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Rhombencephalitis and Coxsackievirus A16

To the Editor: Hand, foot, and mouth disease (HFMD) is a common illness in children and is mainly caused by coxsackievirus A16 (CA16) and enterovirus 71 (EV71). Although its clinical course is usually uneventful and most patients experience a full recovery, serious neurologic complications, including encephalitis, can occur secondarily to HFMD caused by EV71. Such neurological complications occurred during an epidemic in Taiwan in 1998 (*1*). Encephalitis caused by EV71 is characterized by rhombencephalitis, which is a combination of brainstem encephalitis and cerebellitis. Signs and symptoms of rhombencephalitis are irritability, myoclonus, ataxia, and cranial nerve involvement (*1*). In contrast to EV71, HFMD caused by CA16 is associated with few neurologic complications with the exception of infrequent aseptic meningitis (*2*). We report a case of rhombencephalitis that developed in an infant as a complication of HFMD caused by CA16.

HFMD was diagnosed in a 23-month-old girl on the basis of high fever (>40°C, 3 d duration), stomatitis, and multiple papules on her palms, soles, and buttocks. Her illness occurred in the summer of 2007, when sentinel surveillance in the region indicated an epidemic of HFMD caused by both CA16 and EV71. She was admitted to our hospital in Fukoka, Japan, on day 4 of illness because of abnormal eye movement, irritability, and inability to stand. She had intermittent to-and-fro, horizontal oscillations of the eyes (ocular flutter). She also had truncal and limb ataxia and myoclonus in her head and limbs. Brain magnetic resonance imaging (MRI) showed T1-low and T2-high bulbopontine and cerebellar lesions around the fourth ventricle (Figure). Peripheral blood showed a mild leukocytosis (13.13 × 10^9/L) and a C-reactive protein level within reference range (0.9 mg/L). Blood chemistry results were unremarkable. Cerebrospinal fluid (CSF) examination showed mononuclear pleocytosis (74/μL) with normal protein and glucose levels. CA16 was isolated from her stool specimen on day 4 of illness. Based on reverse transcription–PCR, CSF was negative for enterovirus RNA.

Without specific treatment, our patient's fever resolved on day 5 of illness. The myoclonus, ocular flutter, and irritability subsided by day 16, when MRI findings returned to normal. Ataxia disappeared gradually ≈1 month after onset, and no neurologic sequelae occurred. Neutralizing antibody titers against CA16 and EV71 on day 21 of illness were 32 and <8, respectively. Based on the sequence analysis of the partial VP1 region (876 bp), we classified the patient's CA16 strain phylogenetically as genetic lineage C (*3*). This lineage was identical to lineage 2 (*4*), which became the dominant circulating strain in Asia, including Japan, after the late 1990s (98.2% identical to the 1018T/VNM/05 strain isolated in Vietnam

in 2005 [GenBank accession no. AM292441]) (*4,5*).

The patient's symptoms of irritability, ataxia, myoclonus, and ocular flutter 3 days after the onset of typical HFMD manifestations, along with CSF mononuclear pleocytosis and the lesions around the fourth ventricle shown on MRI, led to the diagnosis of rhombencephalitis associated with HFMD. Virologic examination, including virus isolation and antibody assay, suggested that HFMD was caused by CA16 but not by EV71, although the possibility that CA16 infection was coincidental to the rhombencephalitis could not be excluded.

Although rhombencephalitis can be related to various infectious agents (*6*), HFMD complicated by this condition has been exclusively caused by EV71 (*1,7,8*). In Japan, CA16 and EV71 are consistently the 2 major causative agents of HFMD (*9*). EV71 infection is much more frequently associated with serious neurologic complications and fatalities than is CA16 (*2*). Since 1997, several HFMD outbreaks with multiple cases of severe neurologic pathologies have occurred in the Asia-Pacific region including Malaysia, Taiwan, and Western Australia (*1,7,8*). These complications were associated exclusively with EV71.

Why rhombencephalitis developed in our patient with CA16-related HFMD is unclear. One possibility is that the CA16 strain might have acquired neurovirulence by genetic recombination with EV71; phylogenetic evidence supports the possible occurrence of intertypic recombination involving EV71 and CA16 (*10*). Through phylogenetic analysis of the VP1 sequences, we classified the CA16 strain isolated from the patient's stool phylogenetically as genetic lineage C (*3*), a lineage which was identical to lineage 2 (*4*). Genetic recombinations among enteroviruses occur mainly in noncapsid regions (*10*). We did not conduct phylogenetic analysis of the noncapsid regions of the patient's CA16 strain because sequence data on the regions were very limited. Besides the viral factors, host factors, such as immune status and environmental factors, could confer susceptibility to neurologic complications of enteroviral infections.

Rhomobencephalitis associated with HFMD developed in this patient and was caused by CA16. Therefore, neurologic complications, including rhombencephalitis, should be considered even when CA16 is the prevalent virus causing HFMD.

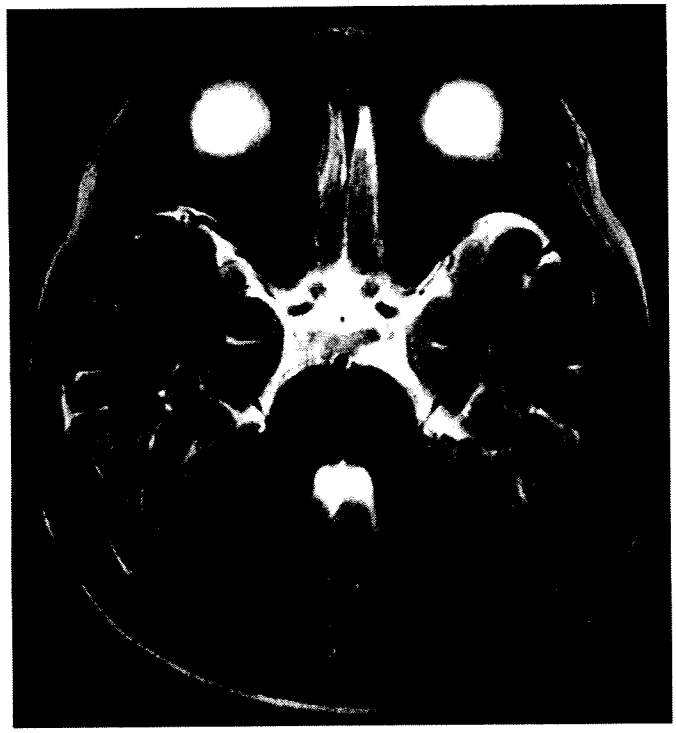

Figure. Axial T2-weighted slice of brain by magnetic resonance imaging, showing hyperintensity lesions in the pons and cerebellum around the fourth ventricle.

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Japanese Encephalitis in Hill and Mountain Districts, Nepal

To the Editor: Nepal, a landlocked country in Southeast Asia with an estimated population of 27 million, is divided administratively into 5 regions; 75 districts comprise 3 ecological zones that run from east to west. Altitude increases from south to north: the 20-district Terai plains in the south, the hill region in the center with 39 districts, and the 16-district mountain regions in the north. Japanese encephalitis (JE) is seasonally endemic to the Terai region, which borders the northern India states of Uttar Pradesh and Bihar. The first outbreak of JE in Nepal was reported in 1978 from the Terai district of Rupendehi (*1*). Since then, JE infection has been reported in animal reservoirs and in humans throughout the Terai region (*1–5*). Although few publications describe the presence of JE outside the Terai, an outbreak of JE in Kathmandu valley in the hill region was confirmed in 1997 (*6*), and a 2006 study reported JE endemicity in Kathmandu Valley (*7*). In recent years, the Ministry of Health and Population in Nepal has introduced public health interventions, including mass immunization campaigns, for JE prevention in these known JE-endemic areas.

JE cases are captured through acute encephalitis syndrome (AES) surveillance conducted by the government of Nepal, with support from the World Health Organization (WHO), through a national sentinel surveillance network. From 2004 through 2006, a total of 108 laboratory-confirmed JE cases were reported from hill or mountain districts (excluding Kathmandu Valley). However, travel histories for case-patients were not available for these years to determine the origin of JE infection. We conducted a study to provide evidence of JE endemicity in hill and mountain districts of Nepal (excluding Kathmandu Valley).

Laboratory-confirmed JE case-patients identified in 2007 who reported residence in 1 of the 52 hill or mountain districts, excluding the 3 hill districts of the Kathmandu Valley, were followed up by surveillance medical officers. All patients (or next of kin if the patient was deceased or unavailable) were visited at home or contacted by telephone to confirm their residence and travel history during the 30 days before the onset of symptoms. Data and sample collection procedures and laboratory methods used for JE diagnosis were as previously reported by Partridge et al. (*7*). Patients were identified by the AES surveillance system if patients' symptoms met the case definition for AES adopted from WHO guidelines (www.who.int/vaccines-documents/DocsPDF06/843.pdf), i.e., acute onset of fever and a change in mental status (e.g., confusion, disorientation, coma, or inability to talk); or if the patient experienced a new onset of seizures (excluding simple febrile seizures) or was identified as having AES, JE, or viral encephalitis. The study population included any person of any age who reported being a resident of 1 of the 52 hill or mountain districts (excluding Kathmandu Valley), who had been seen at any AES reporting site from January 1 through December 31, 2007, and who had been confirmed to have JE antibody by immunoglobulin M capture ELISA on a serum or cerebrospinal fluid (CSF) specimen.

