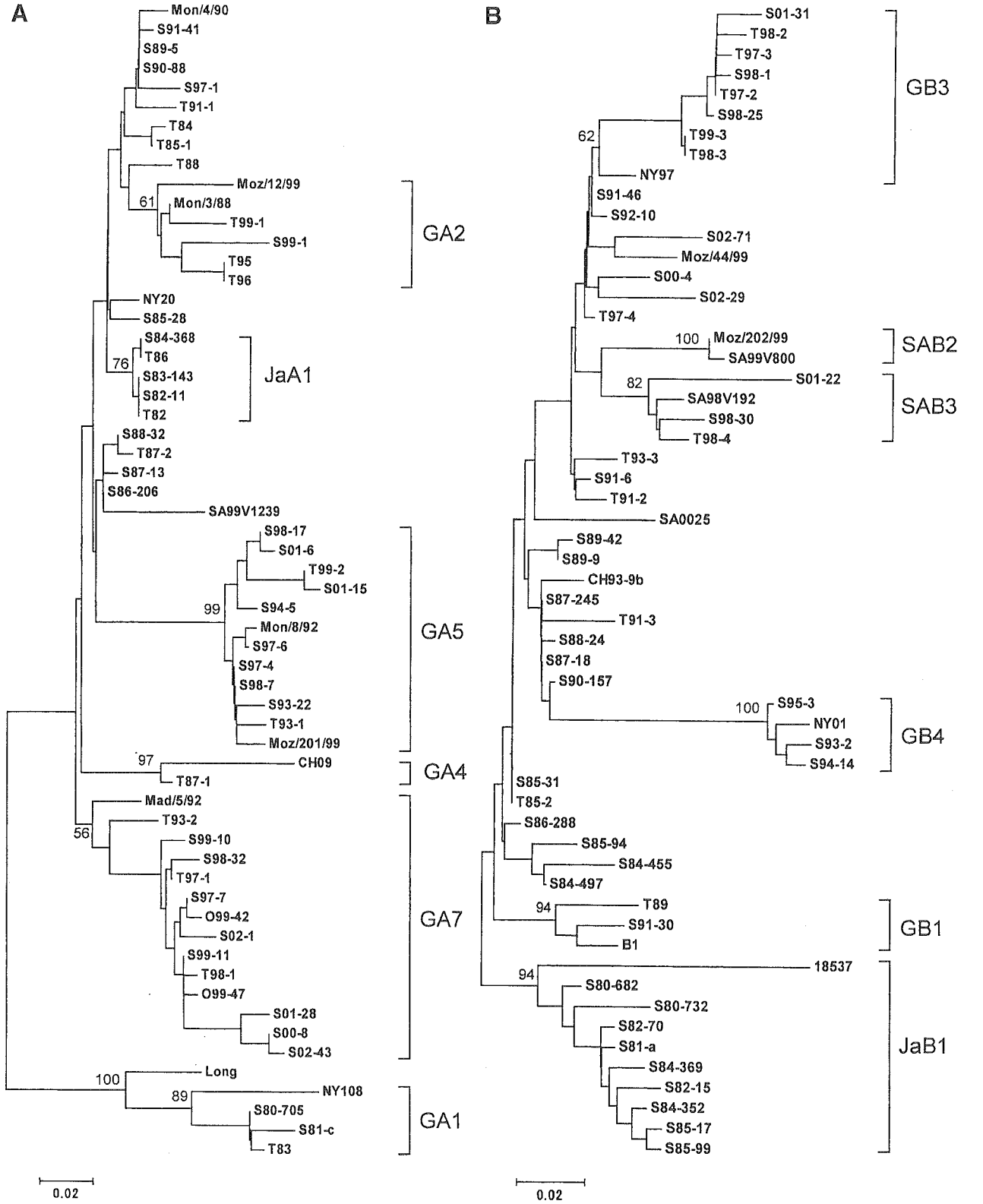


Fig. 1. Phylogenetic tree of Japanese respiratory syncytial virus (RSV) group A (**A**) and B (**B**) G gene nucleotide sequences. Group A Sapporo (1980–2002), Tokyo (1982–1999), and Osaka isolates (1999) are indicated by S80~S02, T82~T99, and O99, respectively. Group B Sapporo (1980–2001) and Tokyo (1985–1999) are indicated by S80~S02 and T85~T99, respectively. Reference sequences of NY108 (GA1), Mon/3/88 (GA2), Mon/4/90 (GA3), CH09 (GA4), Mon/8/92 (GA5), NY20 (GA6), Mad/5/92 (GA7), Moz/12/99 (Cluster A-1), Moz/201/99 (Cluster A-2), and SA99V1239 (SAA1) for group A; B1 (GB1), CH93-9b (GB2), NY97 (GB3), NY01 (GB4), Moz/202/99 (Cluster B-1), Moz/44/99 (Cluster B-2), SA0025 (SAB1), SA99V800 (SAB2), and SA98V192 (SAB3) for group B were obtained from GenBank (NY, New York; Mon, Montevideo; CH, Rochester; Mad, Madrid; Moz, Mozambique; SA, South Africa). Prototype strains Long and 18537 were also included.

Tokyo, and 2 in Osaka) group A and 47 (35 in Sapporo and 12 in Tokyo) group B sequences were analyzed, respectively.

Phylogenetic analysis of 49 field group A isolates and 11 reference strains showed six clusters, that is, GA1, GA2, GA4, GA5, GA7, and one unclassified branches named as JaA1, displaying 56%–99% bootstrap proportions (Fig. 1A). Thirteen isolates, however, could not be assigned to any branch. Each cluster contains 2–14 strains. Different genotypes usually co-circulated in each season. Group A strains isolated in proximate seasons from different communities were often located in the same branch. GA1 cluster consisted of the oldest Sapporo and Tokyo strains, that is, from the first half of the 1980s, and was very close to prototype Long, which was isolated in 1956 in USA. GA2 and GA5 clusters consisted of Sapporo and Tokyo strains from the second half of the 1990s and from after the 1990s, respectively, and both included Western or African reference strains isolated in near seasons. GA7 cluster comprised strains from three Japanese cities and Spain from after the 1990s. JaA1 cluster consisted of strains from the 1980s in Sapporo and Tokyo.

The 47 Japanese group B strains from Sapporo and Tokyo and 10 reference strains clustered into five genotypes, that is, GB1, GB3, GB4, and SAB3, and one unclassified clusters named as JaB1, supported by 62%–100% bootstrapping values (Fig. 1B). However, 22 isolates could not be assigned to any branch. Each cluster contains two to nine strains. Temporal clustering was also observed in Japanese group B strains. For example, GB1 and GB3 clusters consisted of isolates from both cities from around 1990 and from after the second half of the 1990s, respectively, and both contained Western strains isolated in just former seasons. Although GB4 cluster comprised Sapporo, not Tokyo, strains, it contained NY strain isolated in same seasons. SAB3 cluster consisted strains isolated in Sapporo, Tokyo, and South Africa during around 2000. The oldest Sapporo group B strains, that is, from the first half of the 1980s, made one unique branch named as JaB1 showing a linear evolutionary pattern, and clustered with prototype 18537 which was isolated in 1962 in USA.

The sequences were deduced to amino acids. The mean of nucleotide substitution that resulted in amino acid changes among the Japanese isolates was high at 65% and 67% for group A and B, respectively.

The predicted amino acid sequences of isolates in the different genotypes were compared to prototype strain Long and 18537 for group A and B, respectively (Fig. 2). Genotype-specific amino acid substitutions could be identified. Group A prototype Long consisted of 62 amino acids for this region and Japanese group A strains consisted of 61 or 62 amino acids. Group B prototype 18537 consisted of 74 amino acids and Japanese B strains exhibited three different lengths, namely, 74, 77, or 81 residues corresponding to inclusion of three termination codons.

In addition, several Sapporo group B isolates had unique 1–20 longer amino acid lengths than the basic form. They had 3–60 nucleotides insertions in several positions; that is, S85-94, S01-31, S02-29, S00-4, and S02-71 had 3, 15, 9, 60, and 60 nucleotides insertions at nt 706, nt 685, nt 685, nt 727, and nt 727, respectively. These mutant strains grew in the same manner in HEp-2 cell culture as other group B strains (data not shown).

