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).

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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.

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).

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 TCID₅₀ (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 A₂₆₀. 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 A₂₆₀.

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 phylogenetic 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 TCID₅₀ (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 Ct value. Results are expressed as mean $\pm$ 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 μ l reaction volume using a TaqMan Universal PCR Master Mix (Applied Biosystems) containing 2 μ l 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 μ l $^{-1}$). 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 $\pm$ 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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