In 2007, a total of 360 AES cases were reported from 40 hill or mountain districts. Of the 344 reported AES cases for which diagnostic samples were obtained, 90 (26%) were laboratory confirmed as JE from 21 hill and 3 mountain districts. Among laboratory confirmed JE cases, CSF samples were collected from 13 (14%) patients and serum samples from 77 (86%) patients (Table). The largest number of AES and laboratory-confirmed JE cas-

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The strategy for prevention of measles and rubella prevalence with measles–rubella (MR) vaccine in Japan

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ABSTRACT

To eliminate the indigenous measles and rubella virus by 2012 in Japan, the strategy for prevention of measles and rubella prevalence with measles-rubella (MR) vaccine was proposed. Since the vast majority of 1-year old infants are susceptible to measles and rubella, the first MR vaccine should be administered at 1-year old to sustain the herd immunity. Since significant elevation of measles and rubella antibody titers was estimated in a half of children after the second dose, the second dose of MR vaccine within 1 year before elementary school entry is the effective maneuver. Moreover, supplement MR vaccination to the teenage group and 20–29 years' group might be necessary, because the mean measles antibody titers in this group were significantly lower compared with those in the older individuals' groups.

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1. Introduction

Measles is a highly contagious viral illness. The basic reproduction number (R_0) is 12–18 and the herd immunity (H_0) is 83–94% [1]. Measles had been prevalent every 2 years before 1978, when routine measles vaccine was implemented for children in Japan. However, measles prevalence has been noticed every 5 years, because measles vaccination coverage rate is below the herd immunity.

Rubella is also a contagious disease by rubella virus. The basic reproduction number is 6–7 and the herd immunity is 83–85% [1]. Rubella was prevalent every 5 years before starting routine vaccination in Japan. Though vaccine had been implemented for 15-year-old female since 1977, rubella prevalence was not controlled. In 1994 rubella vaccine was implemented for all 1-year-old children. However, rubella prevalence was noticed in 2004, since rubella vaccination coverage rate was also below the herd immunity.

In 2005, measles–rubella (MR) vaccine was licensed in Japan and two dose schedule of MR vaccine has been mandatory since 2006. The first dose is administered at 1-year-old and the second dose is within 1 year before elementary school entry. I reviewed the strategy for the prevention of measles and rubella prevalence with MR vaccine in Japan.

2. Protective antibody titers to symptomatic measles and rubella reinfection

Humoral immunity, cellular immunity and mucosal immunity are participated in the prevention of measles and rubella reinfection. Protective antibody titers to symptomatic measles reinfection are ranging from 31.25 mIU/ml to 500 mIU/ml [2–4]. Samb et al. reported 98% individuals were protective whose antibody titers were greater than 125 mIU/ml, while Orenstein et al. proposed 200 mIU/ml was the protective level [3,5]. Subclinical reinfection is noticed in the vaccinated individuals during measles prevalence. The protective level to subclinical reinfection is also ranging from 500 mIU/ml to 1000 mIU/ml [4]. In rubella 10 IU/ml was proposed as the protective antibody level to symptomatic reinfection [6]. However, the protective antibody level to subclinical reinfection has not been estimated.

3. Decay of maternal antibodies and vaccine-induced antibodies

IgG antibodies are transferred from mother to fetus by an active transport mechanism. Cord blood measles antibody titers at term in the developed countries are 1.38–1.85-fold higher values compared with those in maternal blood, while those in developing countries are 0.92–1.3-fold higher values [7]. Transferred antibodies decayed during infancy. The half-life ranged from 40 to 64 days [7,8]. In Japan, cord blood mean neutralizing (NT) antibody titers to measles virus were $2^{5.86\pm1.81}$-fold (NT antibody titers of $2^{5.86}$-fold were approximately 2900 mIU/ml) and mean hemagglutination inhibition (HI) antibody titers to rubella virus were $2^{7.15\pm1.62}$-fold (HI antibody titers of $2^{7.15}$-fold was approximately 177 IU/ml) [9]. Since greater than 95% of infants are susceptible to measles and rubella at 1-year-old, the first MR vaccine should be administered at the first birthday to sustain the herd immunity.

The vaccine-induced antibodies are also decayed in the absence of natural booster infections. The half-life after the first dose of

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Table 1
Changes of measles mNT antibody after MR vaccination.

NT antibody titers (fold) before vaccination	No change	2-fold elevation	≥4-fold elevation	Total
<2			5	5
2			6	6
4			7	7
8		2	30	32
16		14	33	47
32	11	40	18	69
64	18	16	4	38
128	5	4	1	10
256	1	1		2
Total	35	77	104	216

mNT: microneutralizing method.

Table 2
Changes of rubella HI antibody after MR vaccination.

HI antibody titers (fold) before vaccination	No change	2-fold elevation	≥4-fold elevation	Total
<8			8	8
8			10	10
16		1	12	13
32	1	11	29	41
64	11	30	16	57
128	34	19	1	54
256	19	6		25
512	9			9
Total	74	67	76	217

HI: hemagglutination inhibition method.

measles vaccine ranged from 27 to 42 months and that after the second dose was 61 months [7,10,11]. Meanwhile, Amanna et al. reported the half-life of measles and rubella antibody was 369 years and 85 years, respectively [12]. Since mild measles is observed in some previously vaccinated individuals during prevalence, two doses of measles virus containing vaccine are necessary for measles elimination [13,14].

4. Changes of measles and rubella antibody titers after second MR vaccination within 1 year before elementary school entry

Two-hundred and seventeen children, who had been administered measles vaccine and rubella vaccine separately, were injected MR vaccine (Mearubik®) within 1 year before school entry. In measles, significant elevation of NT antibody titers was estimated in 104 (48.1%) of 216 children and 2-fold elevation was in 77 (35.6%) (Table 1). Significant elevation of antibody titers was observed in 81 (83.5%) of 87 children whose NT antibody titers at the pre-vaccination were less than 16-fold, while significant elevation was noticed in 23 of 119 (19.3%) whose NT antibody titers were greater than 32-fold ($P < 0.0001$, chi-square test).

In rubella, significant elevation of HI antibody titers was detected in 76 (35.0%) of 217 children and 2-fold elevation was 67 (30.9%) (Table 2). Significant elevation of antibody titers was observed in 59 (81.9%) of 72 children whose HI antibody titers at the pre-vaccination were less than 32-fold, while significant elevation was noticed in 17 of 145 (11.7%) whose HI antibody titers were greater than 64-fold ($P < 0.0001$, chi-square test). These results suggested a preexisting high antibody prevented a booster antibody rise and the protective antibody level to subclinical reinfection in the majority might be over 16-fold NT antibody (>800 mIU/ml) in measles and over 32-fold HI antibody (>40 IU/ml) in rubella.

Table 3
The mean measles and rubella antibody titers tested in 2007.

	Method	20–29 years (16)[a]	30–39 years (5)	40–49 years (8)	*P*-Value
Measles	mNT	3.56 ± 1.30[b]	6.80 ± 1.30	7.25 ± 0.46	0.0001
Rubella	HI	5.75 ± 1.18[b]	6.00 ± 1.14	6.63 ± 1.77	0.4741

mNT: microneutralizing method, HI: hemagglutination inhibition method.
[a] Number of tested sera.
[b] $\log_2$ N.

5. Measles and rubella antibody titers in the adult

The mean measles antibody titers in 20–29 years' group were significantly lower compared with those in 30–39 years' group and 40–49 years' group ($P = 0.0001$, ANOVA), while the mean rubella antibody titers were not different in three groups (Table 3). Routine measles immunization has been implemented for 30 years in Japan. In general, the measles antibody levels in the immunized individuals are lower compared with those in the naturally infected individuals [15,16]. These results suggest that supplement measles vaccination to the teenage group and 20–29 years' group might be necessary to prevent measles prevalence in Japan.

6. Conclusion

The transferred maternal measles and rubella antibody are decayed during infants, and the vast majority of 1-year-old infants are susceptible to measles and rubella in Japan. Thus, the first MR vaccine should be administered at 1-year-old to sustain the herd immunity. Since significant elevation of measles and rubella antibody titers was estimated in half of children after the second dose, the second dose of MR vaccine within 1 year before elementary school entry is the effective maneuver. Moreover, supplement MR vaccination to the teenage group and 20–29 years' group might be necessary to eliminate the indigenous measles and rubella virus by 2012.

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Laboratory diagnosis of measles and rubella infection

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ABSTRACT

Current diagnostic laboratory tests were summarized. Measles outbreak was noted in Japan 2007 and large proportion of the patients was high school, college students, and adult patients, caused by D5 Bangkok type. Through molecular epidemiological study, drastic changes in circulating genotypes were demonstrated since 1984 to present, in Japan.

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The WHO has set a goal to reduce the number of measles deaths by 90% by 2010 compared with the 2000 level. To realize the goal of measles elimination, the WHO organized the global measles and rubella (MR) laboratory network (LabNet) to promote laboratory activities to diagnose measles and rubella infection by virus isolation and/or IgM testing for surveillance. Virological surveillance provides informative data on the transmission pathway, for identifying the cases, and documenting the elimination. In many countries, actual lab-based surveillance is systematically organized but there is no such surveillance system in Japan.

