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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

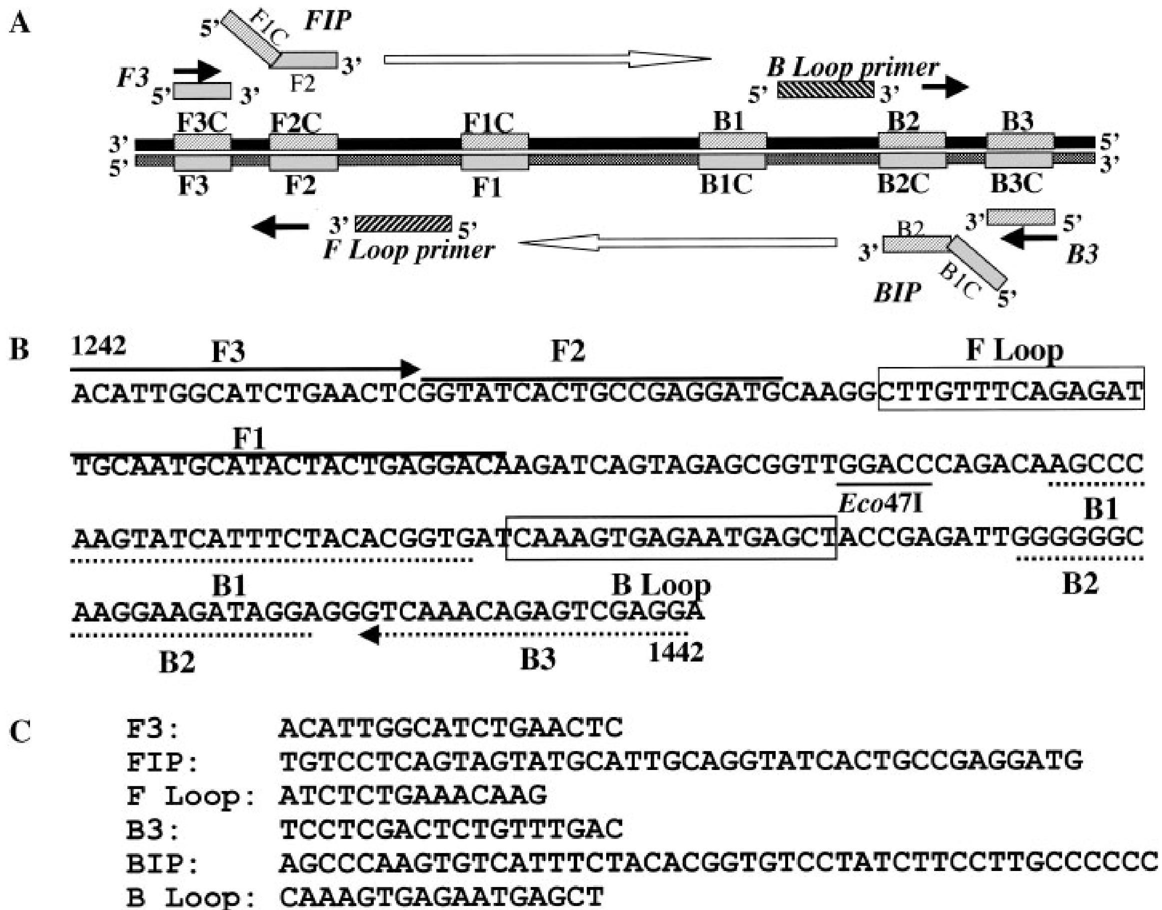


Fig. 1. Primer design for loop-mediated isothermal amplification (LAMP) for the detection of measles genome. Diagram of LAMP primer design (**A**), sequence data from the genome position 1242 to 1442 (**B**) and sequence alignments of the primers (**C**). Eco47I site is underlined to confirm the measles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

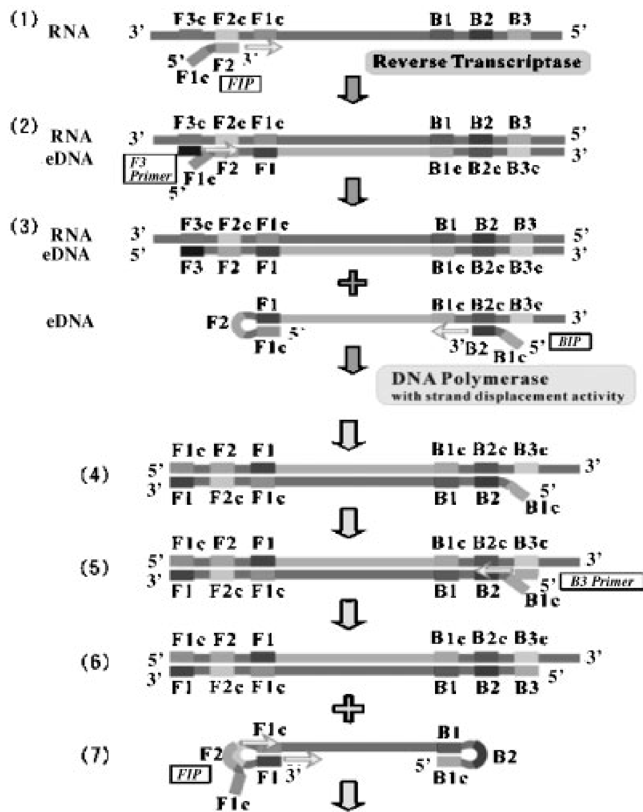


Fig. 2. Diagram of RT-LAMP.