## DISCUSSION

Several factors seem to be important in the circulation and genetic evolution of RSV strains. These include infectivity of the virus, the development of immunological resistance in the community, and viral genetic drift due to spontaneous mutation [Sullender, 2000]. There have been several reports on RSV molecular epidemiology in single or small communities during several epidemic seasons. These studies revealed a predominant lineage in each epidemic season and a change of the predominance over one to three epidemics [Choi and Lee, 2000; Seki et al., 2001]. The evolutionary pattern of

**A**

| | | 240 250 260 270 280 290 298 | |
|---|---|---|---|
| Long | | NTTKTNIITTLLTNNTTGNPKLTSQMETFHSTSSEGNLSPSQVSTTSEHPSQPSSPPNTTRQ* | (62) |
| S80-705 | (GA1) | .....SH..R..E.....L.....P.....I...YL.....P..* | (62) |
| S81-c | (GA1) | .....SH..R..E.....L.....P.....I...YL.....P..* | (62) |
| T83 | (GA1) | .....SH..R..E.....L.....P.....I...YL.....P..* | (62) |
| S99-1 | (GA2) | .....R.....S.I.E..EH...K..L...T.....P....FY.....Y...SP..S...KW* | (62) |
| T95 | (GA2) | .....R.....S.....EH...K..L...T.....Y....YL..SL..S...KW* | (62) |
| T96 | (GA2) | .....R.....S.....EH...K..L...T.....Y....YL..SL..S...KW* | (62) |
| T99-1 | (GA2) | .....R.....S.....EQ...K.NL...T.....P.....Y....YL..SL..S...Y* | (61) |
| T87-1 | (GA4) | .....R..M.....EN...K..L.....P.....Y....Y.....P..S...A* | (61) |
| S93-22 | (GA5) | .I..P..R.....S....LEH..LE..L.....T.....Y....YL...P..S.I.N.* | (62) |
| S94-5 | (GA5) | .I..P..R.....S....L.H...E..L.....T.....Y....YL...P..S.I.D.* | (62) |
| S97-4 | (GA5) | .I..P..R.....S....LEH...E..L.....T.....Y....YL...P..S.I.N.* | (62) |
| S97-6 | (GA5) | .I..P..R.....S....LEH...E..L.....T.....Y....YL...P..S.I.N.* | (62) |
| S98-7 | (GA5) | .I..P..R.....S....LEH...E..L.....T.....Y....YL...P..S.I.N.* | (62) |
| S98-17 | (GA5) | .I..P..R.....S....LEH...E..L.....T.....IY....YL...P..S.I.D.* | (62) |
| S01-6 | (GA5) | .I..P..R.....S....LEH...E..L.....T.....IY....YL...P..S.I.D.* | (62) |
| S01-15 | (GA5) | .I..P..R.....S.K..LEH...Q..L.....T.....IY....YL...P..S.I.D.* | (62) |
| T93-1 | (GA5) | .I..P..R.....S....LEH...E..L.....T.....AY....YL...P..S.I.N.* | (62) |
| T99-2 | (GA5) | .I..P..R.....S....LEH...Q..L.....T.....IY....YL...P..S.I.D.* | (62) |
| S97-7 | (GA7) | ...R...G.....T.....LEY...K..L.....P...P.....Y.....Y....P..S...NE* | (62) |
| S98-32 | (GA7) | ...R...G.....T.....LEY...K.....P...P.....Y.....Y....P..S...SE* | (62) |
| S99-10 | (GA7) | ...R...G.....T.....LEH...K.....P...P.....Y.....Y....P..S...NE* | (62) |
| S99-11 | (GA7) | ...R...G.....T.....LEY...K.....P...P.....Y.....Y....P..S...NE* | (62) |
| S00-8 | (GA7) | ...R...G.....T.....IEY...K.....P...P..P..Y.....Y....P..S...HE* | (62) |
| S01-28 | (GA7) | ...R...G.....T.....IEY...K.....P...P.....Y.....Y....P..S...HD* | (62) |
| S02-1 | (GA7) | ..AR...G.....T.....LEY...K.....P...P.....Y.....Y....P..S...NK* | (62) |
| S02-43 | (GA7) | ...R...G.....T.....IEY...K....L..P...P..P..Y.....Y....P..S...HE* | (62) |
| T93-2 | (GA7) | ...R...R.....T.....EY...R..LY...P...P.....Y.....Y....P..S...N* | (61) |
| T97-1 | (GA7) | ...R...G.....T.....LEY...K.....P...P.....Y.....Y....P..S...NE* | (62) |
| T98-1 | (GA7) | ...R...G.....T.....LEY...K.....P...P.....D.....Y....P..S...NE* | (62) |
| O99-42 | (GA7) | ...R...G.....T...E.LEY...K..L....P...P.....Y.....Y....P..S...NE* | (62) |
| O99-47 | (GA7) | ...R...G.....T.....LEY...K.....P...P.S...Y.....Y....P..S...NE* | (62) |
| S82-11 | (JaA1) | .....R.....T.....EH...K..L...F....P.....Y....YL...L..S...N* | (61) |
| S83-143 | (JaA1) | .....R.....T.....EH...K..L...F....P.....Y....YL...L..S...N* | (61) |
| S84-368 | (JaA1) | .....R.....T.....EH...K..L...F....P.....Y....YL...L..S...N* | (61) |
| T82 | (JaA1) | .....R.....T.....EH...K..L...F....P.....Y....YL...L..S...N* | (61) |
| T86 | (JaA1) | .....R.....T.....EH...K..L...F....P.....Y....YL...L..S...N* | (61) |
| S85-28 | | .....R.....T.....EH...K..L.....S.P.....Y....YL...P.LS...N* | (61) |
| S86-206 | | .....R.....EH...K..L.....P.....Y....YL...P..S...N* | (61) |
| S87-13 | | .....R.....EH...K..L.....P.....Y....YL...P..S..AN* | (61) |
| S88-32 | | .....R.....EH...K..L.....P.....Y....YL...P..SS..N* | (61) |
| S89-5 | | .....R.....T.....EH...K..L.....P.....Y....YL..SP..S...NL* | (62) |
| S90-88 | | .....R.....T.....EH...K..L.....P.....Y....YL..SP..S...NL* | (62) |
| S91-41 | | .....R.....T.....EH...K..L.....P.....Y....YL..SP..S...NL* | (62) |
| S97-1 | | .....R.....T.....EH...K..L.....P....FY.....L..SP..S...NL* | (62) |
| T84 | | .....R.....T.....H...K..L.....P.....Y....Y...SP..S...N* | (61) |
| T85-1 | | .....R.....T.....EH...K..L.....P.....Y....Y...SP..S...N* | (61) |
| T87-2 | | .....R.....EH...K..L.....P.....Y....YL...P..SS.KN* | (61) |
| T88 | | .....R.....T.....EH...K..L.....P.....H....YL..YP..S...N* | (61) |
| T91-1 | | .....R.....T.....H...K..L.....P.S...Y....YL..SP..S...NL* | (62) |

Fig. 2. Amino acid alignment of the G protein for Japanese group A (**A**) and group B (**B**) genotypes. Alignments are shown relative to prototype sequences Long and 18537, respectively. Amino acid length numbers for these regions are indicated in the brackets. Stop codons are expressed by (*).

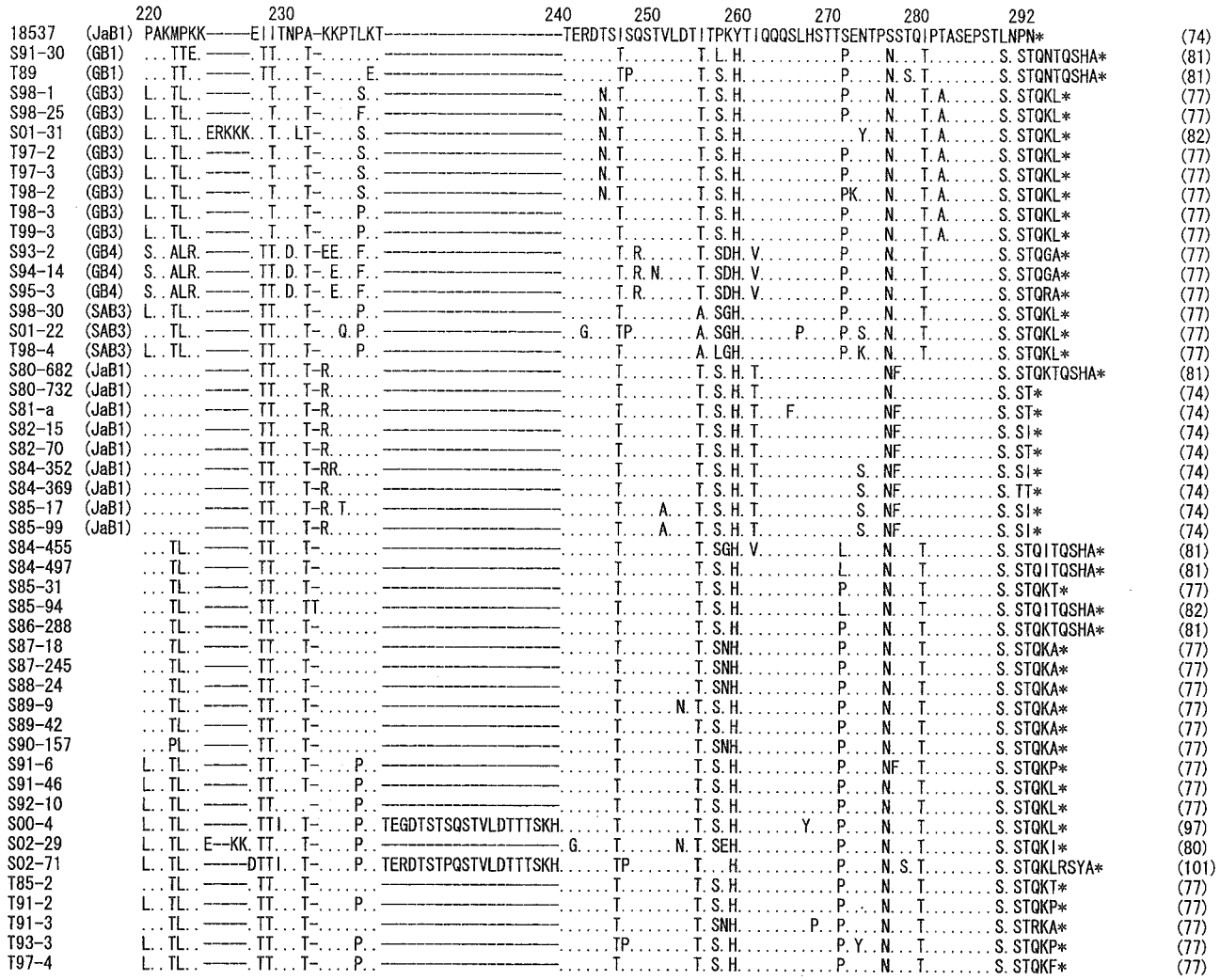


Fig. 2. (*Continued*)

RSV correlates with temporal rather than geographic variation [Garcia et al., 1994; Cane and Pringle, 1995]. On the other hand, Peret et al. [1998] showed that RSV outbreaks were community based in nature in a study on isolates during a single epidemic season in five distinct communities. To clarify the distribution pattern of RSV epidemic strains more precisely, a genetic analysis was undertaken of many RSV isolates from several communities over long-term epidemic seasons.