Virus infection is diagnosed by several serological examinations using paired sera obtained in the acute and convalescent phases. The hemagglutination inhibition (HI) test is employed for the detection of antibodies against rubella virus, but became obsolete for antibodies against measles virus, because of their lower sensitivity and difficulty in the acquisition of fresh green monkey red blood cells (RBC). The neutralization test (NT) identified the protective antibodies and should be used for the detection of protective immunity against measles. But the NT is time-consuming, complicated, and requires experience. IgG enzyme linked immunosorbent assay (EIA) is sensitive, easy to perform, and used for sero-epidemiological study. IgM EIA is employed for diagnosis using single serum samples. IgG EIA antibody shows a good relationship with the NT but the protective level of IgG EIA is not known. In a 2007 outbreak of adult measles, a low-level positive antibody titer of IgG EIA was insufficient to protect against infection.

Virus isolation is traditionally performed for the laboratory-based confirmation of measles and rubella virus infection, but the sensitivity of virus isolation is influenced by the timing, procedure of sample collection, and condition of sample transport. Measles virus is isolated, using B95a or SLAM/Vero cells with high sensitivity when the sample is obtained during the period of measles rash before pigmentation. As for rubella virus isolation, it takes 3–4 weeks to obtain the results after three blind passages in RK13 or Vero cells, confirmed by hemadsorption or immuno-fluorescent staining.

Recently, several molecular diagnostic laboratory approaches for the detection of virus genome have been developed, such as the reverse transcription-polymerase chain reaction (RT-PCR), hybridization, and real-time PCR. Loop-mediated isothermal amplification (LAMP) is characterized by auto-cycling DNA synthesis with *Bst* DNA polymerase having high strand-displacement activity. It requires specifically designed primers and results are obtained within 1 h under isothermal condition at 63–65 °C without any temperature shifts. Approximately 0.1 TCID50 of measles and rubella virus genome could be detected by RT-LAMP, showing a higher sensitivity than RT-nested PCR.

The number of reported measles patients increased in March 2007 and this measles outbreak expanded throughout Japanese districts, peaking in the middle of May. Most patients were adolescents in high school and university students, with a much lower proportion of young infants. A relatively large proportion of the adult measles cases showed a previous immunization history and, thus, typical measles symptoms were not observed, only demonstrating mild fever and eruption. Virus isolation and detection of measles genome by RT-LAMP and RT-PCR were investigated from clinical samples in very mild cases with fever and rash. Virus isolation was unsuccessful but measles virus genome was detected by RT-LAMP even in these very mild cases.

Virological examinations support the characterization of circulating measles and rubella virus strains to monitor the transmission and antigenicity. The WHO recognizes 23 genotypes of measles virus and 7 genotypes with 3 provisional genotypes of rubella virus. In each nationwide outbreak, different genotypes were isolated in Japan: C1 (before 1984), D3 (1985–1990 and 1997–2000), D5 (1990 and afterward) and H1 (after 2000). Measles virus strains in 2007

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were of the D5 genotype belonging to Bangkok.THA/93, different from the D5 Palau.BLA/93 lineage identified during 1990–2005. The D5 Bangkok type was detected in Phnom Penh in 2002, and Taiwan 2003. D5 in Japan 2007 was suggested to have been imported from outside, rather than resulting from the accumulation of mutations from D5 of 1990–2005, and caused a nationwide outbreak. Moreover, it was exported to other countries via the vector of Japanese tourists visiting overseas. As for rubella surveillance, sporadic outbreaks were demonstrated and more than 10 cases of congenital rubella syndrome were reported in 2004–2005. Molecular surveillance for rubella virus was also conducted: 1a (1960s), 1E (1987 outbreak), and 1D (2004 sporadic outbreak). In Japan, dynamic genotypic shifts were therefore demonstrated.

A catch-up campaign was implemented using combined measles and rubella vaccine to immunize teenagers of 13 and 18 years of age for the next 5 years from April 2008. We should also implement the systemic surveillance based on the laboratory examinations.

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Laboratory and Epidemiology Communications

Importation of the Evolving Measles Virus Genotype D9 to Yamagata, Japan from Thailand in 2009

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The measles control project led by the World Health Organization (WHO) is ongoing with a current target of measles elimination in the WHO Western Pacific Region, including Japan, by 2012 (1). Laboratory-based surveillance plays an important role in the control of measles. Although IgM enzyme-linked immunosorbent assay (ELISA) is used as the standard method for confirming measles cases (2), virus isolation and genome detection by reverse-transcription PCR (RT-PCR) have also been carried out at most of the public health laboratories in Japan. WHO currently recognizes 23 genotypes of the measles virus (MV) (3). We reported an outbreak of measles caused by genotype D9 (D9) at a junior high school in Yamagata, Japan in 2004 (4).

We recently experienced an imported measles case caused by D9 in Yamagata Prefecture, Japan in 2009. The suspected case was an unvaccinated 7-month-old boy. He visited Bangkok, Thailand with his parents and returned to Japan at Narita International Airport on March 1st, and then moved to Yamagata City by airplane on March 2nd. He developed fever, cough, rhinorrhea, and rash on March 16th, 2009. He was clinically suspected of having measles. Throat swab and blood specimens were collected on March 19th for virus isolation, genome detection, and sequence analysis as described previously (4). There were no suspected cases of secondary infection.

MV (MVi/Yamagata/Jpn/12.09) was isolated using Vero/hSLAM and B95a cell lines from the blood specimen and the MV genome was detected in both throat swab and blood specimens. The blood specimen was positive for IgM antibody against MV by ELISA. The N gene sequences of the isolated MV and of the genomes detected in the specimens were identical. A BLAST search showed that this sequence (GenBank accession no. AB509376) was similar to D9 and identical to the three strains reported in Thailand (FJ356073, FJ356075, and FJ356077). The results along with the patient's history indicated that he was infected with MV D9 in Thailand. There was a 2.9% (13/456) divergence in the N gene and a 1.3% (24/1,854) divergence in the H gene (AB509377) between MVi/Yamagata.Jpn/12.09 and the D9 reference strain Victoria.AUS/12.99. The sequence divergence of greater than 2.5% in the N gene fulfilled the requirement for the classification as a new genotype; however, the divergence of 2.0% in the H gene did not (5). There was a 1.3% (6/456) divergence in the N gene between MVi/Yamagata.Jpn/12.09 and D9 isolates in Yamagata in 2004. These results and a phylogenetic tree based on registered D9 sequences as shown in Fig. 1 suggest that the D9 strains have been evolving.

Virological surveillance has revealed that, in Japan, the predominant genotype was C1 before 1985, D3 in the 1987-1988 outbreak, D5 in 1991-1993, H1 in 2002-2003, and D5

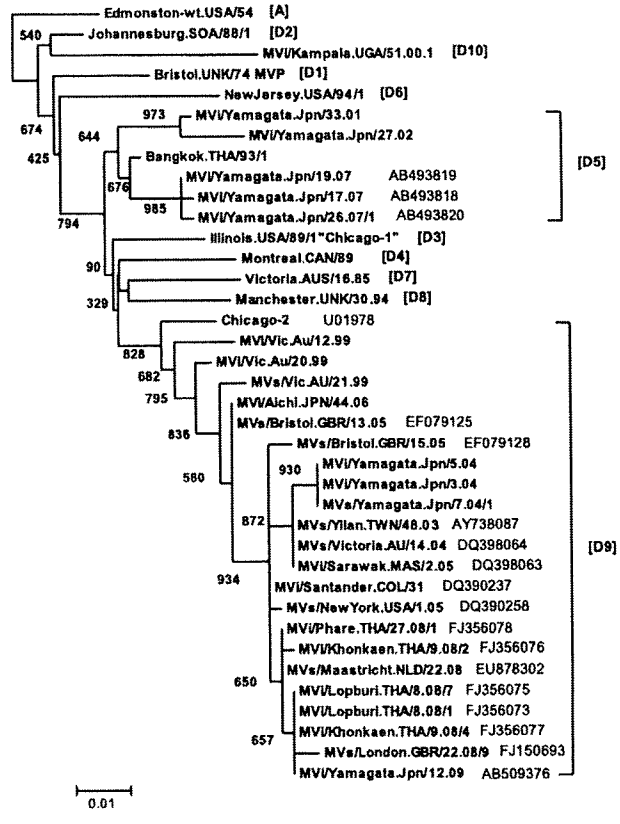

Fig. 1. Phylogenetic tree of genotype D measles viruses isolated in Yamagata, Japan in 2001-2009, genotype A and D reference strains (4-6,8), and genotype D9 strains registered in GenBank (accession numbers are shown after the strain name). The tree was based on the nucleotide sequences (456 bps) of the nucleoprotein and was generated by the CLUSTAL W program. The scale indicates 1% nucleotide differences. The numbers are the boostrap probabilities (%).

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again since 2006 (1,6). The genotypes of MVs detected in Yamagata have been consistent with the changes in the national surveillance data (4). Minor genotypes have also been reported in Japan along with the predominant MV genotype. In 2004, we reported an MV outbreak caused by D9, which had not previously been reported in Japan (4). In May of 2008, a D4 strain was isolated from a patient returning from Israel, and possible imported cases due to H1 were reported in February and March of that year in Osaka, Japan (7). These findings suggest frequent importation of MVs into Japan from abroad.