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

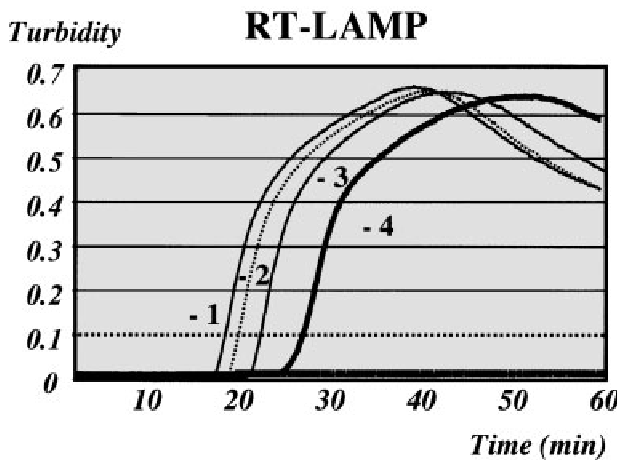
We have already reported the construction of N protein expression plasmid for reverse genetics [Kumada et al., 2004]. Coding region of the N gene was cloned in pBleuscript SK II-vector at the downstream of T7 promoter. Plasmid was linearized by *Spe* I digestion and RNA was transcribed by T7 RiboMAX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

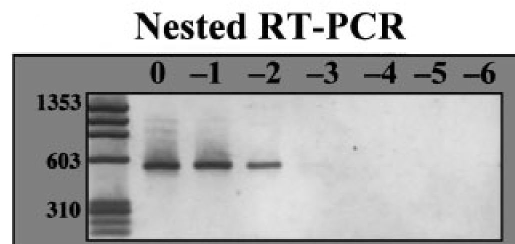
RESULTS

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 2×10^3 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/





MVi/Tokyo. JPN/87-K [D3] 2×10^3 TCID50/200ul

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 2×10^3 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 6×10^3 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 8×10^4 TCID50/200 μl, and 3×10^4 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

