Evaluation of Real-Time RT-PCR Compared with Conventional RT-PCR for Detecting Human Metapneumovirus RNA from Clinical Specimens

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SUMMARY: Human metapneumovirus (hMPV) is an etiologic agent of respiratory tract infections. In this study, we compared the sensitivity and specificity of real-time reverse transcription (RT)-polymerase chain reaction (PCR), conventional RT-PCR, and nested PCR in detecting hMPV genes. A total of 146 clinical specimens from 143 patients who showed acute respiratory tract infection symptoms were tested by real-time RT-PCR, conventional RT-PCR, and nested PCR targeting for the fusion gene. We detected hMPV RNA from 14 (9.6%) clinical specimens (real-time RT-PCR, 8; conventional RT-PCR, 5; and nested PCR, 13). When conventional RT-PCR was the reference standard, the sensitivity and specificity of real-time RT-PCR were 100 and 97.9%, respectively. When nested PCR was the standard, the sensitivity and specificity of real-time RT-PCR were 53.8 and 99.2%, respectively. Therefore, real-time RT-PCR was more sensitive than conventional RT-PCR but less so than nested PCR. Phylogenetic analysis showed that the real-time RT-PCR detected four genetic sublineages of hMPV. These results taken together indicate that real-time RT-PCR is an efficient method for detecting four genetic sublineages of hMPV from clinical specimens.

INTRODUCTION

Human metapneumovirus (hMPV) is a causative agent of acute respiratory tract infections. Since its discovery in 2001, this virus has been shown to infect males and females of all ages and induce clinical symptoms ranging from upper to lower respiratory tract illnesses such as bronchiolitis, bronchitis, and pneumonia (1-4). hMPV is an single-strand negative sense RNA virus belonging to the family Paramyxoviridae, subfamily Pneumovirinae, genus Metapneumovirus. hMPV has been shown to separate genetically into groups A and B, each of which consists of two sublineages (5,6).

Several methods have been developed to detect hMPV from clinical specimens. To detect hMPV proteins, indirect fluorescent assay or direct fluorescent assay using specific antibodies can be used (7,8). Gene amplification tests such as conventional reverse transcription (RT)-polymerase chain reaction (PCR) and real-time RT-PCR for detecting viral RNA have also been used (9). Virus isolation using cultured cells takes 3 or more weeks and is not as sensitive compared with conventional RT-PCR. Therefore, conventional RT-PCR is an efficient way to detect hMPV in clinical specimens. Use of a real-time RT-PCR assay for nucleocapsid (N), phosphoprotein (P), polymerase (L), or fusion (F) genes has been reported recently (10-13). However, few evaluations comparing real-time RT-PCR with conventional RT-PCR or nested PCR have been conducted (14). In this study, we designed a real-time RT-PCR method that detects four genetic sublineages of hMPV and compared it with conventional RT-PCR and nested PCR in terms of sensitivity and specificity.

We also examined whether real-time RT-PCR could detect individual parts of genetic sublineages of hMPV.

MATERIALS AND METHODS

Clinical specimens: From June 2006 to February 2008, 146 clinical specimens derived from 143 patients with acute respiratory tract infections were collected from attending sentinel pediatric clinics in Osaka City, Japan. Specimens derived from outbreaks at infant nursery or elementary schools were excluded. The age distribution of the patients was <0.5 years (n = 16), 0.5 - 1 years (n = 20), 1 - 3 years (n = 45), 3 - 5 years (n = 15), 5 - 10 years (n = 28), >10 years (n = 15), and unknown (n = 4). Specimens were composed of 88 nasal mucus samples, 35 throat swab samples, 18 sputum samples, 3 tracheal aspirate samples, and 2 mouthwash samples.

RNA extraction and RT-PCR: Viral RNA from clinical specimens was isolated using QIAamp Viral RNA Mini Kit (Qiagen Inc., Valencia, Calif., USA) according to the manufacturer’s instructions. RT was performed using SuperScript III RNase H- reverse transcriptase (Invitrogen Corp., Carlsbad, Calif., USA) with random hexamer primers. RT-PCR and nested PCR for hMPV F were conducted as described previously (15).

Real-time RT-PCR: To establish the real-time RT-PCR method, we first constructed the positive standard sample. hMPV RNA (JPOC06-1) was extracted and cDNA was synthesized as previously reported (15). A full-length segment of the F gene was amplified by KOD-plus DNA polymerase (TOYOBO Corp., Osaka, Japan) using forward primer 5'-GGAGTTCCACCATGTGGAAAGTGTCACCATGGGAAGTGGTG-3' and reverse primer 5'-CCGGTCGAGATTTGCTGGTAGATGAGAGCC-3'. The amplicon was subcloned into the EcoRI-XhoI sites of a pcDNA3.1-MCS-HA vector, which was constructed by inserting an HA-tag into the XhoI-XbaI sites of the pcDNA3.1 vector (Invitrogen). Real-time RT-PCR for detect-
The F gene was performed with TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, Calif., USA) using such primers as hMPV F (forward), 5´-ARYTGCCRATCTTTGGBGTYATAG-3´ (corresponding to 3,866-3,889 nt of 00-1 isolate); hMPV F (reverse), 5´-TYTKACAATACCAAYCCCTTGRTCYTC-3´ (3,964 - 3,988 nt of 00-1); and hMPV-F (Probe), 5´- (FAM) MAARGCAGCYCCYTCTTGYTCMGRA (BHQ-1)-3´ (3,909 - 3,933 nt of 00-1). The probe was labeled on the 5´ end with the fluorescent dye FAM and on the 3´ end with BHQ-1. The target sequence of the real-time RT-PCR was planned in the amplified region by nested PCR (15). The real-time RT-PCR conditions were as follows: 50°C for 2 min and then 95°C for 10 min, then 40 cycles of 95°C for 15 s, and 56°C for 1 min using ABI PRISM 7700 Sequence Detection Systems (Applied Biosystems).

