Detection of human metapneumovirus and respiratory syncytial virus by duplex real-time RT-PCR assay in comparison with direct fluorescent assay

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Abstract

Human respiratory syncytial virus (hRSV) and human metapneumovirus (hMPV) are important respiratory pathogens of small children and adults. The present study aimed to design a sensitive real-time RT-PCR assay for the detection of hRSV and hMPV in comparison with direct fluorescent assay (DFA) and to determine the incidence of hMPV and hRSV as causative agents of respiratory infections in a Finnish population. For DFA detection of hMPV antigen, four commercial antibodies were evaluated. The duplex real-time RT-PCR assay achieved a sensitivity of $10^3$ copies/mL of specimen for hRSV and hMPV type A viruses and $10^4$ copies/mL for type B hMPV. The detection rate of the RT-PCR assay was compared with those for DFA detection of hMPV and hRSV in analyses of 350 nasopharyngeal aspirates sent to HUSLAB, Helsinki University Hospital, for routine virus diagnostics during November 2007 to June 2008. Of the samples analyzed, 43 (12.3%) were positive for hRSV by DFA and an additional 13 specimens (3.7%) were positive for hRSV by RT-PCR. Only four samples (1.1%) were found to be positive for hMPV RNA by RT-PCR, with two of them also positive by DFA. The duplex real-time RT-PCR assay described in the present study can therefore be applied for efficient identification of hMPV and hRSV in clinical specimens and collection of information on the epidemiology and clinical outcome of these viruses.

Keywords: Direct antigen assay, human metapneumovirus, human respiratory syncytial virus, realtime, respiratory tract infection, RT-PCR

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Introduction

Human respiratory syncytial virus (hRSV) and human metapneumovirus (hMPV) are known as respiratory pathogens of small children. However, recent studies provide increasing evidence that hRSV and hMPV also cause a wide spectrum of respiratory symptoms in adults [1–4].

Diagnosis of hRSV is commonly based on antigen detection using commercially available point-of-care tests and direct fluorescence assay (DFA). For hMPV detection, the traditional virus isolation in cell culture is trypsin-dependent and requires long incubation times, and untypical cell lines [5]. Furthermore, antibody responses have not been useful in the diagnostics of acute hMPV infections [6]. Recently, commercial monoclonal antibodies for detection of hMPV antigens in clinical samples have become available, promoting the rapid detection of the virus [7–9]. Limitations of the traditional culture-based diagnostics and increasing knowledge of the genetics of hRSV and hMPV have led to development of a number of real-time RT-PCR-based assays enabling rapid and specific detection of the viruses in clinical specimens [10–13].

Overlapping seasonal occurrence of hRSV and hMPV with other respiratory viruses [3,4,14–16] and the inability to clinically distinguish between hRSV and hMPV infections or illnesses seen with other respiratory viruses [2,4,15–18] emphasize the need for a sensitive and rapid detection of these viruses. We describe a duplex real-time RT-PCR assay for simultaneous detection of hMPV and hRSV in clinical samples. In addition, data from an evaluation of four commercially available monoclonal antibodies for DFA detection
of hMPV in clinical samples is presented in comparison with the real-time RT-PCR assay.

Materials and Methods

Clinical samples and quality controls
Of the clinical specimens sent to HUSLAB, Helsinki University Hospital, for DFA detection of adenovirus, influenza A and B viruses, hRSV and parainfluenza viruses 1–3 during a period of November 2007 to June 2008, 24 bronchoalveolar lavage (BAL) samples and 326 nasopharyngeal aspirates (NPAs) were available for the present study. Samples originated from both immunocompetent and immunocompromised patients. Of the 350 samples analyzed, 148 (42.3%) were from patients aged less than 3 years, 102 (29.1%) from those aged 3–18 years and 100 (28.6%) from those over 18 years of age. Also examined were 24 quality assurance samples from the 2006 and 2007 Metapneumovirus & Respiratory Syncytial virus RNA Proficiency Programmes (QCMD, Glasgow, UK), five quality control samples containing adenoviruses 4, 5 and 7 from the 2006 Adenovirus Programme and three samples containing echoviruses 16 and 30 and coxsackievirus B3 from the 2006 Enterovirus Programme of the same organizer. Nucleic acids from all samples were isolated by QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) and RNAs were instantly subjected to duplex RT-PCR assay.