Analysis of chronological and geographical changes in circulating genotypes reveals that MV D9 strains have evolved and circulated internationally. Although an increase in vaccination coverage is of primary importance for control of measles epidemics, careful surveillance of MV circulation and transmission pathways using laboratory-based surveillance techniques such as genotyping is also of importance.

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Laboratory and Epidemiology Communications

An Outbreak of Measles Classified as Genotype H1 in 2008 in Osaka Prefecture

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Despite the availability of highly effective vaccines, measles has remained endemic in Japan (1,2). From February to March in 2008, seven patients from two families were clinically diagnosed with measles in the southern region of Osaka Prefecture. The two families had no apparent contact with each other. We examined clinical specimens from three of seven patients and detected three measles viruses, which were classified as genotype H1. This report summarizes the results of this investigation.

Case 1

Patient 1: The index patient was a 36-year-old man and a resident of Osaka Prefecture. He presented with a fever (39.3°C), nasal discharge, and conjunctivitis on February 15. On February 19, he had developed a rash and was clinically diagnosed with measles.

Patient 2: The index patient's child was a 9-month-old girl. She was vaccinated against measles on February 20, one day after her father was diagnosed with measles. However, on February 26, she developed a fever (38.8°C), pharyngitis, and a cough, which were followed by a rash on February 29. This patient's throat swab and blood sample were collected on March 7 and were subjected to viral detection using measles virus-specific reverse transcription (RT)-nested polymerase chain reaction (PCR).

Patient 3: The index patient's wife was a 34-year-old woman who had an unknown vaccination history against measles. She had a fever, cough, headache, and nausea on February 27, followed by a rash on March 3. The patient's serum was initially negative for measles-specific immunoglobulin M (IgM) and G antibodies on February 27 and 29, respectively; however, she subsequently seroconverted (IgM 5.8, enzyme immuno-assay; cut-off > 0.8) on March 7. A throat swab and blood sample were also collected on March 7 and were subjected to viral detection.

Patient 4: A 38-year-old woman, a sister of Patient 3, presented on March 8 with a fever that was followed by a rash, and she was subsequently also clinically diagnosed with measles. On March 17, this patient's serum was positive for measles IgM (6.2).

Case 2

Patient 5: The index patient was an unvaccinated 27-month-old boy with a fever (38.3°C), cough, pharyngitis, and nasal discharge on March 8, followed by development of a rash on March 12. A throat swab was collected for examination on March 13, by which time the rash and fever faded. On March 15, the patient's serum was positive for measles IgM (9.8).

Patient 6: The brother of Patient 5 was an unvaccinated 3-year-old boy who developed a fever and cough on March 15, followed by a rash on March 19. This patient was clinically diagnosed with measles.

Patient 7: The sister of Patient 5 was an 8-month-old girl who developed a fever and cough on March 16, followed by a rash on March 20. This patient was also clinically diagnosed with measles.

Throat swabs and blood specimens were collected from three patients (Patients 2, 3, and 5). To detect the measles virus, patients' samples were tested by measles virus-specific RT-nested PCR, and viral isolation was carried out by sample inoculation onto B95a cells. The virus-specific hemagglutinin (H) and nucleocapsid (*N*) protein genes were amplified in all samples, whereas the virus was isolated in B95a cells from a blood sample from Patient 2. For genotyping, 520-bp fragments including the 3′ terminus of the *N* gene were sequenced directly using an ABI PRISM 3130 DNA sequencer (Applied Biosystems, Foster City, Calif., USA), and the 3′ terminal 456-nt sequence of the *N* gene was determined. All of the nucleotide sequences were identical to each other. Surprisingly, these sequences were identical to that of another strain detected in China (MVs/Hong Kong.CHN/36.07/1) [EU368828]. Phylogenetic analysis showed that the sequences detected in the patients belonged to genotype H1 (Fig. 1), represented by Hunan.CHN/93/7 (*N* gene accession no. AF045212). The detected viruses were designated as MVs/Osaka.JPN/10.08/4 in the case of Patient 2, MVs/Osaka.JPN/10.08/6 for Patient 3, and MVs/Osaka. JPN/11.08 for Patient 5, according to the WHO nomenclature. The sequence data generated in the present report were submitted to the DNA Data Bank of Japan and were given the accession nos. AB457180, AB457181, and AB457182, respectively.

Outbreaks of measles in Japan during the past 2 years were primarily caused by D5 genotype viruses (2,4). In Osaka Prefecture, 24 measles viruses were genetically detected in 2007, and all of these belonged to genotype D5. As regards the isolation of genotype H1 measles virus in Japan, isolates of this genotype were reported in Tokyo and Kawasaki City in 2001, followed by isolates in Osaka City and all over Japan in the years 2002 to 2004 (2-4). However, the *N* gene sequences detected in this study shared little homology with those of other genotype H1 strains isolated in Japan (Fig. 1). Originally, the H1 genotype was considered to be indigenous to China and Korea (3); the measles viruses reported in the

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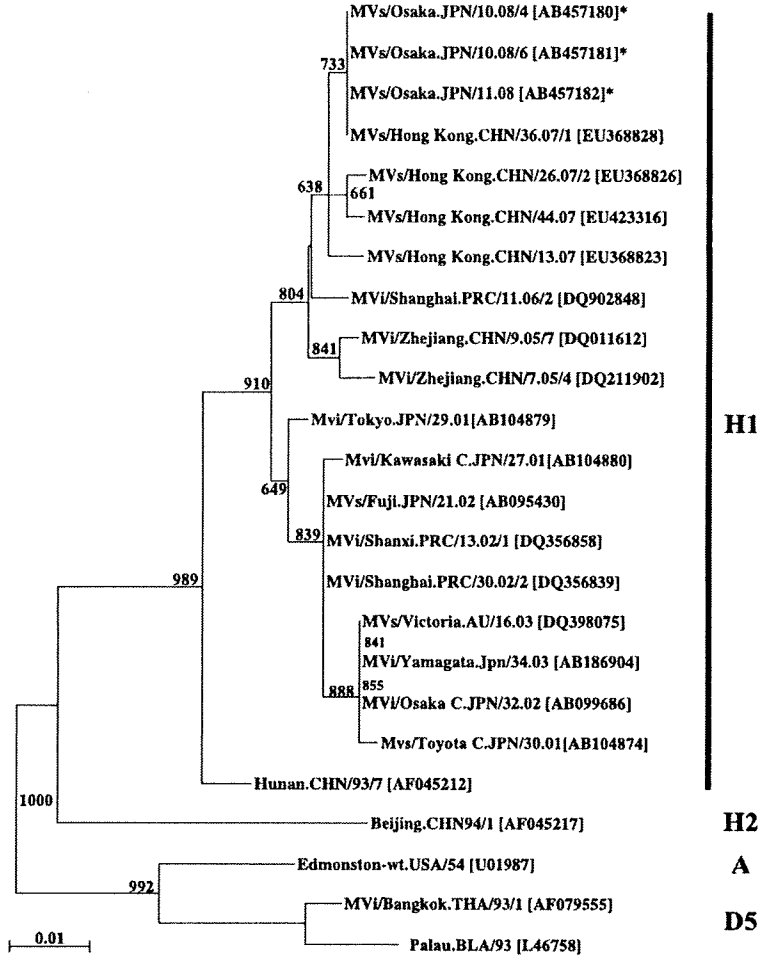

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method with the 3′ terminal 456-nt of the *N* gene. The GenBank accession number for each DNA sequence is given in parentheses. The number on each branch indicates the occurrence in 1,000 bootstrap replicates. The scale bar represents 0.01 substitutions per site. The asterisk indicates the sequence originated from this study.

present paper had a sequence identical to that of MVs/Hong Kong.CHN/36.07/1.

Index Patient 1 had no history of overseas travel, but he had sometimes visited Kansai International Airport on business. Thus, his infection with the type H1 measles virus raised the possibility of direct contact between Patient 1 and infected travelers at that international airport who were from measles-endemic countries. Index Patient 5 did not attend a nursery school or kindergarten, nor did he have any contact with the patients in Case 1. Consequently, the source of measles infection in these cases was not identified. However, these findings suggest that the H1 wildtype measles virus was imported from an Asian country to Osaka, Japan in recent years, and that the disease was circulating endemically in the southern region of Osaka Prefecture. Further measles surveillance will reveal the distribution of genotype H1 measles viruses. Careful epidemiological studies of laboratory-diagnosed measles viruses should be carried out on samples collected in the area around international airports in order to protect against the import and export of measles.

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Short Communication

Epidemiological and Molecular Studies of Measles at Different Clusters in Hokkaido District, Japan, 2007

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SUMMARY: In 2007 eight epidemic clusters (more than 15 cases in each) and other sporadic cases of measles occurred in Hokkaido district, Japan. A total of 850 cases were identified. Approximately half of them were ≥15 years of age, resulting in a huge public health problem in the community associated with school closings, and 31% of the cases reported that they already had a history of vaccination. Of 28 isolates of the measles virus detected, all were identified as genotype D5, identical to the type isolated in other areas of Japan, suggesting that a highly homologous measles virus circulated in Japan. The occurrence pattern of measles patients and molecular epidemiology indicated that the measles virus that spread in Hokkaido district might not be indigenous.