Phylogenetic analysis: For hMPV-positive samples by conventional RT-PCR or nested PCR, a phylogenetic analysis for the F gene (275 nt: 3,793 - 4,067 nt of the 00-1 isolate) was performed as described elsewhere (15).

RESULTS

Detection of hMPV RNA in the clinical specimens: We detected 14 hMPV RNA in the 146 clinical specimens (9.6%) by real-time RT-PCR, conventional RT-PCR, and nested PCR. Of the 14, 10 (71.4%) specimens were derived from patients under the age of 3 years. hMPV-positive specimens consisted of 10 nasal mucus, 2 nasal swab, and 2 sputum specimens.

Real-time RT-PCR versus conventional RT-PCR or nested PCR: To evaluate the specificity and sensitivity of real-time RT-PCR, conventional RT-PCR, and nested PCR for hMPV, we compared the results of using the three processes to examine the same group of specimens. Of the 14 hMPV RNA, 8 were detected by real-time RT-PCR, 5 by conventional RT-PCR, and 13 by nested PCR (Table 1). When the F gene was performed with TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, Calif., USA) using such primers as hMPV F (forward), 5´-ARYTGCCRATCTTTGGBGTYATAG-3´ (corresponding to 3,866-3,889 nt of 00-1 isolate); hMPV F (reverse), 5´-TYTKACAATACCAAYCCCTTGRTCYTC-3´ (3,964 - 3,988 nt of 00-1); and hMPV-F (Probe), 5´- (FAM) MAARGCAGCYCCYTCTTGYTCMGRA (BHQ-1)-3´ (3,909 - 3,933 nt of 00-1). The probe was labeled on the 5´ end with the fluorescent dye FAM and on the 3´ end with BHQ-1. The target sequence of the real-time RT-PCR was planned in the amplified region by nested PCR (15). The real-time RT-PCR conditions were as follows: 50°C for 2 min and then 95°C for 10 min, then 40 cycles of 95°C for 15 s, and 56°C for 1 min using ABI PRISM 7700 Sequence Detection Systems (Applied Biosystems).

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consisted of the following: Cluster A2 (n = 4), B1 (n = 1), and B2 (n = 8) (Fig. 1). To add the strain of Cluster A1, we took the JPOC04-108 strain as reported previously (15). Real-time RT-PCR-positive strains were indicated by asterisks as follows: Cluster A1 (n = 1), A2 (n = 1), B1 (n = 1), and B2 (n = 5). Therefore, real-time RT-PCR used in this study could detect four genetic sublineages of hMPV.

**DISCUSSION**

Recently, the use of real-time RT-PCR assays to target hMPV genes has been reported. Some research groups constructed two or more pairs of primer and probe sets to detect four genetic sublineages of hMPV, and other groups used one pair of specific sequence primers (11,13,16). In this study, we designed a single set of primers and a TaqMan probe with wobble sequences for the F gene and showed that our process was able to detect four genetic sublineages of hMPV from clinical specimens. The detection rate in this research was 9.6% overall (all three methods) and 5.5% by real-time RT-PCR. The positive rate by real-time RT-PCR is consistent with the findings of other research groups (6.4 - 9.4%) (13,14). In most of the reported studies the authors performed real-time RT-PCR by one-step RT-PCR and constructed standard samples as viral RNA or RNA transcripts. In this study, we made our assay after separating RT-reaction products and used plasmid DNA as the standard sample. Thus, our system did not provide an absolute quantification, but a relative one. Cote et al. suggested that the N or L gene would be more suitable than other genes for gene amplification (10). Although we only investigated the F gene, we detected four genetic sublineages, and our detection rate was not much lower than that seen in other studies. Therefore, the F gene provides adequate regions for gene amplification.

Kikuta et al. reported that the viral load was not influenced by age, gender, or severity of illness (16). However, the number of positive samples in real-time RT-PCR in our study was small, and further analysis is needed.

In conclusion, the real-time RT-PCR we constructed was more sensitive than conventional RT-PCR but less sensitive than nested PCR. However, the specificity of real-time RT-PCR was equivalent to that of conventional RT-PCR and nested PCR. Moreover, no cross-reaction was observed in this study (data not shown). Therefore, real-time RT-PCR is a useful method for detecting hMPV in clinical specimens.

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