Production of viral RNAs
The hRSV strain B/Wash/18537/’62 (CH18537) (ATCC No. VR-1401) was obtained from the American Type Culture Collection (Manassas, VA, USA). The hMPV type B1 (strain NL/1/99) and type A1 (strain NL/1/00) were obtained as quality control samples from QCMD.

For production of viral RNAs, the viral templates were amplified in an RT-PCR reaction using primers RSV-CF, RSV-CR, MPV-CF and MPV-CR as described in Table 1 and transcribed with T3 RNA polymerase (Ambion, Austin, TX, USA) and the RNAs purified with RNeasy Mini Kit (Qiagen) and analyzed by agarose gel electrophoresis. The RNA concentrations were determined by measuring optical density at 260 nm using NanoDrop Technologies Inc., Wilmington, DE, USA).

Real-time RT-PCR
Two primer sets from the nucleoprotein genes of hRSV (primers RSV-F and RSV-R) and hMPV (primers MPV-F and MPV-R) were designed (Table 1). The reaction mixture for separate RT-PCR reactions consisted of 300 μM dNTPs (600 μM for dUTP), 50 mM Bicine, 115 mM KAc, 0.01 mM EDTA, 60 nM Rox, 3.0 mM MnAc, 5 U rTth DNA polymerase and 0.5 U uracil N-glycosylase in a total volume of 50 μl (TaqMan® EZ RT-PCR Kit; Applied Biosystems, Foster City, CA, USA). For the duplex RT-PCR, increased concentrations of 4.5 mM MnAc and 2 U of Ampli Taq Gold polymerase (Roche Diagnostics, Basel, Switzerland) were used. A concentration of 500 nM was used for primers RSV-F, RSV-R and MPV-R, and 700 nM for hMPV-F. The optimal concentrations for combined MPV-A and MPV-B probes and RSV-probe were 100 nM and 150 nM, respectively. Ten microlitre of the viral template was used in a total volume of 50 μl RT-PCR reaction.

Amplification was performed on a Stratagene MXP3000 in duplicate (Stratagene, La Jolla, CA, USA). An UNG-treatment of 2 min at 50°C was followed by RT-reaction at 60°C for 40 min and inactivation of UNG at 95°C for 5 min. The cycling conditions were: denaturation at 94°C for 20 s and annealing and extension at 60°C for 60 s. These steps were repeated 45 times.

<table>
<thead>
<tr>
<th>Virus genus and target gene</th>
<th>Oligonucleotide designation</th>
<th>Sequence (5’ to 3’) and orientation*</th>
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<tbody>
<tr>
<td>hRSV, nucleoprotein</td>
<td>Primer RSV-CF&lt;sup&gt;b&lt;/sup&gt;</td>
<td>GATGGGGCAAATATGGAAACA, +</td>
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<tr>
<td></td>
<td>Primer RSV-CR</td>
<td>GATTGCAAATCGTGTAGCTGT, +</td>
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<td></td>
<td>Primer RSV-F</td>
<td>TGGAAACATACGTGAACAARCTTCA, +</td>
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<tr>
<td></td>
<td>Primer RSV-R</td>
<td>GCACCCATATTGTWAGTGATGCA, +</td>
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<tr>
<td></td>
<td>Probe RSV</td>
<td>GCACCCATATTGTWAGTGATGCA, +</td>
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<tr>
<td></td>
<td>Primer MPV-CF&lt;sup&gt;b&lt;/sup&gt;</td>
<td>AATGTCTCTTCAAGGGATTCAC, +</td>
</tr>
<tr>
<td></td>
<td>Primer MPV-CR</td>
<td>GTTCTGCAGCTTCTTTTTCTTC, +</td>
</tr>
<tr>
<td></td>
<td>Primer MPV-F</td>
<td>TCATATAAGCATGCTATATTAAAAAGGCTGT-BHQ, +</td>
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<td></td>
<td>Primer MPV-R</td>
<td>CCTATYTCTGCAGCATATTTGTAATCAG, +</td>
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<td></td>
<td>Probe MPV-A</td>
<td>GTTCTGCAGCTTCTTTTTCTTC, –</td>
</tr>
<tr>
<td></td>
<td>Probe MPV-B</td>
<td>TCTATATAAGCATGCTATATTAAAAAGGCTGT-BHQ, +</td>
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*+, sense; -, anti-sense.
<sup>1</sup>T3 RNA polymerase promoter sequence AATTTAACCCCTACTAAAGGGAGA before viral sequence.
<sup>2</sup>Adapted and modified from Maertzdorf et al. (2004).
Precision study
A precision study was performed using dilution series of the in vitro transcribed RNAs corresponding to $1 \times 10^2$, $1 \times 10^3$, $1 \times 10^6$ and $1 \times 10^8$ RNA transcripts per RT-PCR reaction. For intra-assay variability, five parallel reactions were run on one plate, whereas interassay variability was assessed by running one dilution series on four consecutive days.