Measles virus (MV) is the monotypic virus in the genus *Morbillivirus* within the family *Paramyxoviridae*, and is the etiological agent of acute and highly contagious infection in humans (1). Infection with MV has clinical signs and symptoms such as cough, coryza, high fever, maculopapular rash and conjunctivitis, can cause death in some cases, and is often complicated with pneumonia and/or encephalitis. In 2001, a national-scale epidemic of measles in Japan occurred, with an estimated 286,000 cases, mainly among infants and young children (2). Consequently measles control was intensified all over the country (through a nationwide public awareness campaign including the establishment of a measles surveillance system by each prefecture and the promotion of timely vaccination with a first dose of live MV-containing vaccine), resulting in a dramatic decrease in cases. In recent years, measles cases have been reported in small numbers (seven cases in 2005 and three cases in 2006) in Hokkaido district. However, in the spring of 2006, a local, small epidemic of measles occurred in certain regions of Kanto district and further spread to Tokyo by the end of 2006. Subsequently, it spread through the whole country, including the Hokkaido district during the "Golden Week" holidays in May 2007 (3).

From 1996-2007, measles surveillance in Japan consisted of aggregating cases reported from two sentinel (pediatric and adult) surveillance systems. In Hokkaido district, there were 143 pediatric sentinel facilities and 23 hospitals in the adult sentinel surveillance system in 2007. Additionally, the Department of Health and Welfare, Hokkaido Government, recommended that all health practitioners report any clinical or laboratory-confirmed cases to local health officials. Therefore, the number of reported cases appeared to be close to the actual number of measles cases. The vaccination status of the cases was mainly confirmed with vaccination cards or through recall of the cases or of their parents. A database of 2007 measles cases was constructed by the Department of Health and Welfare with the cooperation of local health officials. We selected data regarding the residency, sex, age, date of onset of illness, clinical signs and symptoms and vaccination history.

The first cluster of measles occurred in Muroran city, located approximately 150 km southwest of Sapporo, the capital of Hokkaido. The initial patient officially reported was a 53-year-old woman; the date of onset of clinical illness was 23 April 2007. Subsequently, 97 cases <15 years of age and 145 cases ≥15 years of age were documented by the end of July 2007, comprising a notable peak (Fig. 1A). The second cluster occurred in Kushiro city, located approximately 300 km east of Sapporo, and lasting for approximately 2 months from July to August with 47 cases <15 years of age and 93 cases ≥15 years of age (Fig. 1B). Including other small clusters and sporadic cases, a total of 850 measles cases were ultimately reported in Hokkaido district during 2007: 449 measles cases (52.8%) <15 years of age and 401 cases (47.2%) ≥15 years of age (Fig. 1C). Fortunately, no deaths were associated with this measles epidemic. As noted, approximately half of the measles cases were ≥15 years of age, with the most reported cases between 10 and 14 years of age, leading to short-term school closures (complete or partial) including one elementary school, one junior high school, six high schools and one university.

Of the 850 documented cases, 264 (31.1%) were shown to have already been vaccinated, and 428 (50.4%), including persons ineligible for routine vaccination (i.e., <1 year of age), had no measles vaccination. The vaccination history of the remaining 158 (18.6%) cases was unclear due to the absence or uncertainty of their records.

The World Health Organization recommended that vaccination coverage of children should exceed 95% (4). Since 2002 the Department of Health and Welfare has investigated the rate of vaccination coverage for children when routine physical examinations are carried out at 18 months and 3 years of age. The rate of vaccination coverage at 3 years of age increased to 97.7% in 2006, compared to 93.6% in 2002. Eventually, the number of patients with measles between 1 and 4 years of age was small, just 86 cases (10.1%), in 2007.

The age distribution and vaccination history of the patients

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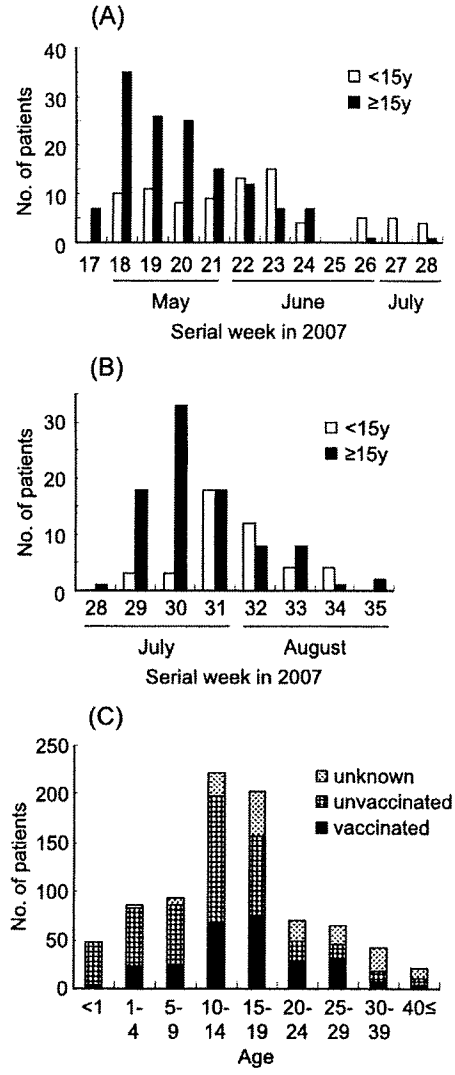

Fig. 1. (A) Measles epidemic in Hokkaido district, Japan in 2007. Initially the epidemic occurred in Muroran city from April to July. (B) Second epidemic in Kushiro city from July to August. (C) Age distribution and vaccination coverage of all measles cases (total 850).

shown in Fig. 1C revealed that in this epidemic the measles infection mainly targeted both unvaccinated and once-vaccinated adolescents in addition to unvaccinated young children. This suggested that one-dose vaccination was indeed insufficient to entirely prevent the infection and emphasized receiving the second dose of the vaccination to give children without sufficient immunity an additional opportunity to be protected against the infection. In contrast, it was reported recently that antibody responses after live viral infection had long half-lives of 50 years or more (5). However, the authors also found that it was unknown whether vaccine-induced immunity is stable for as long as that induced by the natural infection. Thus, for the sake of future vaccination design it is important to clarify the duration of antibody responses and to determine the timing of the additional vaccination.

To investigate the molecular epidemiology of the measles epidemic, throat swabs were obtained from 40 cases in eight different clusters including the cities of Muroran and Kushiro. Viral RNA was extracted directly by using the QIAamp viral RNA Mini Kit (Qiagen, Germantown, Md., USA) according to the manufacturer's manual. A one-step RT-PCR kit (Qiagen) was used to amplify 574 nucleotides coding for the COOH-terminus of the nucleoprotein (N) and then a nested second PCR was conducted to amplify 533 nucleotides of the *N* gene as described by Morita et al. (6). Water instead of RNA or first RT-PCR product was added to the reaction mixture as a negative control in each PCR reaction. The nucleotide sequence was determined by the Applied Biosystems 3130*xl* genetic analyzer (Applied Biosystems, Foster City, Calif., USA) using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems). Sequence data of the *N* gene of MV corresponding to 150 amino acids of the COOH-terminus of the N protein were aligned using the CLUSTAL X program (7), and the genotype was assigned on the basis of a phylogenetic tree constructed with MEGA 3 software (8). The reliability of the tree was estimated using 1,000 bootstrap replications.

Measles viral RNA was detected in 28 (70.0%) cases by the RT-PCR. The PCR product was identified as MV by the sequence data and was indicated the genotype D5 completely identical each other. Based on 450 nucleotides coding for the COOH-terminus of MV N, phylogenetic analysis was performed on five isolates collected in May (Hokkaido.JPN/20.07/21 and Hokkaido.JPN/20.07/27; two from Muroran city), June (Hokkaido.JPN/23.07/57 from Tomakomai city), August (Hokkaido.JPN/33.07/74 from Kushiro city) and December (Hokkaido.JPN52.07/11 from Monbetsu city), and the representative genotype D5 strains as shown in Fig. 2. The phylogenetic tree showed that D5 strains were divided into two major groups: Bangkok.THA 93 and Palau.BLA 93. Japanese isolates including those of Hokkaido in 2007 were clustered in the Bangkok lineage, which differs from those spread in Japan prior to 2002 (Fig. 2). These data suggested that genotype D5 of the MV circulating in Japan was highly homologous in its nucleotide sequence, leading to the genetic stability of MV during this period. The molecular epidemiological data for the Hokkaido isolates of MV in 2007 differed from those for the isolates from Sapporo in 2001 but were homologous to those of the Gunma isolates in 2007 and the measles epidemic that occurred in Hokkaido after the epidemic in Kanto district, indicating that the recent MV isolated in Hokkaido district might not be indigenous.

As mentioned above, the Hokkaido isolates obtained between April and December were highly homologous. This coincided with a report that field isolates collected at different times often show very low levels of nucleotide divergence (9,10). Additionally, while genetic variation of MV is common in countries with endemic transmission, no variation has been observed in countries at or near the measles elimination stage (11-14). Therefore, the findings of our study demonstrated that, mainly due to the increase in successful vaccination, a movement from endemic to epidemic transmission of MV appeared to occur in Japan.