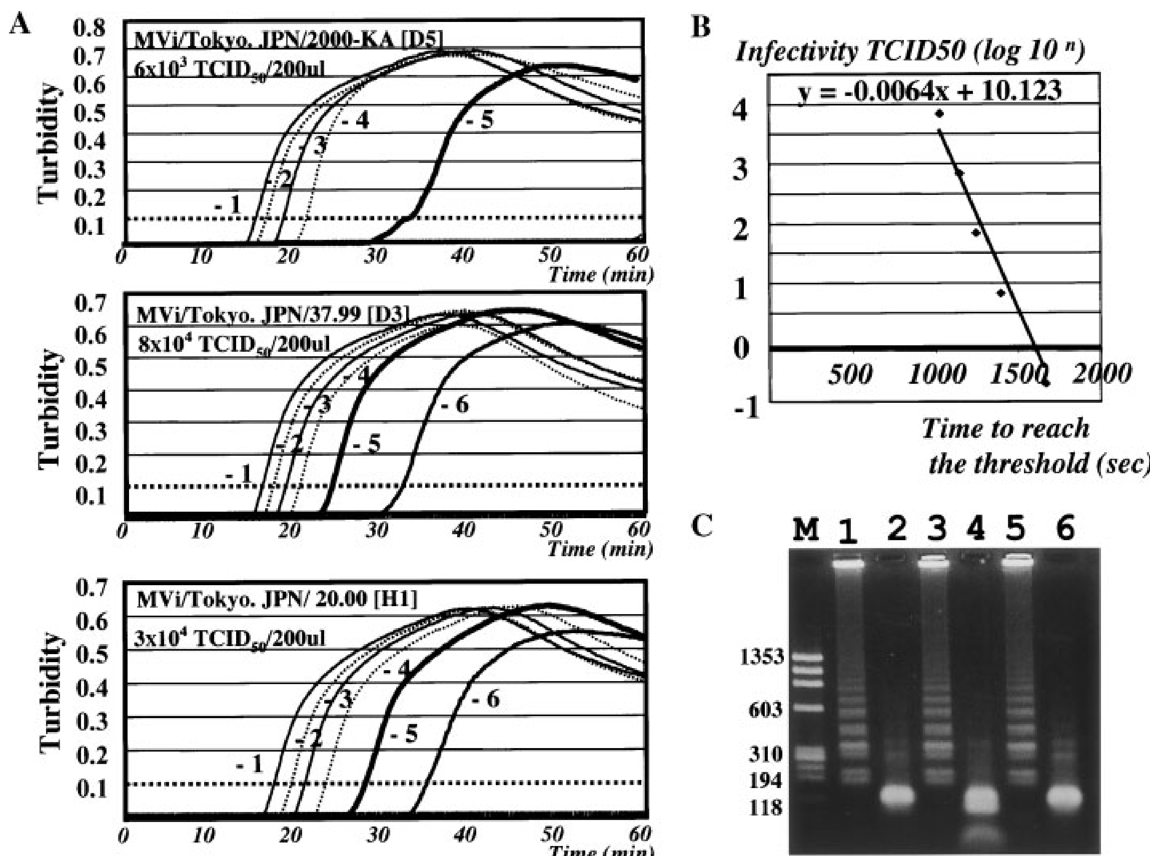


Fig. 4. Sensitivity of RT-LAMP in different genotypes. MVi/Tokyo.JPN/2000-KA (genotype D5), MVi/Tokyo.JPN/37.99 (genotype D3), and MVi/Tokyo.JPN/20.00 (genotype H1) were used. RNA was extracted from 200 μl of virus culture medium and re-suspended in 25 μl. Five micro liters was subjected to RT-LAMP (**A**). 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

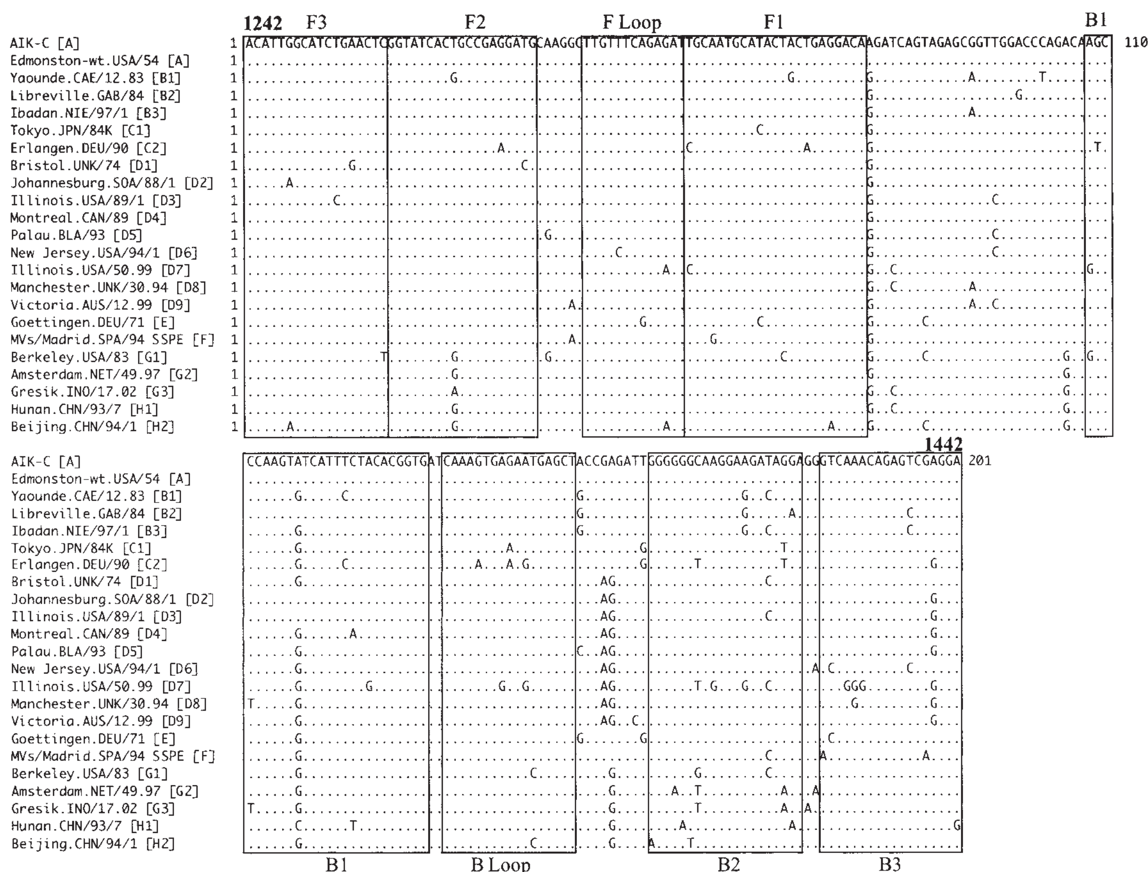


Fig. 5. Sequence alignment from F3 to B3 region (from genome positions 1242 to 1442). Primers regions are shown in frames and all reference sequences were referred to GeneBank (ref of WHO, 2001).

[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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