DFA
Cells of the clinical samples were concentrated by centrifugation, placed on two slides, dried and fixed in acetone. One slide was tested for adenovirus, influenza A and B viruses, hRSV and parainfluenza viruses 1–3 as primarily requested, using the Light Diagnostics™ Respiratory DFA Viral Screening & Identification Kit (Millipore, Billerica, MA, USA). The other slide was stored at $-70^\circ$C and tested later for the presence of hMPV antigen using four antibodies: Anti-human Metapneumovirus (hMPV) (Argene, Verniolle, France), D$^3$ DFA Metapneumovirus Identification Kit (Diagnostic Hybrids, Inc., Athens, OH, USA), OXOID hMPV (Imagen™; Oxoid Ltd, Cambridge, UK) and Human Metapneumovirus (hMPV) DFA Reagent (Light Diagnostics™; Chemicon International Inc., Temecula, CA, USA).

Results

Detection limit and precision of the real-time RT-PCR
For the separate RT-PCR reactions, a detection limit of 50 RNA transcripts of hRSV per RT-PCR reaction was achieved in repeated experiments. For hMPV subtypes A and B, a sensitivity of 50 and 150 RNA transcripts was obtained, respectively. Combining the separate RT-PCR assays as a duplex RT-PCR resulted in decreased sensitivity and repeated detection of 100 RNA transcripts per reaction, corresponding to $10^3$ copies/mL of sample, for hRSV was achieved. For hMPV subtype A and B viruses, 100 and 1000 RNA transcripts, equaling $10^2$ and $10^4$ copies/mL of specimen, respectively, were detected.

In the precision study, intra-assay coefficients of variation of the cycle threshold (Ct) values for hRSV and hMPV subtype A and B viruses were 3.63%, 3.99% and 3.99%, and interassay coefficients of 3.78%, 5.59% and 5.80% were obtained, respectively.

DFA
All four commercial antibodies showed a clear positive signal for hMPV subtypes A1, A2, B1 and B2 on control slides of the D$^3$ DFA Metapneumovirus Identification Kit, and no nonspecific staining was detected on negative control slides. However, when 56 NPA samples were studied, two of the antibodies, Anti-human Metapneumovirus (Argene) and OXOID hMPV (Imagen™), gave nonspecific signals in 27 and seven samples, respectively. These nonspecific signals were seen in samples positive for RSV, PIV2, InfA, InfB and adenovirus by DFA, but also in samples negative by DFA and negative for hMPV and hRSV in the RT-PCR assay. The remaining 294 samples were screened with the two hMPV antibodies from Diagnostic Hybrids, Inc., and Light Diagnostics™. These reagents performed with equal sensitivity and specificity.