The resurgence of measles in Japan in 2007 led to the change of the sentinel surveillance system to nationwide mandatory case-reporting in January 2008, and all health practitioners were required to report any clinical or laboratory-confirmed case to local health officials (2). Moreover, in addition to the usual two-dose vaccination program for children aged 1 and 6 years old (before entrance to elementary school), a 5-year vaccination catch-up campaign was initiated in April 2008, targeting cohorts aged 13 and 18 years. We conclude that, in Japan, the implementation of high vaccination coverage of children and adolescents seems to be

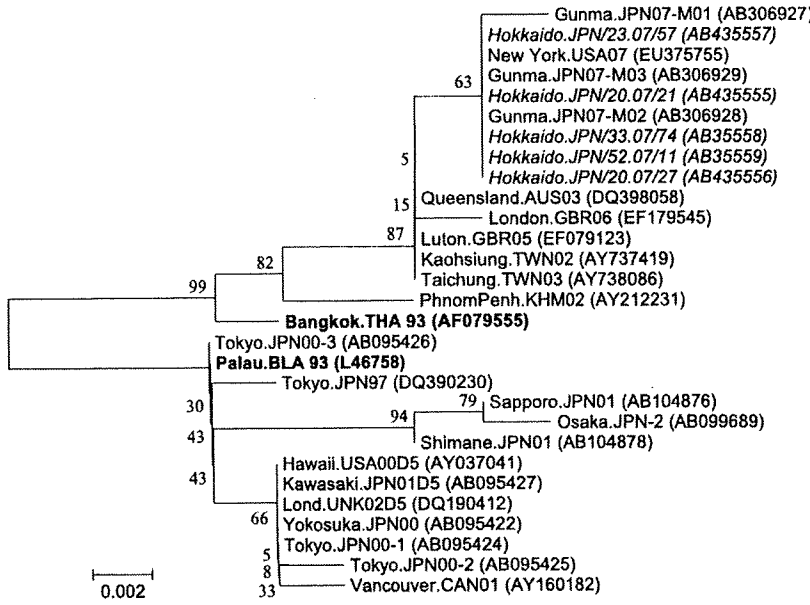

Fig. 2. Phylogenetic tree of 450 nucleotides coding for COOH-terminus of N protein of MV. Prototype MV strains were shown with their genotype written as bold type and isolates in Hokkaido district were shown as italics. Numerals at the branch showed bootstrap values in percent. GenBank accession numbers of each strain were shown in parenthesis.

necessary to prevent future outbreaks.

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Development of an assay for the detection and quantification of the measles virus nucleoprotein (N) gene using real-time reverse transcriptase PCR

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We developed a new quantification method for the measles virus (MeV) nucleoprotein (N) gene using real-time reverse transcriptase PCR. This method allowed us to quantify 10^1–10^7 copies per reaction (corresponding to 5×10^{-1}–5×10^5 copies μl^{-1}) of the MeV N gene. We also quantified the MeV N gene from the throat swabs of 22 patients with measles as well as the MeV genotypes A, D3, D5, D9 and H1 in viral suspensions derived from MeV-infected cells. As a result, 3.9×10^3–5.2×10^6 copies ml^{-1} and 7.4×10^7–2.0×10^8 copies ml^{-1} of the MeV genomes (N gene) were detected in the throat swabs and viral suspensions, respectively. No other viruses (enteroviruses, respiratory syncytial virus, human metapneumovirus or mumps virus) were detected in the assay. The results suggest that this method is applicable to the detection and quantification of some genotypes of MeV.

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INTRODUCTION

The measles virus (MeV), genus *Morbillivirus*, family *Paramyxoviridae*, causes acute and highly contagious measles infection in humans (Griffin, 2007). The case fatality rate in developing countries is generally in the range of 1–5 %, but may be as high as 25 % in populations with high levels of malnutrition and poor access to health care (http://www.who.int/mediacentre/factsheets/fs286/en/print.html). Therefore, the World Health Organization has focused on the infection as an eliminative disease (WHO & United Nations Children's Fund, 2001; WHO, 2006).

A genetic detection method for MeV, i.e. reverse transcriptase nested PCR (nested RT-PCR), is widely used (Morita *et al.*, 2007). While this method may be very sensitive, it can take more than 8 h to detect MeV. Moreover, cross-contamination during the RT-PCR processes may cause significant problems (Llop *et al.*, 2000; Drago *et al.*, 2004). To improve these problems, recently a real-time reverse transcriptase PCR (real-time RT-PCR) method using specific primers and probes has been applied for the detection of various virus genomes (Mackay *et al.*, 2002). This method is highly sensitive and specific, and quantitative.

In this study, we developed a sensitive and quantitative assay for the detection of the MeV nucleoprotein (N) gene in clinical specimens (throat swabs) and viral suspensions derived from MeV-infected cells (Vero/SLAM cells).

†These authors contributed equally to this work.

Abbreviations: C_t, threshold cycle; RT-PCR, reverse transcriptase PCR.

The GenBank/EMBL/DDBJ accession numbers for the measles virus nucleoprotein gene sequences are AB447494–AB447515.

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METHODS

Clinical specimens. Throat swabs were obtained from 22 patients with measles. The samples were centrifuged at 3000 *g* for 30 min at 4 °C and the supernatants were used in this study (Morita *et al.*, 2007). The specimens were stored at −80 °C until used. Detailed data concerning patients, copies of the MeV N gene in throat swabs and genotypes are shown in Table 1.

Viruses, virus propagation and RNA extraction. Five MeV strains (genotype A, CAM-70; genotype D3, MVi/Okinawa.JPN/31.01[D3]; D5, MVi/Okinawa.JPN/03.03[D5]; D9, MVi/Yamagata.JPN/3.04[D9]; and H1, MVi/Okinawa.JPN/14.03[H1]) and the 22 throat swab specimens were propagated in SLAM (signalling lymphocyte activation molecule, CD150)-expressing Vero cells (Vero/SLAM cells, kindly donated by Dr Y. Yanagi, Faculty of Medicine, Kyushu University) with Opti-MEM (Invitrogen). Vero and Vero/SLAM cells were maintained as previously described (Ono *et al.*, 2001). Each virus suspension titre was 10^6–10^7 $TCID_{50}$ $(0.1\ ml)^{-1}$ (tissue culture infective dose 50%). MeV RNA was extracted from 200 µl of the throat swab specimen or the viral suspension using a High Pure Viral RNA kit (Roche Diagnostics). The extracted RNA was then suspended in 50 µl DNase/RNase-free water with 1 U RNase inhibitor (Applied Biosystems) μl^{-1}.

Design of primers and probe. We aligned nucleotide sequences of the N gene from various genotypes of the reference MeV strains (genotype A, B1, B2, B3, C1, C2, D1–D10, E, F, G1, G2, G3, H1 and H2), as previously described (WHO, 2005; Morita *et al.*, 2007). Based on these data, we designed new primers and a TaqMan probe using Primer Express (R) version 1.5 software (Applied Biosystems) (Thomas *et al.*, 2007) (Table 2).

Preparation of control plasmid. To prepare the control plasmids, the N gene (position 1368–1616, 249 bp) in the following genotypes was amplified by PCR using the newly designed primers: genotype A, CAM-70 (GenBank accession no. U03650); genotype D3, MVi/Okinawa.JPN/31.01[D3] (AB435245); D5, MVi/Okinawa.JPN/03.03[D5] (AB435246); D9, MVi/Yamagata.JPN/3.04[D9] (AB186905); and H1, MVi/Okinawa.JPN/14.03[H1] (AB435247). The products were cloned into a pCR2.1-TOPO vector (Invitrogen) and purified with a High Pure Plasmid Isolation kit (Roche Diagnostics), according to the manufacturer's instructions. The concentration of the plasmid was determined by measuring the A_{260}. The DNA sequence was confirmed by sequencing, using a Big Dye Terminator version 1.1 Cycle Sequencing kit (Applied Biosystems) (Morita *et al.*, 2007).

Synthetic RNA of the N gene. Synthetic RNA of the MeV N gene (full-length, genotype A, position 108–1685, 1578 nucleotides) was prepared using the T7 MEGAscript kit (Applied Biosystems) as previously described (Hummel *et al.*, 2006). Synthetic RNA was treated with DNase and purified. The size of the synthetic RNA was determined by ethidium bromide staining on denatured agarose gel. The concentration of the synthetic RNA of the N gene was determined by measuring the A_{260}.