In the present study, field isolates from two Japanese urban communities, Tokyo and Sapporo, which are 1,000 km apart, over approximately 20 epidemic seasons were evaluated with reference strains in Western and African countries. Although only a limited number of strains and limited epidemic seasons were evaluated, some interesting findings were obtained.

In comparison with reference strains, Japanese group A strains formed five known clusters and one original cluster. On the other hand, Japanese group B strains formed four known clusters and one original branch. Almost all branches of phylogenetic trees consisted of isolates from the two communities during similar and limited epidemic seasons. Of particular importance is that the group A and B strains which were allocated to known genotypes were usually isolated at almost the same time as each reference strains from Western and African countries. These findings suggest that Japanese RSV strains have circulated with a geographical and temporal clustering pattern while participating in genetic evolution in a global setting.

On the other hand, considerable number of Japanese group A and B strains could not be assigned to any RSV genotypes identified previously in Western or African countries. This might have resulted partially from technical matters, such as the location of G sequences selected to make a phylogenetic tree in the present study. However, it is still considered that there are some other unidentified genotypes in Japan and also in the world, and Japanese strains, especially group B, have evolved steadily and individually in each community

with repeat epidemics, as shown by Venter et al. [2001] in South Africa.

Higher mean percentages of nucleotide changes resulting in amino acid changes in the present study (65% for group A and 67% for group B) suggest an immunological pressure in these genetic evolutions [Garcia et al., 1994; Cane and Pringle, 1995].

In the present study, several wild mutant group B strains are described which have G protein with 1–20 extra amino acid sequences in the C-terminal third. They had 3–60 nucleotide insertions at several nucleotide positions. These mutations were also confirmed in the analysis of nasopharyngeal secretion specimens. Therefore, it is rather unlikely that these mutant strains resulted from in vitro technical matters such as the process of tissue culture. A nosocomial outbreak of the mutant strain with 20 amino acid longer G protein (S00-4) was identified in a day care center in 2000–2001 epidemic season [Nagai et al., 2004]. In Buenos Aires, Argentina, three similar group B strains with 60 nucleotides insertions at the G protein gene were isolated in 1999 [Trento et al., 2003]. The sequences of extra nucleotides and insertion site are nearly identical with those of the Sapporo strains (S00-4 and S02-71). They possibly represent the progenitors of the mutant viruses.

Mutant group A strains with altered G protein length were not found in this study, and to our knowledge there has been no reports of such mutant strains, although Cane and Pringle [1995] reported one exceptional mutant group A strain with a truncated G protein. In vitro, some escape mutant group A strains have been produced with neutralizing monoclonal antibodies and characterized [Garcia-Barreno et al., 1990; Rueda et al., 1991].

Interestingly, almost all mutant group B strains with change of nucleotide length were isolated during a couple of recent years. Therefore, the susceptibility of the G gene C-terminal third to mutation may be one of the characteristics of recent RSV group B field strains. One group B wild strain with an over 30 amino acid truncated G protein was isolated recently in South Africa [Venter et al., 2002]. Further emergence of such wild mutant strains should be monitored in the future.

The G protein is one of the targets for the neutralizing and protective immune response [Norrby et al., 1987; Rueda et al., 1991]. The G protein, especially its C-terminal third, is the most variable both between and within the two subgroups and appears to accumulate amino acid changes with time [Garcia et al., 1994; Sullender, 2000]. Changes in this portion sufficient to alter the amino acid lengths, but without apparent functional deficit, may result in escape from immunological responses and present one possible way whereby the group B strains can survive and cause repeated epidemics in one community.

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# A Simple Method for the Detection of Measles Virus Genome by Loop-Mediated Isothermal Amplification (LAMP)

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Approximately 20,000–30,000 measles patients were reported in a surveillance of infectious diseases because of low vaccine coverage of 80% in Japan. Among them, some were thought to be secondary vaccine failure (SVF) with generally mild or non-typical measles illness and sometimes became a source of further transmission. We have developed a new, sensitive, and rapid method to detect the measles virus genome by reverse transcription loop-mediated isothermal amplification (RT-LAMP). We examined 50 nasopharyngeal secretion (NPS) samples that were obtained during the 1999 outbreak and stored at −70°C and fresh NPS, lymphocytes and sera from 11 patients in 2003. Total RNA was extracted from the samples and subjected to reverse transcription-polymerase chain reaction (RT-PCR) and RT-LAMP. We detected the genomic RNA corresponding to at least 0.01–0.04 TCID50, 30–100 copies in samples by RT-LAMP within 60 min after extraction of RNA, and all four genotypes isolated in Japan were equally amplified. Specific DNA amplification was monitored spectrophotometrically by real time turbidimeter and the quantity of RNA was calculated. Measles virus genome was detected in 44 of 50 stored NPS by RT-PCR and in 49 by RT-LAMP. The vaccine strain was discriminated from wild strains after sequencing the LAMP products. RT-LAMP is a useful rapid diagnostic method for the detection of measles virus without any special apparatus, showing higher sensitivity than RT-PCR, and expected to be applied for hospital-based infection control and for laboratory-based measles surveillance. ***J. Med. Virol. 76:406–413, 2005.*** © 2005 Wiley-Liss, Inc.

**KEY WORDS:** measles virus; reverse transcription-polymerase reaction (RT-PCR); reverse transcription-loop-mediated isothermal amplification (RT-LAMP); rapid diagnosis



## INTRODUCTION

Measles virus is a single stranded negative sense RNA virus, belonging to the genus Morbillivirus, family Paramyxoviridae, order Mononegavirales. It consists of 15,894 nucleotides, coding six structural proteins; nucleoprotein (N), phosphoprotein (P), membrane (M), fusion (F), hemagglutinin (H), and large (L) proteins [Griffin et al., 2001]. Two glycoproteins of F and H are components of outer surface envelope proteins and cooperatively play an important role in the initial attachment of virus to the cells and following virus-cell fusion [Wild et al., 1991; Griffin et al., 2001]. Genome RNA is surrounded by N, P, and L proteins and they comprise the ribonucleoprotein complex (RNP) [Griffin et al., 2001]. Measles virus is considered to have monotypic characteristics but genetic variation has been described for the wild types. The standardized nomenclature for describing the genetic characteristics of wild-type measles virus isolates is demonstrated using 8 clades, designated A, B, C, D, E, F, G, and H, with 22 genotypes [WHO, 2001]. The differentiation of measles virus genotypes is based on the sequence results of the most variable region of 500 nucleotides at the 3′end of the N gene or full-length H gene. In our previous

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reports [Nakayama et al., 1995; Yamaguchi, 1997; Takahashi et al., 2000], the measles virus strains isolated in Japan since 1984 were classified into four different genotypes, C1 (before 1985), D3 (1985–1990), D5 (after 1990), Chicago-type D3 (1997–1999), and H1 genotype (after 2000) [Zhou et al., 2003].

Measles is still a major killer among infants worldwide and the total number of cases is estimated to be more than 30 million despite approximately 80% of vaccine coverage. Meanwhile, measles-related deaths are estimated to be 0.77 million [WHO, 2002; Stein et al., 2003]. Recently, several problems have come into existence, such as sporadic measles outbreaks, an increase in the number of secondary vaccine failure (SVF) and transmission in high school or among university students and adults [Helfand et al., 1998; WHO, 2002; Nakayama et al., 2003]. Some modified measles patients with SVF were not correctly diagnosed without any virological examinations because of their non-typical measles illnesses and caused further transmission among susceptible individuals.