Patient samples
Of the 350 clinical specimens analyzed, 54 (15.4 %) and 4 (1.1 %) were found to be positive for hRSV and hMPV in RT-PCR, respectively (Table 2). The age range of the hRSV positive patients was from 1 month to 80 years (age median 2 years); 30 of the samples (55.6 %) were from children under the age of 3 years and ten (18.5%) were from patients over 18 years of age. Forty-one of the RT-PCR-positive samples and two of the RT-PCR-negative samples were positive by DFA. Of the four specimens positive for hMPV by RT-PCR, DFA was clearly positive for two samples and negative for one (Table 2). The other RT-PCR positive sample was considered DFA-negative for hMPV with discrepant DFA staining for all four reagents used; hRSV RNA was detected in this sample, whereas the DFA result for hRSV was negative. The age range of the hMPV positive patients was from 3 months to 44 years, with half of the patients being under the age of 3 years and the other half being over 20 years of age. Monthly proportions of RT-PCR-positive samples are shown in Fig. 1.

<table>
<thead>
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<th>TABLE 2. Results of the analysis of the clinical samples</th>
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<tr>
<td><strong>Duplex RT-PCR</strong></td>
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<td>Result</td>
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<td>Negative</td>
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<td>hRSV</td>
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<td>hMPV</td>
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<td>hRSV, hMPV</td>
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$^a$Discrepant staining with the direct fluorescent assay reagents used.

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ture of the patient. To simultaneously detect hRSV and
test for the prevalent viruses compatible with the clinical pic-
test for all respiratory viruses, but rather tests are chosen to
available. However, it is rarely necessary to simultaneously
detection of hMPV and hRSV [21].

hMPV positive samples
hRSV positive samples
Samples available to the study

FIG. 1. Monthly distribution of RT-PCR positive samples over the
course of the present study. hRSV, human respiratory syncytial virus;
hMPV, human metapneumovirus.

Quality control samples
Analysis of the quality control samples found 11 and 12 sam-
positive for hMPV and hRSV, respectively, with one of
samples being positive for both viruses. Two of the qual-
ity control specimens appeared negative. The results
obtained were consistent with the preference assay results
of the final reports by the organizer and both A and B sub-
types of hMPV and hRSV were detected at all provided dilu-
tions. All samples of the adenovirus and enterovirus
programmes were negative in the RT-PCR assay.

Discussion

Identification of hRSV and hMPV relies on the detection of
viral antigens and viral nucleic acids in clinical samples. The
use of RT-PCR for the detection of hRSV and hMPV has
been described in several studies and has been found to have
greater sensitivity than DFA and viral culture
[6,7,10,11,19,20]. However, DFA offers a rapid and simple
alternative to molecular assays, thus enabling rapid diagnosis.
Therefore, a combination of the two methods, with DFA as
a first line test followed by RT-PCR for DFA-negative sam-
plies, may be the best approach for achieving rapid and sensi-
tive detection of hMPV and hRSV [21].

PCR-based tests enabling the detection of a large panel of
respiratory viruses from clinical samples are commercially
available. However, it is rarely necessary to simultaneously
test for all respiratory viruses, but rather tests are chosen to
test for the prevalent viruses compatible with the clinical pic-
ture of the patient. To simultaneously detect hRSV and
hMPV, viruses causing clinically indistinguishable illnesses, par-
ticularly in small children, we designed two primer pairs from
the nucleoprotein genes of these viruses because this gen-
ome region has been reported to facilitate the sensitive
detection of hMPV [12,22]. Because the routine diagnostics
of respiratory viruses in our laboratory is largely based on
DFA detection of viral antigens, we also compared the RT-
PCR results with commercially available antibodies for hMPV
detection.

The sensitivity of the duplex RT-PCR assay was $10^3$
opies/mL of sample for hRSV and subtype A hMPV and
$10^6$ copies/mL for subtype B hMPV, which are values similar
to those obtained in other studies [23,24]. This is satisfac-
tory for clinical diagnosis because these viruses are usually
abundant in nasopharyngeal samples. It has been reported
that multiplexing often results in decreased sensitivity of
PCR and the detection limits of a multiplex RT-PCR for dif-
ferent viruses may differ notably [24]. In our assay, the
higher detection limit for type B hMPV may result from the
internal structures of the RNA transcript and from a single
mismatch generated in the production of the viral RNA tran-
script of virus strain NL/1/99 at the binding site of the pri-
mer MPV-F. Therefore, the actual sensitivity for subtype B
hMPV may be $<10^4$ copies/mL. The real-time RT-PCR assay
detected both hMPV and hRSV in one clinical sample and in
one quality control sample. This implies the ability to detect
coinfections of these viruses, although the effect of co-infec-
tion on sensitivity and precision of the assay was not studied.
The assay proved to have a high specificity for identifying all
positive quality control samples containing varying dilutions
of hMPV and hRSV subtypes A and B, and negative control
specimens, including those of the adenovirus and enterovirus
programmes, were negative. Furthermore, the 24 clinical
samples that were positive for other respiratory pathogens
by DFA were negative in RT-PCR for hMPV and hRSV
(Table 2), demonstrating the specificity of the RT-PCR assay.