Procedures of quantitative real-time RT-PCR. The reverse transcription mixture contained 10 µl template RNA from the throat swab specimens, 4 µl random hexamer (20 pmol), 4 µl 5× RT-PCR buffer, 1 µl PrimeScript RT Enzyme Mix I (Takara) containing 10 units RNase inhibitor μl^{-1} (Applied Biosystems) and 1 µl DNase- and RNase-free distilled water. The samples were incubated for 15 min at 37 °C then for 5 s at 85 °C. PCR amplification was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems) under the following conditions: initial uracil-*N*-glycosylase (UNG) amplicon

Table 1. Patient data, copies of the N gene of measles virus in throat swab, and genotype

Patient	Strain	Onset of rash	Sampling date	Copies*	Genotype	Vaccination	GenBank accession no.
1	MVs/Okinawa.JPN/15.08/3	4 April 2008	4 April 2008	4.4×10^5	D5	Unknown	AB447511
2	MVs/Okinawa.JPN/16.08/1	14 April 2008	14 April 2008	2.8×10^5	D5	Unknown	AB447513
3	MVs/Okinawa.JPN/16.08/2	18 April 2008	14 April 2008†	6.8×10^3	D5	Unknown	AB447512
4	MVs/Okinawa.JPN/16.08/3	16 April 2008	17 April 2008	8.4×10^4	D5	Unknown	AB447514
5	MVs/Okinawa.JPN/18.08/1	29 April 2008	29 April 2008	1.6×10^5	D5	Yes	AB447515
6	MVs/Okinawa.JPN/21.07	23 May 2007	23 May 2007	7.4×10^4	D5	Yes	AB447510
7	MVs/Okinawa.JPN/37.06	11 September 2006	12 September 2006	8.9×10^4	D5	No	AB447495
8	MVs/Okinawa.JPN/38.06	19 September 2006	22 September 2006	5.3×10^5	D5	No	AB447494
9	MVs/Okinawa.JPN/39.06/1	27 September 2006	27 September 2006	5.2×10^6	D5	Unknown	AB447496
10	MVs/Okinawa.JPN/39.06/2	27 September 2006	27 September 2006	1.2×10^5	D5	Yes	AB447497
11	MVs/Okinawa.JPN/39.06/3	28 September 2006	28 September 2006	3.4×10^6	D5	No	AB447498
12	MVs/Okinawa.JPN/39.06/5	28 September 2006	28 September 2006	2.6×10^5	D5	No	AB447499
13	MVs/Okinawa.JPN/40.06/1	1 October 2006	5 October 2006	1.2×10^6	D5	Yes	AB447500
14	MVs/Okinawa.JPN/40.06/2	6 October 2006	6 October 2006	1.3×10^4	D5	No	AB447501
15	MVs/Okinawa.JPN/41.06/1	9 October 2006	10 October 2006	1.1×10^6	D5	No	AB447502
16	MVs/Okinawa.JPN/41.06/2	9 October 2006	10 October 2006	3.6×10^5	D5	No	AB447503
17	MVs/Okinawa.JPN/42.06/1	21 October 2006	20 October 2006†	6.0×10^4	D5	No	AB447504
18	MVs/Okinawa.JPN/45.06/1	7 November 2006	7 November 2006	2.0×10^4	D5	Unknown	AB447505
19	MVs/Okinawa.JPN/45.06/2	6 November 2006	7 November 2006	1.3×10^5	D5	Unknown	AB447506
20	MVs/Okinawa.JPN/45.06/3	7 November, 2006	7 November 2006	3.9×10^3	D5	Unknown	AB447507
21	MVs/Okinawa.JPN/48.06	30 November 2006	2 December 2006	1.1×10^5	D5	No	AB447508
22	MVs/Okinawa.JPN/50.06	14 December 2006	15 December 2006	1.6×10^5	D5	Yes	AB447509

*Copies are expressed as ml^{-1} of throat swab.
†Sampling was performed the day that fever appeared.

Table 2. Primer and probe sequences used for MeV real-time RT-PCR

Primer or probe	Sequence (5′→3′)*	Polarity†	Location‡
Primers			
MV-F	CAS RGT GAT CAA ART GRR ARY GAG CT	+	1368–1393
MV-R	YCC TGC CAT GGY YTG CA	−	1600–1616
Probe§			
MV-T	FAM-TCY GAT RCA GTR TCA AT-MGB-NQF	−	1530–1546

*Mix bases in degenerate primers and probes are as follows: S=G/C, R=A/G, Y=C/T.
†+, Sense; −, antisense.
‡Corresponding nucleotide position of the MVi/Edmonston-wt.USA/54[A] strain full-length genome (GenBank accession no. AF266288).
§Probe is labelled with 6-carboxyfluorescein (FAM) reporter dye at the 5′-end, and with minor groove binder (MGB)-non-fluorescent quencher (NQF) at the 3′-end of the oligonucleotide.

degradation (50 °C for 2 min) and denaturation of UNG at 95 °C for 10 min to activate DNA polymerase, then 50 cycles of amplification with denaturation at 95 °C for 15 s, and annealing and extension at 58 °C for 1 min. Amplification data were collected and analysed with Sequence Detector software version 1.3 (Applied Biosystems). A 10-fold serial dilution of standard cDNA plasmids (10^7–10^1 copies of genotype A, D3, D5, D9 or H1) was used for the quantification of copies as a standard curve assay.

Genotyping of MeV. We performed genotyping of MeV isolated from the clinical samples. N gene amplification and nucleotide sequencing were performed as previously described (Takeda *et al.*, 1999; Morita *et al.*, 2007). Nucleotide sequences of the partial N gene of the MeV were analysed phylogenetically using the CLUSTAL W program available on the DNA Data Bank of Japan (DDBJ) homepage (http://clustalw.ddbj.nig.ac.jp/top-e.html) and TreeExplorer (Version 2.12) (http://evolgen.biol.metro-u.ac.jp/TE/). Evolutionary distances were estimated using Kimura's two-parameter method and phylogenic trees were constructed using the neighbour-joining method (Saitou & Nei, 1987). The reliability of the tree was estimated using 1000 bootstrap replications.

Specificity assay. To examine the specificity of the real-time RT-PCR assay, eight enterovirus samples (echovirus types 9 and 18; coxsackie viruses A16, B2 and B5; enterovirus 71; and poliovirus types 1 and 3) and 18 respiratory virus samples (five strains of respiratory syncytial virus, five of mumps virus, four of human metapneumovirus, two of influenza subtype A and two of influenza subtype B) were tested. The titres of these viruses ranged from 10^3 to 10^5 $TCID_{50}$ $(0.1\ ml)^{-1}$.

RESULTS AND DISCUSSION

Optimization of primer and probe concentrations in the present assay

It is suggested that primer and probe concentrations affect sensitivity and specificity of real-time RT-PCR (Hashimoto *et al.*, 2007). Thus we optimized the primer and probe concentrations as previously described (Hummel *et al.*,

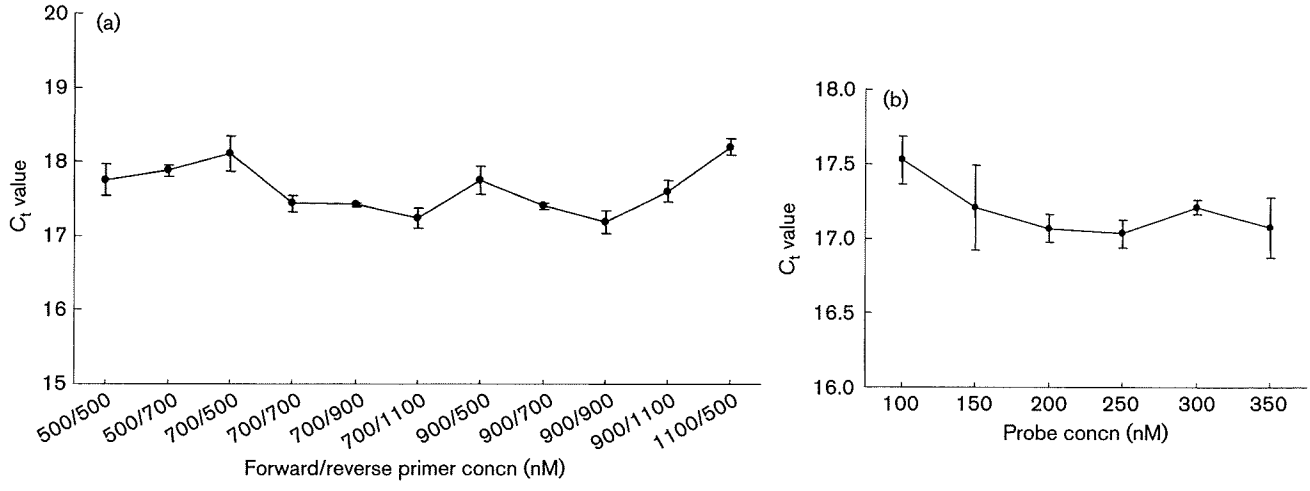

Fig. 1. Optimization of primer and probe concentrations in the present assay. Here, 10^7 copies of synthetic RNA of the N gene (full-length, genotype A) were used. The *x*-axis indicates the concentrations of primer or probe and the *y*-axis indicates the C_t value. Results are expressed as mean ± SD from three independent experiments. Detailed procedures are described in the text.

2006). The C_t (threshold cycle) values derived from the amplification plots for the present primer (ranging from 500 to 1100 nM) and probe (ranging from 100 to 350 nM) are shown in Fig. 1(a) and Fig. 1(b), respectively. In the present assay, 900 nM of both forward and reverse primers and 250 nM of probe showed the lowest C_t values. From these data, quantitative real-time PCR was carried out in a 20 µl reaction volume using a TaqMan Universal PCR Master Mix (Applied Biosystems) containing 2 µl cDNA, 900 nM each primer (MV-F and MV-R) and 250 nM TaqMan MGB probe (MV-T).