The diagnosis of virus infection is traditionally performed by virus isolation and serological examinations, but these methods are time-consuming and not appropriate for clinical setting. In some virus laboratories, molecular-based diagnostic methods, such as reverse transcription-polymerase chain reaction (RT-PCR) and hybridization were employed. Recently, real time RT-PCR has been developed, showing its rapidity and quantitative features [Ozoemena et al., 2004; Schalk et al., 2004]. Approximately 100–280 copies of the measles virus genome were detected [Ozoemena et al., 2004; Schalk et al., 2004], but it is not appropriate as a rapid diagnostic tool for clinical use because it requires a specific apparatus. A more sensitive and specific method for DNA amplification method, loop-mediated isothermal amplification (LAMP), was developed by one of the authors [Notomi et al., 2000]. This method employed *Bst* DNA polymerase with strand displacement activity and a set of four specially designed primers that recognize a total of six different sequences in the target DNA. The key reaction is the construction of a 5′ and 3′ end loop dumb-bell structure and multi-branched stem-loop products are amplified through repetition of the reactions. The distinctive features of LAMP are rapidity, high sensitivity, high specificity, and simplicity; these are required for the rapid diagnosis. We developed this new method for the detection of the measles virus genome by real-time reverse transcription-loop-mediated isothermal amplification (RT-LAMP) targeting the measles N gene and compared its sensitivity with nested RT-PCR reported previously [Nakayama et al., 1995; Zhou et al., 2003].

## MATERIALS AND METHODS

### Measles Virus Strains

All measles virus strains used in this study were isolated in Japan from 1984 to 2002; genotype A [Edmonston, AIK-C], genotype C1 [MVi/Tokyo.JPN/84-E], genotype D3 [MVi/Tokyo.JPN/87-K], Chicago-type D3 [MVi/Tokyo.JPN/37.99(Y)], genotype D5 [MVi/Tokyo.JPN/2000-KA], and Genotype H1 [MVi/Tokyo.JPN/20.00(S)]. They were isolated from nasopharyngeal swabs (NPS) or peripheral blood mononuclear cells (PBMC) by Vero cells before 1985 and B95a cell cultures after 1987 [Nakayama et al., 1995; Yamaguchi, 1997; Takahashi et al., 2000; Zhou et al., 2003]. To examine the sensitivity of RT-LAMP, we used 50 nasopharyngeal secretion (NPS) samples stored at −70°C obtained from natural measles and fresh samples were obtained from 11 patients; 8 were suspected as having SVF and 3 had vaccine-associated illness.

### RT-PCR

Total RNA was extracted from 200 μl of virus culture fluid or clinical samples with a magnetic bead RNA extraction kit (TOYOBO Co. Ltd., Osaka, Japan), and the RNA pellet was suspended in 25 μl of distilled water. It was subjected to nested RT-PCR and RT-LAMP targeted at the COOH terminus of the N protein region known as the most variable region [WHO, 2001]. The measles virus genome was first converted to cDNA with N-430(+) primer (5′-ATTAGTAGTGATCAATCCAGG-3′) with AMV reverse transcriptase (Life Technologies, Inc., Gaithesburg, MD). The first PCR was performed with a set of N-850(+) (5′-TAGAAACTATGTATCC-TGCT-3′) and MPX(−) (5′-AGGCCTGATTGAACCAT-GAT-3′), and the nested PCR was done with N1200(+) (5′-GATCCAGCATATTTTAGATTAG-3′) and NP-P2(−) (5′-AGGGTAGGCGGATGTTGTTCT-3′). PCR was performed using 1.25 U of *Taq* DNA polymerase (TaKaRa BioMedicals, Tokyo, Japan) by TaKaRa thermal cycler (TaKaRa BioMedicals) with 30 rounds of thermal cycling conditions; denature at 93°C for 1 min, re-annealing at 58°C for 1 min, and extension at 72°C for 2.5 min. PCR products were confirmed by electrophoresis through 1.5% agarose gel stained with ethidium bromide, as previously reported [Nakayama et al., 1995; Yamaguchi, 1997; Zhou et al., 2003].

### Measles Virus RT-LAMP

LAMP method was characterized by auto-cycling strand displacement DNA synthesis with *Bst* DNA polymerase (New England Biolabs, Beverly, MD) and a specially designed set of primers. The principle of primer design is shown in Figure 1A and the LAMP primer is targeted for the N region similar to the RT-PCR region from the genome position 1242 to 1442 (Fig. 1B). We synthesized six LAMP primers recognizing eight different regions, referred to the software program for LAMP primer design (Eiken Chemical Co. Ltd., Tokyo, Japan); two outer primers (F3 and B3), two inner primers, a forward inner primer (FIP) and a backward inner primer (BIP), and two loop primers (Loop F and Loop B), and primer sequences are shown in Figure 1C. The FIP contains the complementary alignment of F1 linked with the F2 sequence (F1C + F2), and BIP contains the complementary sequence of B1 sequence linked with the

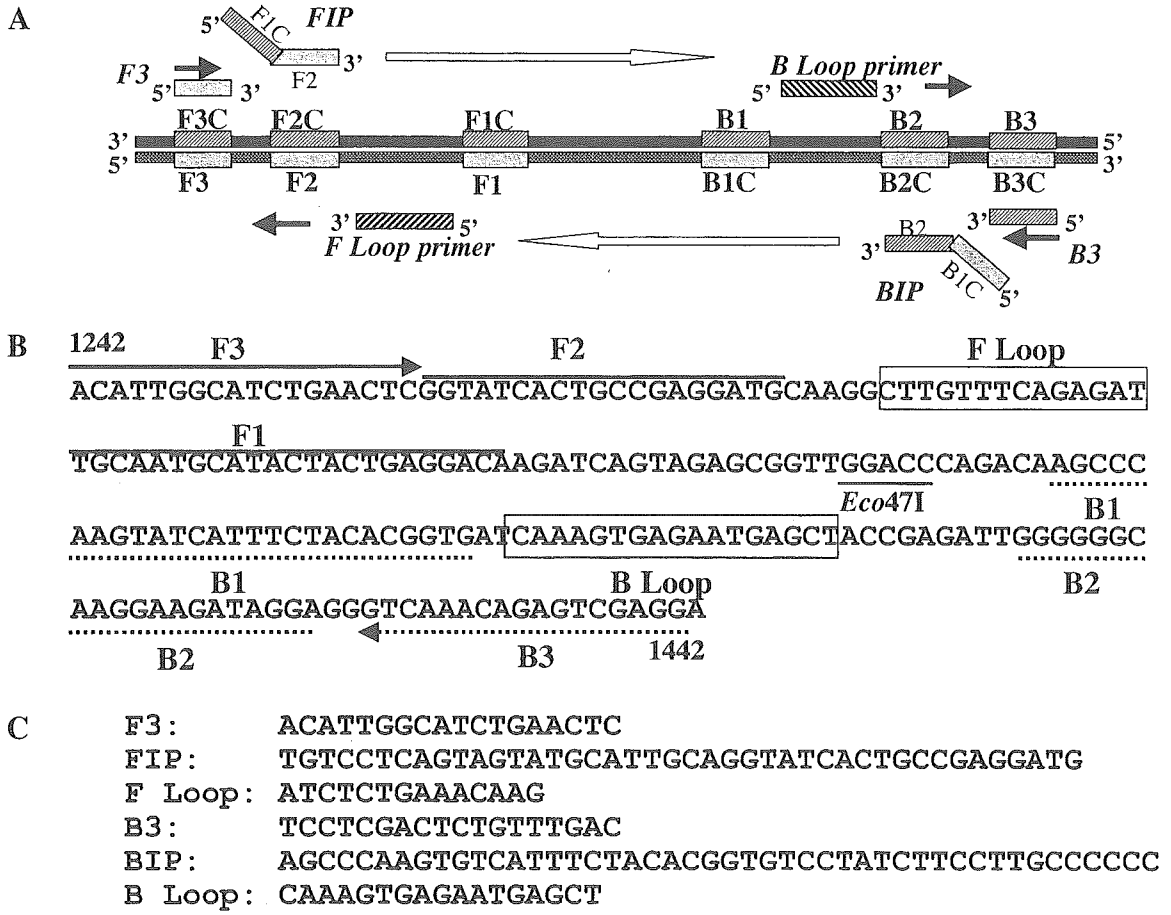


Fig. 1. Primer design for loop-mediated isothermal amplification (LAMP) for the detection of measles genome. Diagram of LAMP primer design (**A**), sequence data from the genome position 1242 to 1442 (**B**) and sequence alignments of the primers (**C**). Eco47I site is underlined to confirm the measles virus genome amplification by reverse transcription-loop-mediated isothermal amplification (RT-LAMP).

B2 (B1C + B2). Basically, these four primers amplified the target DNA and we synthesized two additional loop primers F and B located between F1 and F2, and between B1 and B2, respectively. The addition of two loop primers enhances the specificity and reactivity [Nagamine et al., 2002]. For the LAMP reaction, the mixture was made up to a total of 25 μl of reaction mixture, containing 40 pmol (each) of FIP and BIP, 5 pmol (each) of F3 and B3, 20 pmol (each) of Loop F and Loop B, 1.4 mM each dNTPs, 0.8M betaine, 20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween 20, 1 U AMV reverse transcriptase (New England Biolabs), 8U *Bst* DNA polymerase (New England Biolabs), and 5 μl of sample RNA. The reaction mixture was subjected to real-time turbidimeter LA200 (TERAMECS, Kyoto, Japan) [Mori et al., 2004] and the LAMP reaction was carried out at 63°C for 60 min. The turbidity was scanned every 6 sec.