Of the 350 clinical samples analyzed in the present study,
43 (12.3%) were positive for hRSV using the routine hRSV
antigen test and the real-time RT-PCR detected the virus in
an additional 13 specimens (3.7%). Two samples negative for
hRSV by RT-PCR were positive by DFA. It is not known
whether these results are the result of nonspecific staining in
DFA or false RT-PCR-negatives resulting from degradation
of viral RNA, inhibitors of amplification or primer mis-
matches. Of the samples analyzed, two were considered
positive for hMPV by DFA and an additional two were posi-
tive in RT-PCR. Although the relatively high proportion of
positive results in adults obtained in the present study pro-
vides further evidence that hMPV and hRSV are respiratory
pathogens of adults, it also reflects the study samples, of
which only 42.3% were from small children who are the
most susceptible to these viruses. Our material included
samples from immunocompromised adults and children, and
it is well documented that both hRSV and hMPV are impor-
tiant pathogens among these patients [25]. Three of the four hMPV positive patients were diagnosed with an underlying haematological malignancy and one of these patients, a 2-year-old girl, died 1 month later with unresolved lung tissue damage seen at post mortem. Two adult haematological patients and an otherwise healthy 3-month-old boy recovered from the infection and had no subsequent documented respiratory episode. Although the low number of hMPV positive specimens may reflect a low incidence of hMPV in clinical disease, the current opinion is that asymptomatic carriage of the virus is uncommon and the presence of hMPV RNA in respiratory samples is associated with clinical infection [2,6,17,21].

The reported incidence of hMPV and hRSV ranges widely, from 1% to 31% and 4% to 41%, respectively, in different study populations [2,3,7,13,17,26,27]. The highest detection rates are generally obtained in studies of inpatients and pediatric patients, during high prevalence months and in specimens negative for other respiratory viruses. The incidence rate of 15.4% for hRSV in our study population is in agreement with previously documented data, although the 1.1% incidence rate of hMPV is low considering the samples were collected during high prevalence months. However, the prevalence of hMPV is known to vary from year to year and the study period November 2007 to June 2008 may have represented a milder winter [3,4,14,16,28]. Nevertheless, the results obtained in the present study are consistent with another Finnish study reporting an incidence rate of 1.3% in children [17] as well as with previous studies reporting a greater abundance of hRSV compared to hMPV [2,3,10,14,26], although a higher prevalence of hMPV has also been reported [24]. In the present study, hMPV infections occurred in February and March 2008, whereas hRSV infections were found from December 2007 to April 2008 (Fig. 1). Although this was not an epidemiological study, these findings are in agreement with reports on hMPV prevalence peaking later in the year than hRSV infections [3,15,16]. Although dual infections with hMPV and hRSV and co-infections with other respiratory viruses are commonly reported in clinical studies [4,14,18,27,29–31], we found only one co-infection of hMPV and hRSV.

In conclusion, the real-time RT-PCR assay described in the present study meets the current need for the rapid and sensitive detection of hRSV and hMPV in cases of severe respiratory diseases of unknown aetiology. The results obtained in our study suggest that hMPV has a relatively small role as a causative agent of respiratory illness compared to hRSV but testing pediatric and immunocompromised patients with respiratory symptoms for the presence of hMPV seems justified. The simultaneous detection of hRSV and hMPV will facilitate studies on the clinical presentation of these viruses, including cases of co-infection.

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Transparency Declaration

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The authors have no conflicts of interest to declare.

References