Linearity, sensitivity and specificity of the real-time RT-PCR

To assess the linearity of the quantitative real-time RT-PCR, we prepared 10-fold serial dilutions (10^7–10^1 copies) of cDNA plasmids (genotype A, D3, D5, D9 or H1), measured using newly designed primers and a probe. A representative standard curve and amplification plots using the standard plasmid (genotype A) are shown in Fig. 2(a, b). Good linearity was obtained from 10^1 to 10^7 copies per reaction (R^2=0.9996) using other genotype-cloned plasmids (genotype D3, D5, D9 or H1) (data not shown). Next, to address the issue of sensitivity of the present assay, we prepared 10-fold serial dilutions (10^7–10^1 copies) of synthetic RNA of the MeV N gene (genotype A) and measured copy numbers as previously described (Fig. 3a) (Hummel *et al.*, 2006). As a result, a good coefficient factor (R^2=0.9987) was obtained from 10^1 to 10^7 copies per reaction (corresponding to 5×10^{-1}–5×10^5 copies μl^{-1}). The results suggest that the reliable measurement range of the present assay is 10^1–10^7 copies per reaction. In addition, we prepared viral RNA (corresponding to 10^1–10^5 copies per reaction) of some MeV genotypes (genotype A, D3, D5, D9 or H1) using a dilution series of the viral suspensions and quantified copy numbers of their N gene. Good linearities (from 10^1 to 10^5 copies per reaction) as well as synthetic RNA were obtained for all genotypes (Fig. 3b). No other viruses such as enteroviruses, respiratory syncytial viruses, mumps viruses and influenza viruses (subtypes A and B) were detected with the present assay. The literature does contain some reports regarding the detection and quantification of MeV using sensitive real-time PCR methods (Hummel *et al.*, 2006; Thomas *et al.*, 2007). For example, Thomas *et al.* (2007) developed a quantification assay for the haemagglutinin (H) gene in MeV using a real-time PCR method and Hummel *et al.* (2006) developed a real-time RT-PCR method targeting multiple genes [nucleoprotein (N), fusion (F) and H genes]. In these methods, the sensitivity limits were two and ten copies per reaction, respectively (Hummel *et al.*, 2006; Thomas *et al.*, 2007). Thus these values were very similar to those of our method, even though the target gene and/or region of MeV were different. Taken together, the results suggest that our method is sensitive, specific and quantitative for the MeV N gene, and is applicable to the quantification of various MeV genotypes.

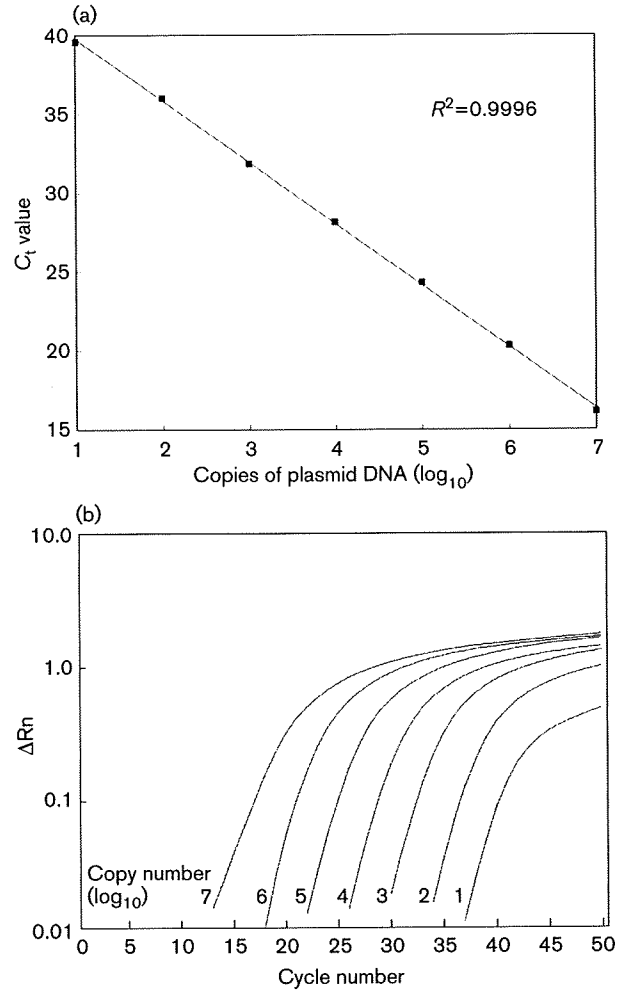

Fig. 2. Standard curve and amplification plots of quantification of the nucleoprotein (N) gene of MeV. A 10-fold serial dilution (10^7–10^1 copies per reaction) of cDNA plasmid (genotype A) was used. (a) The *x*-axis indicates the logarithmic concentration (log_{10}) of the cDNA plasmid and the *y*-axis indicates the C_t value. (b) The *x*-axis indicates the C_t value and the *y*-axis indicates ΔRn (adjusted fluorescence intensity). Detailed procedures are described in the text.

Isolation, genotype and quantification of MeV from throat swabs of patients with measles

MeV was isolated from all specimens and the genotype was confirmed as D5 by phylogenetic analysis (Table 1). In addition, throat swabs from patients with measles contained 3.9×10^3–5.2×10^6 copies ml^{-1} of the N gene. No significant differences were found between intervals of onset of rash and sampling date and copies of MeV in the throat swab specimens. In these patients, the lowest number of copies of MeV from raw data was 15.4 copies per reaction (corresponding to 3.9×10^3 copies ml^{-1} in throat swab, multiplication factor 250), suggesting that this value represents a reliable zone of the standard curve in the

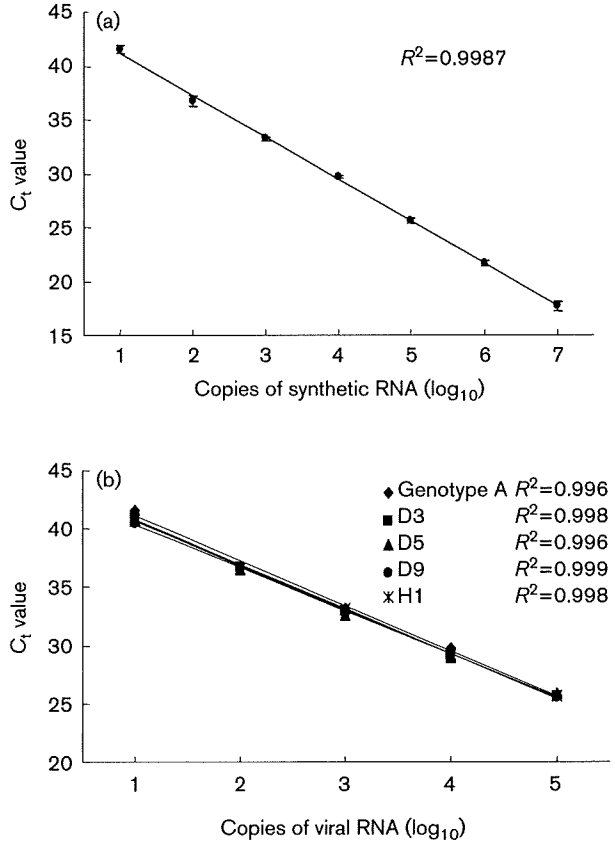

Fig. 3. Linearity of the real-time RT-PCR assay. (a) A 10-fold serial dilution (10^7–10^1 copies per reaction) of synthetic RNA of the MeV N gene (full-length, genotype A) was used. (b) A 10-fold serial dilution (10^5–10^1 copies per reaction) of viral RNA of each genotype (genotype A, D3, D5, D9 or H1) was used. In Fig. 3(a, b), the *x*-axis indicates the logarithmic concentration (log_{10}) of the synthetic or viral RNA and the *y*-axis indicates the C_t value. Results are expressed as mean ± SD from three independent experiments. Detailed procedures are described in the text.

present assay. In this study, we did not measure other MeV genotypes (D3, D9 or H1) in the throat swab specimens since we were unable to obtain them from patients with measles. Thus we quantified the A, D3, D5, D9 and H1 genotypes of the MeV N gene in viral suspensions derived from MeV-infected Vero/SLAM cells and some plasmid controls. As a result, each viral suspension contained 7.4×10^7–2.0×10^8 copies ml^{-1}. In Japan, over the past 10 years, domestic measles outbreaks have occurred in 1998, 2001, 2006 and 2007 (Kubo *et al.*, 2002; Zhou *et al.*, 2003; Nakajima *et al.*, 2003; Morita *et al.*, 2007). The predominant MeV genotype detected during these outbreaks was D5, although a small number of other genotypes including D3, D9 and H1 were also detected. Thus it may be important to confirm detection of these MeV genotypes in Japan. In the present study, in order to detect and quantify MeV genotypes and to prevent cross-reactions of the N gene in other paramyxoviruses such as mumps virus, respiratory syncytial virus and parainfluenza viruses, we targeted a specific region of the MeV N gene. However, the sequences of this region (the PCR target region) may be variable (Griffin, 2007). As such, it may be necessary to modify the primers/probe sequences in the future when mismatches are found to occur in them. Furthermore, we did not examine one-step real-time RT-PCR in this study, a method which may be advantageous when large numbers of samples are screened (Hummel *et al.*, 2006). Thus in future work we will consider the need to introduce this technique in the present assay. The present results indicate that the newly developed assay may be applicable for the detection and quantification of MeV from clinical specimens (throat swabs) and viral suspensions.

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