The diagram of LAMP is shown in Figure 2. Measles genome is a negative sense RNA and then first converted to cDNA with F2 portion of FIP primer in Figure 2(1) by AMV reverse transcriptase. F3 primer extends the cDNA synthesis with displacement of RNA-cDNA double strand in Figure 2(2). The reverse transcription process produces two kinds of structures; RNA-cDNA complex from F3 to B3 portion and single strand cDNA primed by FIP primer in Figure 2(3). This cDNA forms 5′end loop structure. BIP primer anneals to 3′ end of cDNA and extends DNA synthesis in Figure 2(3)(4). B3 primer attaches the B3 portion and detaches the double strand DNA in Figure 2(5). Thereafter, double strand DNA and dumb-bell loop structure of single strand DNA are produced in Figure 2(6)(7). This dumb-bell loop structure is basic product for further extension of LAMP reaction and FIP primer binds to the 3′ end of single strand loop region in Figure 2(7). Similar DNA synthesis with displacement activity continues with cycling reaction and multi-branched loop structures are synthesized [Notomi et al., 2000].

As the LAMP reaction progresses, the reaction by-products pyrophosphate ions bind to magnesium ions and they form white precipitates of magnesium pyrophosphate. Light (650 nm) emitted by light emitting diodes passes through PCR tubes containing the LAMP solution and illuminates the photodiode on the opposite side. The turbidity is calculated based upon the ratio between the intensity of light received by photodiode and emitted light intensity. Thus, measurement of the

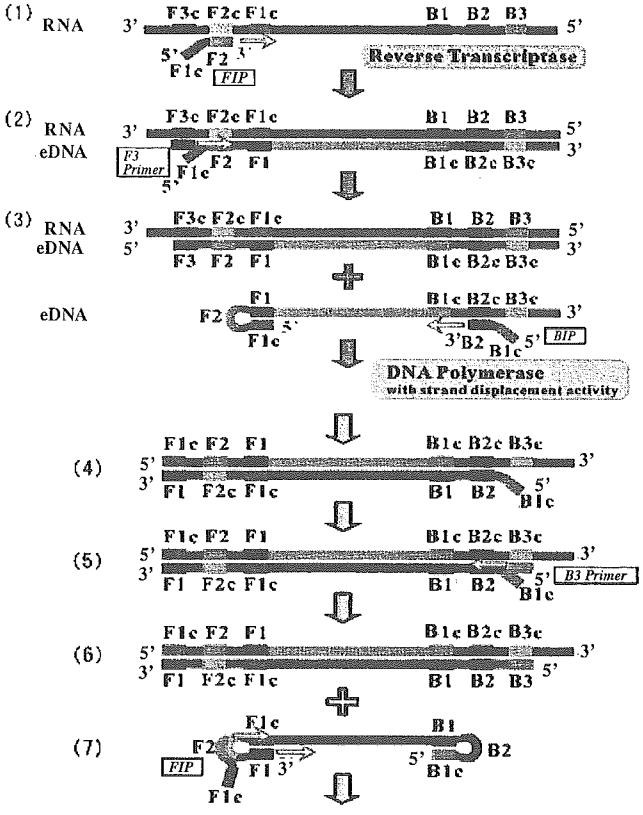


Fig. 2. Diagram of RT-LAMP.

turbidity closely related to the amplification of DNA and the turbidity >0.1 was considered as LAMP positive [Mori et al., 2004].

### Sequencing of the LAMP Products

The LAMP product was purified by a magnetic bead DNA purification kit (TOYOBO Co. Ltd.) and was sequenced with the F2 primer by dye terminator method using ABI 377A sequencer (Applied Biosystems, Foster city, CA).

### Construction of the N Protein Expression Plasmid

We have already reported the construction of N protein expression plasmid for reverse genetics [Kumada et al., 2004]. Coding region of the N gene was cloned in pBleuscript SK II-vector at the downstream of T7 promoter. Plasmid was linearized by *Spe* I digestion and RNA was transcribed by T7 RiboMAX Express Large Scale RNA Production System (Promega, Madison, WI). The transcribed RNA was used as a template for RT-LAMP.

## RESULTS

### Sensitivity of LAMP

Mvi/Tokyo.JPN/87-K strain of genotype D3 was used to examine the sensitivity of the LAMP method and the results are shown in Figure 3. The virus contained $2 \times 10^3$ TCID 50/200 μl. RNA was extracted from 200 μl and suspended in 25 μl. The RNA was serially diluted by 1:10 and 5 μl was used for nested RT-PCR and RT-LAMP. The threshold for a positive reading of the spectrophotometric value was defined as 0.1 [Mori et al., 2004]. Measles virus genome was detected at a $10^{-4}$ dilution by RT-LAMP and at $10^{-3}$ dilution by nested RT-PCR. At least 0.04 TCID 50 genome was detected by RT-LAMP. We synthesized the measles N gene RNA and the sensitivity was examined. The detection limit was estimated as 30–100 copies of RNA in the sample (data not shown).

We compared the sensitivity of LAMP for different genotypes of measles virus and the results of MVi/Tokyo.JPN/2000-KA (genotype D5), MVi/Tokyo.JPN/

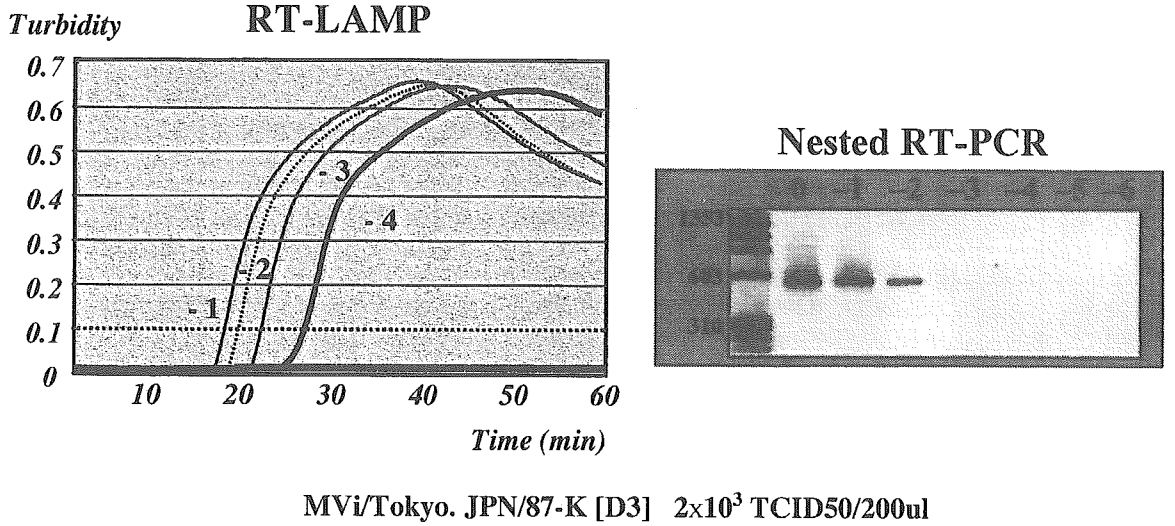


Fig. 3. Comparison of the sensitivity of RT-LAMP and nested reverse transcription-polymerase chain reaction (RT-PCR). MVi/Tokyo.JPN/87-K (genotype D3) was used. RNA was extracted from 200 μl of $2 \times 10^3$ TCID50 and re-suspended in 25 μl. Five micro liters was subjected to RT-LAMP and nested RT-PCR.

37.99(Y)(genotype D3), and MVi/Tokyo.JPN/20.00(S) (genotype H1) are shown in Figure 4A. The infectivity of genotypes D5 was $6 \times 10^3$ TCID50/200 μl and RT-LAMP showed positive for $10^{-5}$ dilution. The detection limit was estimated as 0.012 TCID50. D3 and H1 contained $8 \times 10^4$ TCID50/200 μl, and $3 \times 10^4$ TCID50/200 μl, respectively. RT-LAMP was positive for $10^{-6}$ dilution of both genotype strains and detection limit was estimated as 0.016–0.006 TCID50 with similar sensitivity. All genotypes (A, C1, D3, D5, and H1) were equally amplified.

We analyzed the correlation between the time (in seconds) to reach the threshold >0.1 of turbidity and infectivity (TCID50). The result using MVi/Tokyo.JPN/20.00(S)(genotype H1) is shown in Figure 4B. A linear correlation was obtained: y (TCID50) = $-0.0064 \times$ (seconds) +10.123. Using the equation, we calculated the virus genome quantity related to the infectivity of the samples.

Multi-branched stem loop structure is the characteristics of LAMP and LAMP products demonstrated the typical ladder pattern. Specific amplification was confirmed that the ladder-like LAMP products became a single band after digestion with specific restriction enzyme. Eco47I site is demonstrated in Figure 1B. The results of electrophoresis are shown in Figure 4C. LAMP products of D5, D3, and H1 exhibited a ladder pattern in lanes 1, 3, and 5 and after digestion with Eco47I they became a single DNA band (lanes 2, 4, and 6).

### Detection Rate by RT-PCR and LAMP

We used 50 NPS samples from the patients diagnosed clinically as having a measles infection in 1999 and they were stored at −70°C for 4 years. The results of the RT-PCR and LAMP are shown in Table I. Measles virus was not isolated from the stored samples but the genome was detected in 49 by RT-LAMP and in 44 by nested RT-PCR. Fresh samples were obtained from 11 patients. Measles virus was isolated from two NPS samples and the measles genome was detected in 8 from 11 NPS by nested RT-PCR and in 9 by RT-LAMP. It was also detected in all PBMC and serum samples by RT-LAMP with higher sensitivity than by RT-PCR.

### Sequence Analysis of LAMP Products of Measles Infection

Wild-type measles virus genotypes are now classified into 22 genotypes [WHO, 2001] and we depicted the sequence alignments of 22 reference strains in the target

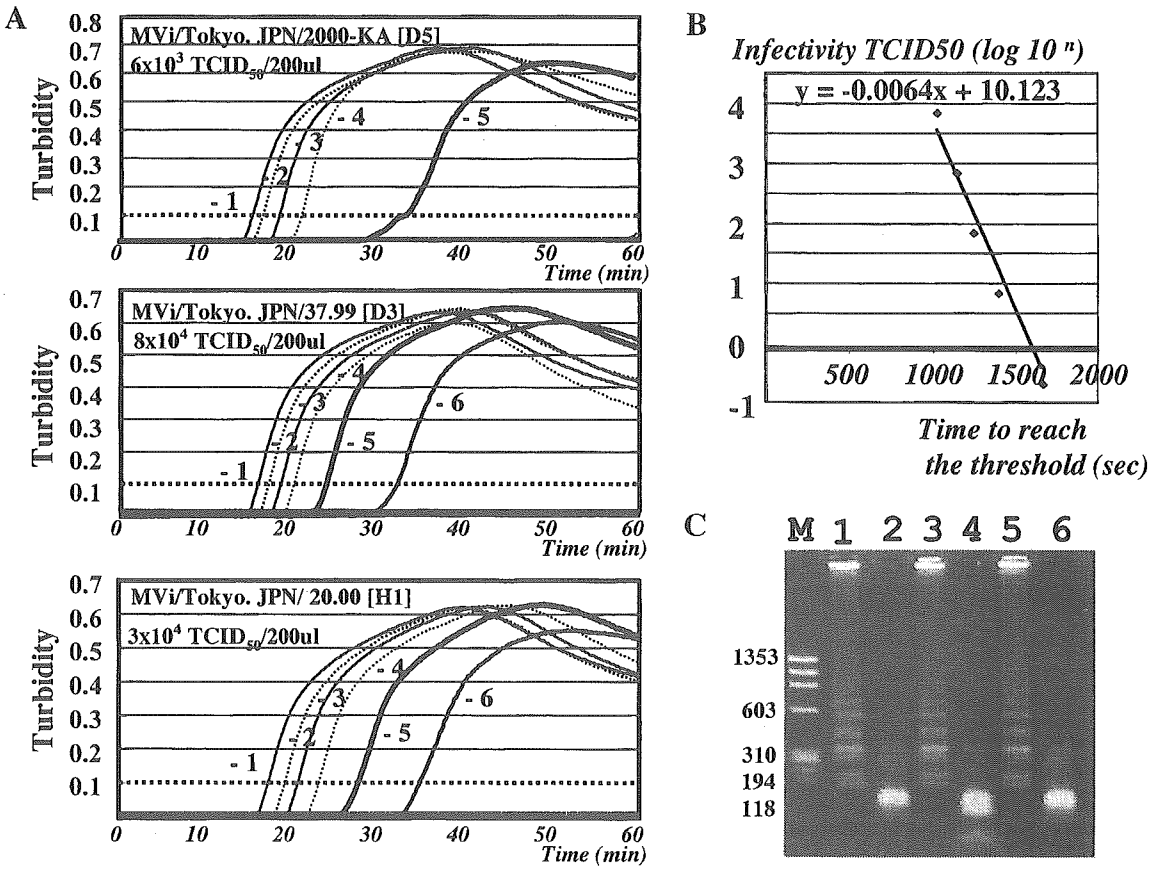


Fig. 4. Sensitivity of RT-LAMP in different genotypes. MVi/Tokyo.JPN/2000-KA (genotype D5), MVi/Tokyo.JPN/37.99 (genotype D3), and MVi/Tokyo.JPN/20.00 (genotype H1) were used. RNA was extracted from 200 μl of virus culture medium and re-suspended in 25 μl. Five micro liters was subjected to RT-LAMP (**A**). LAMP reaction times were monitored to reach the threshold >0.1 in turbidity and the correlation between the logarithmic titers of infectivity and reaction time is shown (**B**). The results of electrophoresis of LAMP products are shown (**C**). LAMP products of D5, D3, and H1 are shown in lanes 1, 3, and 5 and those after digestion with Eco47I in lanes 2, 4, and 6.

TABLE I. Detection of Measles Virus Genome by Nested Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Reverse Transcription-Loop-Mediated Isothermal Amplification (RT-LAMP)

| Samples | Virus isolation | Nested RT-PCR | RT-LAMP |
|---|---|---|---|
| Stored NPS (n = 50) | — | 44 | 49 |
| NPS (n = 11) | 2 | 8 | 9 |
| PBMC (n = 6) | 0 | 4 | 6 |
| Serum (n = 5) | ND | 3 | 5 |

NPS, nasopharyngeal secretion; PBMC, peripheral blood mononuclear cells.

region of the N gene (Fig. 5). Two hundred one nucleotides were amplified from genome position 1242 to 1442 by RT-LAMP.

After immunization with live measles vaccine, approximately 10% of the recipients developed febrile reactions and rash. We obtained NPS, PBMC, and sera from three patients with vaccine-associated illness. In three recipients, measles genome was detected from PBMC and plasma. LAMP products were purified and sequenced by F2 primer. They were identified as genotype A and the others were H1 wild genotype circulating in Japan.

## DISCUSSION

In Japan we still have annual outbreaks and especially school outbreaks are reported in teenagers and adults [Nakayama et al., 2003]. They had a past history of immunization and did not show the typical measles illness [Helfand et al., 1998; Mossong et al., 1999, Lievano et al., 2004]. They were initially diagnosed as having toxic dermatitis, a drug allergy, or an unknown viral infection. When they were diagnosed as having measles infection by conventional serological examination, they extended the infection to persons who were in contact with them [Mossong et al., 1999]. In this standpoint of view, rapid virological diagnostic kits are expected for the diagnosis of the patients with vaccine-modified non-typical measles.

Virus isolation takes more than 1 week, even using sensitive B95a cells [Kobune et al., 1990]. As for the serological response, the detection of IgM EIA antibodies was employed in a clinical setting, but the negative for IgM EIA does not always imply negativity in terms of virus infection [Griffin and Bellini, 2001]. A recent development in the molecular approach was applied for a clinical diagnostic tool. We reported the use of nested RT-PCR for the detection of the measles virus genome, and it showed a high sensitivity and specificity

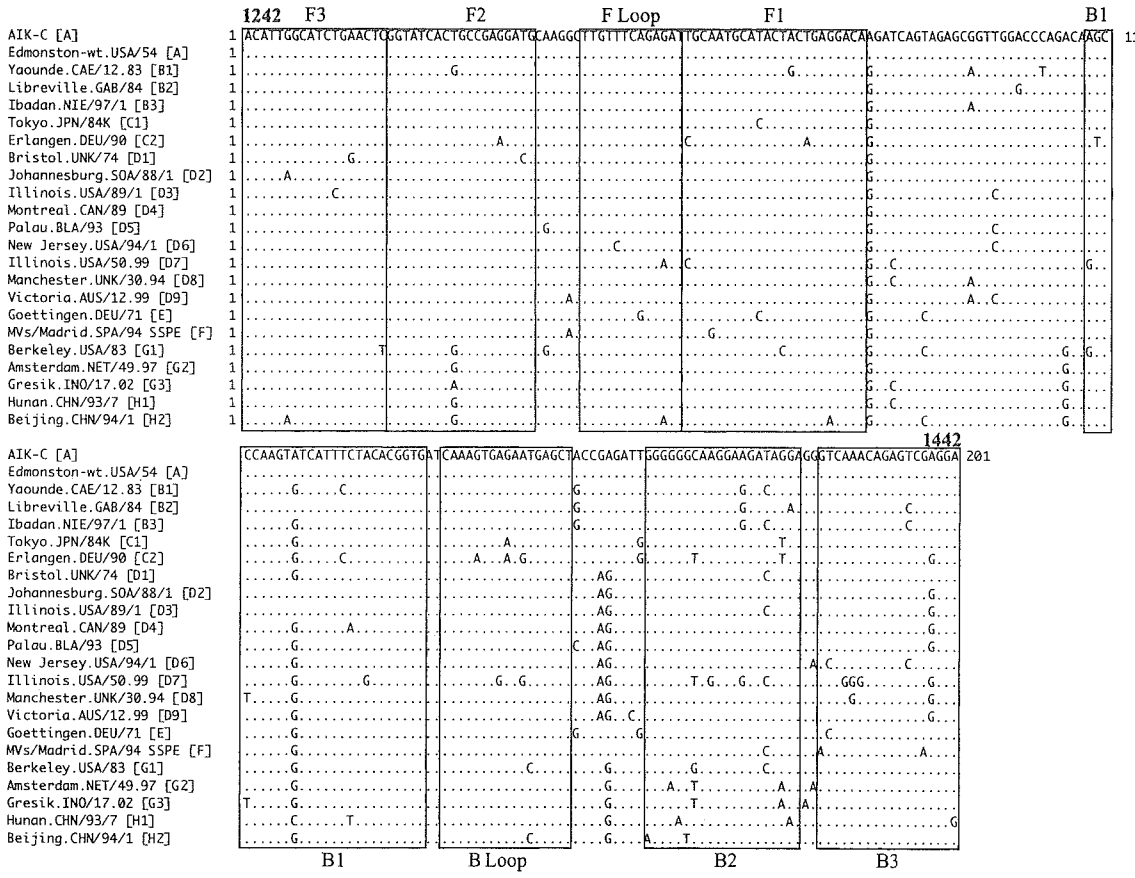


Fig. 5. Sequence alignment from F3 to B3 region (from genome positions 1242 to 1442). Primers regions are shown in frames and all reference sequences were referred to GeneBank (ref of WHO, 2001).

[Nakayama et al., 1995; Afzal et al., 2003]. RT-PCR procedures established at various laboratories have differed in sensitivity by as much as 1,000-fold [Afzal et al., 2003]. Recently, several authors have reported real-time RT-PCR, which has the additional advantage of being a quantitative method, but this method was not appropriate for rapid bedside diagnosis [Ozoemena et al., 2004; Schalk et al., 2004]. Ozoemena et al. [2004] reported that the nested PCR method proved to be 10 to 100 times more sensitive than *Taq*Man PCR method, but they preferred *Taq*Man RT-PCR because of its advantages of contamination control, automation, and real time quantitative features. The sensitivity of the system depends on the selection of primers and probe alignments [Ozoemena et al., 2004].

LAMP was developed to amplify the target DNA without any temperature shifts for denature, annealing, and extension. LAMP has been applied for the detection of many kinds of infectious agents, mainly for the DNA virus of human herpesvirus (HHV) 6 and 7, varicella-zoster virus (VZV), or the bacterial genome [Iwamoto et al., 2003; Kuboki et al., 2003; Maruyama et al., 2003; Ihira et al., 2004; Okamoto et al., 2004; Yoshikawa et al., 2004]. Yoshikawa et al. [2004] reported that the VZV genome was amplified with a detection limit of 500 copies by LAMP. Recently, RT-LAMP method was reported for the detection of West Nile virus [Parida et al., 2004], SARS corona virus [Thai et al., 2004], and mumps virus [Okafuji et al., 2005]. SARS coronavirus was detected with high sensitivity of 0.01 pfu detection limit by RT-LAMP and 100-fold higher sensitivity of nested RT-PCR system. We developed RT-LAMP for the detection of the measles virus genome and compared the sensitivity of RT-LAMP with that of RT-PCR for the detection of measles virus. Measles RT-LAMP had 10-fold higher sensitivity than nested RT-PCR and the detection limit was approximately 0.01 TCID 50, 30–100 copies, similar to SARS RT-LAMP system. The measles virus genome was detected in 49 of 50 stored samples by RT-LAMP but in 44 by nested RT-PCR. It was also efficiently amplified from clinical samples of NPS, PMBC, and sera. Genotypes A, C1, D3, D5, and H1 were amplified without any differences in the detection limits. We could not examine all 22 genotypes. From the results of sequence alignments in Figure 5, several mutations were observed in eight primer regions and some were located at the 3′ end of each primer. It might influence the sensitivity of RT-LAMP, but we could deal with it by minor-modification of primers. RT-LAMP procedure is simple operation in a single tube and time-saving and we can obtain the results within 1 hr after the extraction of the virus genome. LAMP method for the detection of the genome of pathogenic agents is a useful tool in hospital-based rapid diagnosis. We calculated the quantity of genome in the sample by monitoring the spectrophotometric value and there was a linear correlation between the genome quantity and reaction time to reach the threshold. The quantitative RT-LAMP will contribute much to the better understanding about the different pathophysiology of virus infection [Okafuji et al., 2005].

The LAMP system has clinical benefits of high sensitivity, specificity, rapidness, and simplicity, which are required for its usage as a rapid diagnostic tool. This system will no doubt come into wide use as a genetic diagnostic tool.

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# Proposal for genetic characterisation of wild-type mumps strains: Preliminary standardisation of the nomenclature

Brief Report

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**Summary.** Though mumps virus (MuV) is a monotypic virus, genetic variation between strains has been described. Viruses have been placed into genotypes designated A–L based on the nucleotide sequence of the small hydrophobic (SH) gene, which is the most variable gene in the mumps genome. Molecular characterisation of MuV is an important component of mumps surveillance because it can help identify the transmission pathways of the virus as well as distinguish between wild-type and vaccine strains. Here, we propose a standardized nomenclature and an analysis protocol for the genetic characterisation of mumps strains to facilitate expansion of molecular epidemiological studies. In addition to assigning standard

reference strains for the recognized genotypes of MuV, a convention is proposed for naming for strains and criteria to designate a new genotype.

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Mumps virus (MuV) is a member of the genus *Rubulavirus* of the family *Paramyxoviridae*. Its genome is a single-stranded, negative sense, non-segmented RNA of 15,384 nucleotides. The genome contains seven transcription units that encode open reading frames (ORFs) for the nucleocapsid (N), the V protein (and after editing the phospho- (P) protein), the matrix (M), the large (L), the fusion (F), the haemagglutinin-neuraminidase (HN) and the small hydrophobic (SH) proteins [3].

Mumps is a common and highly transmissible viral disease with infection resulting in bilateral parotitis. Infections are not usually fatal, but the neurological complications of mumps can be severe. Aseptic meningitis occurs in about 10% of cases. In 0.02–0.03% of cases, encephalitis may occur and require hospitalization. In fact, mumps encephalitis accounted for 36% of viral encephalitis cases before the introduction of vaccine. In the pre vaccine era, annual incidence rates for mumps were 200–700/100,000 and rates of up to 6000/100,000 have been reported in military and community outbreaks. In countries that have successfully introduced the MMR vaccine or monovalent mumps vaccine, incidence rates for mumps are usually less than 1/100,000. MMR is currently used in 23/25 developed countries and 19/22 of countries with economies in transition. However, MMR is only used in 24% (40/168) of developing countries, and very little is known about the burden of mumps disease in countries without mumps vaccination programmes.

Laboratory diagnosis is important for confirmation of mumps infection, as there are other causes of parotitis. The standard methods of serum antibody detection, virus antigen detection in the cells obtained from throat swab and virus isolation from throat swab or urine are still commonly used though in specialised laboratories techniques are now available for the diagnosis of mumps infection by detection of IgM antibody or mumps RNA in oral fluid [8]. Mumps specific PCR products are subsequently sequenced for strain characterisation [2, 7, 8].

MuV is serologically monotypic. Nevertheless, distinct clusters of wild-type viruses have been described and reported to be co-circulating globally. Strain diversity was identified based on a comparison of the nucleotide sequences of the SH gene [23]. The SH gene shows the greatest amount of nucleotide sequence variation on the genome of MuV although the function of the SH protein remains unclear. Phylogenetic comparison of SH gene sequences has been used to classify MuV strains into a number of genotypes. Genotypes A–L (with names proposed in this report) have been described by investigators over recent years [1, 2, 6–13, 15–23].

Investigation of the chronological and geographical distribution of mumps genotypes has shown that MuV genotyping has a valuable role in mumps surveillance [2, 6, 8, 10, 11, 16, 18, 19]. Genetic analysis is the only means available to distinguish vaccine from wild-type viruses. Sequence information is also important for assessing the safety of mumps vaccines since it has been shown that some of the mumps vaccines are more reactogenic than others [4, 5]. The molecular data can also be used to track transmission chains of the virus [8].

Therefore, countries that have introduced mumps vaccination should consider initiating molecular characterization of wild-type mumps viruses as an important component of mumps surveillance.

At present, there is no internationally agreed nomenclature and standard analysis for describing the genetic characteristics of wild-type MuV. Strains have been assigned to various genotypes on the basis of differing sequence windows containing from 270 to 318 nt of the SH gene. Genotypes have been assigned by the investigating laboratory and this lack of standardisation has for example, led two groups, one in Japan [20] and one in Sweden [18] to propose two different strains of MuV as the genotype J at the same time. Scientists from ten laboratories around the world, who have contributed to the genetic characterisation of MuV, propose here a standardization of international nomenclature based on previous reports and experiences of the authors. A system for genetic characterisation of mumps strains that will facilitate future molecular epidemiological studies and avoid confusion in the future designation of new genotypes has also been proposed.

SH genes of several hundred MuV strains have been sequenced. The entire SH gene, 316 nt sequence containing the protein encoding region of 171 nt (57 amino acids) is proposed as the minimum amount of sequence information required to assign a genotype to an unknown strain. Based on a comparison of the published MuV SH sequences available in GenBank and previous proposals, genotypes A–L have been designated. Nucleotide sequence divergence was up to 19% between 30 selected MuV strains, representing genotypes A–L (Fig. 1). The phylogenetic tree (Fig. 1) presents all currently assigned MuV genotypes. The tree was drawn based on 316 nt of the entire SH gene including the non-coding region using the Clustal and MegAlign program of the DNASTAR package (Fig. 1a). Software such as bootstrap analysis (1,000 times) using the Neighbour-joining method (PAUP 4.0 Beta10 package) should be applied in assigning strains to new genotypes (Fig. 1b). Other phylogenetic programmes can also be used for data analysis and genotyping when the reference strains that we propose are employed.

The first isolate from each genotype is proposed to be the reference strain for that genotype. Furthermore, in the phylogenetic trees one recent isolate has also been included when available. The known geographic distributions of these genotypes are listed in Table 1. Some of the 12 chosen reference strains (highlighted in Table 1) may have never been successfully isolated and have been sequenced directly only from clinical specimens. These strains will be replaced in the reference tree by related cell culture isolates if and when they become available. As the Vero cell line is sensitive to mumps virus and available in most laboratories, we suggest that newly proposed reference strains should be isolated in (or adapted to) Vero cells and stored as early passage material.

The strain names should provide basic information as well as be easy to use. The three letters of UN ISO3 country code (http://unstats.un.org/unsd/methods/m49/m49alpha.htm), year of detection, ID number of the strain and genotype identified are essential, and MuVs and MuVi indicated at the beginning will represent the strains identified directly in clinical samples or in cell culture isolates respectively, e.g. MuVs-GBR05-320-D or MuVi-CHN06-608-H. Previously published strains can retain their original names as a prefix to the full description.

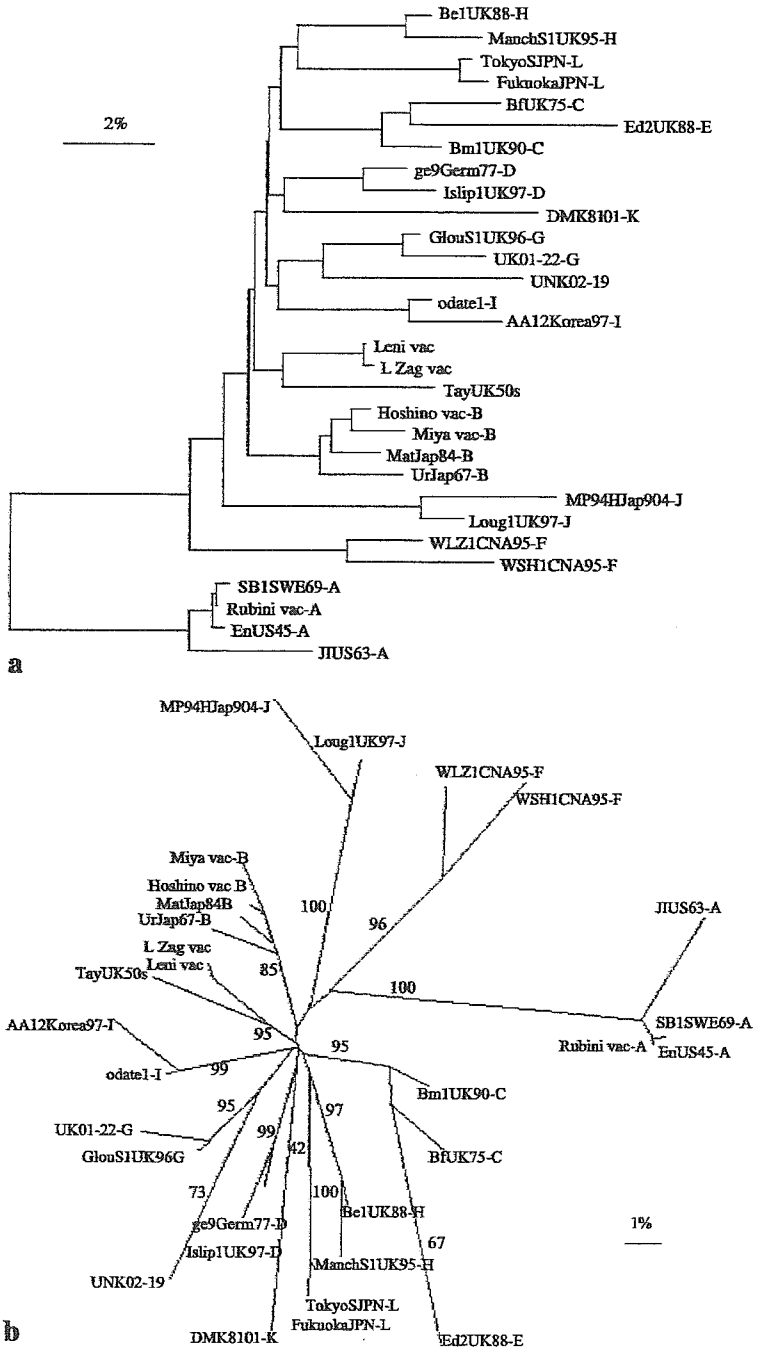


**Fig. 1.** Phylogenetic tree presenting all current assigned reference strains of mumps virus genotypes including one recent strain when it is available. Genotypes were indicated at the end of each strain. The tree was drawn based on 316 nt of the entire SH gene including the non-coding region (**a**) using the Clustal and Megalign program of the DNASTAR package and (**b**) using the bootstrap analysis (1,000 times) of the Neighbour-Joining method (PAUP 4.0 Beta10 package)

**Table 1.** The proposed reference strains and global distribution of mumps genotypes

| Genotype | Reference strain | GB accession no | Country (IS03) and year identified |
|---|---|---|---|
| A | **End/USA45*** | D90231 | USA45, 50, 63; SWE69, 93; CHE74; |
| | SBL-1/SWE69* | D00663 | DEU87, 92; CAN88 |
| | JL/US63 (vaccine) | D90232 | |
| | Rubini (vaccine) | X72944 | |
| B | **Urb/Jap67** | D90236 | JPN67-95; GBR89, 90 |
| | Mat/Jap84 | D90233 | |
| | Miya (vaccine)* | D90234 | |
| | Hoshino (vaccine) | AB003414 | |
| C | **Bf/UK75*** | X63709 | GBR75, 80s, 90, 98-2000; SWE80s, 92; |
| | Bm1/UK90 | DS26771.DAT | DEU87, 92, 93; CHE95; PRT96; LTU98-00 |
| D | **Ge9/Gem77** | DS26771.DAT | DEU77; PRT96; GBR96, 97, 99; |
| | Islip1/UK97 | AF142766 | LTU99; DEN80s, 90s, 01; JPN93 |
| E (C) | **Ed2/UK88** | X63711 | GBR88 |
| F | **WLZ1/CNA95** | Z77158 | CHN95; GBR 99; SWE71, 72, 84; |
| | WSH1/CNA96 | Z77160 | |
| G | **Glouc1/UK96*** | AF142764 | GBR91, 96-05; JPN99-05 |
| | UK01-22 | AY380075 | |
| H | **Be1/UK88** | DS26771DAT | GBR88, 95, 96, 98-01; |
| | ManchS1/UK95* | AF142771 | CHE 95, 98-00; KOR99; JPN97 |
| I | **Odate-1** | D86174 | JPN93; KOR97-01 |
| | AA12/Korea97* | AF180374 | |
| J | **MP94H/JNP94** | AB03417 | JPN94, GBR97 |
| | Loug1/UK97 | AF142770 | |
| K | **DK81/01 (DMK81)** | AF365891 | DNK81-88 |
| L | **Fukuoka49/JPN00*** | AB105483 | JPN00-01 |
| | Tokyo S-III-10/JPN01* | AB105480 | |
| ** | Leningrad 3 (vaccine) | AY493374 | RUS53 |
| | L-Zagreb (vaccine) | AJ272363 | |
| ** | Tay/UK50s* | AF142774 | GBR50s |
| ** | UNK02-19* | AY380077 | GBR02 |

*Isolate available; **Reference strains for potential new genotypes

The proposal presented here was established and finalised through international cooperation of the 13 authors. In future, a new genotype should only be assigned when the following criteria are met:

(1) The sequence of at least the entire 316 nt of the SH gene is unique when compared to all existing reference strains;
(2) The divergence (variation rate) based on the 316 nt of the entire SH gene is >5% when compared to all of the established reference strains;
(3) At least two identical or very similar strains are identified representing the proposed new genotype. New genotypes should be designated if a case can be made for sustained transmission/detection of viruses with significantly divergent SH gene sequences;
(4) Two other mumps lab/groups must have been contacted for confirmation to avoid duplication of genotype;
(5) Submit the sequence data to GenBank and indicate the proper strain name and data source (derived from cell culture isolate or directly from clinical sample).

In summary, we proposed the establishment of a standardisation of the nomenclature of MuV strains and the criteria for the assignment of strains to specific genotypes to facilitate molecular epidemiological investigations using these criteria. Genotypes A–L are proposed based on sequence diversity of the SH gene. Sequence analysis of other genes such as the HN or the C terminus of the N protein may also be useful in monitoring MuV evolution and tracking its transmission. Ongoing communication and collaboration between virologists working on MuV are necessary to enhance the role of molecular techniques in surveillance and control of mumps infection. A standardized nomenclature will make it easier for virologist to communicate with public health officials who are involved in mumps control activities.

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