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Real-Time Reverse Transcriptase PCR Assay for Improved Detection of Human Metapneumovirus

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Abstract

Background—Human metapneumovirus (HMPV) is a paramyxovirus with multiple genetic lineages that is a leading cause of acute respiratory disease. Several RT-PCR assays have been described based on limited available sequence data.

Objectives—To develop a broadly reactive real-time RT-PCR assay for HMPV that allows for a rapid, sensitive, and specific detection in a clinical or research setting.

Study Design—Three published assays for HMPV were modified based on analysis of multiple HMPV sequences obtained from GenBank. Original and modified assays were tested against prototype HMPV strains from each genetic sublineage, multiple isolates of HMPV from different years, a collection of clinical specimens, and commercial validation panels.

Results—A number of potential sequence mismatches with diverse HMPV strains were identified. Modifications were made to oligonucleotides to improve annealing efficiency. Primers and probes based on newer sequence data offered enhanced detection of all subgroups, especially for low titer specimens. The new primers and probe detected multiple clinical isolates of HMPV collected over a twenty-year period. The modified assay improved detection of HMPV in a panel of clinical specimens, and correctly identified HMPV samples in two commercial validation sets.

Conclusions—We report a modified real-time RT-PCR assay for HMPV that detects all genetic lineages with high sensitivity.

Keywords

Human metapneumovirus; diagnostics; real-time RT-PCR

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BACKGROUND

Human metapneumovirus (HMPV) is a paramyxovirus isolated from children with lower respiratory infection in the Netherlands in 2001[1]. HMPV is a leading cause of acute respiratory infection (ARI) in children and adults, with symptoms similar to those caused by other respiratory viruses[2-14]. Several RT-PCR assays to detect HMPV have been published[14-21]. However, there are two major genetic lineages of HMPV, each with minor sublineages that exhibit substantial diversity[22-28]. Published assays have been based on limited sequence data and some vary in sensitivity between viral lineages[16,21]. Due to the differential transcription of paramyxoviruses, N is the most highly transcribed gene[30] and both HMPV F and N genes are relatively conserved[23,24,27,31]. Thus, the fusion (F) and nucleocapsid (N) genes have been widely used as RT-PCR targets[14-21,29].

OBJECTIVES

We sought to develop an improved HMPV real-time RT-PCR assay based on the most recent available sequence data to enhance detection of diverse strains of HMPV from all four lineages.

STUDY DESIGN

Sequence analysis

Published full-length and partial N and full-length F gene sequences were obtained from GenBank or strains sequenced in our laboratory[8,12,27,31,32]. Viral sequences and published primer and probe sequences were aligned with MacVector 11 (MacVector). GenBank sequences used are listed in Supplemental Table 1. Primers and probe were designed using Primer Express 2.0 (Applied Biosystems).

Viruses, clinical specimens, and reference panels

Plaque-purified prototype HMPV strains were cultured and titered on LLC-MK2 cells[33,34]. Viruses isolated in the Vanderbilt Vaccine Clinic[8,12] were passaged until cytopathic effects were visible and confirmed by immunofluorescence using anti-HMPV antisera[33]. Clinical specimens were obtained from adult and pediatric patients with ARI. Nasal and throat swabs were collected and combined in transport medium (Beckton Dickinson) and aliquoted into MagMAX Lysis/Binding Solution Concentrate (Applied Biosystems) or MagNAPure LC Total Nucleic Acid Isolation Kit lysis/binding buffer (Roche), snap frozen, and stored at −80°C. Reference proficiency panels were obtained from the Quality Control For Molecular Diagnostics (QCMD)[35,36] and extracted by the same methods.

RNA extraction

RNA was extracted from thawed specimens according to the manufacturer's protocols for the MagMAX-96 Viral RNA Isolation Kit (Applied Biosystems) or MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche).

Real-time RT-PCR assay

One-step reactions were prepared using AgPath-ID One-Step RT-PCR kit (Ambion) according to the manufacturer's instructions with 1 μM forward and reverse primers and 0.25 μM probe. Cycling parameters were 50°C × 30 min, 95°C × 10 min and 45 cycles of 95°C × 15 sec and 60°C × 30 sec, with fluorescence data collected during the 60°C annealing/extension step. Primer/probe sets were also tested using SuperScript III Platinum One-Step qRT-PCR kit (Invitrogen) according to the manufacturer's instructions with the same primer/probe concentrations; results were similar to the AgPath-ID kit (not shown).

Amplifications were performed using the StepOne Plus (Applied Biosystems). All clinical specimens were tested in a separate real-time RT-PCR assay for RNAse P to ensure RNA integrity and exclude PCR inhibition[19]. To generate RNA runoff transcripts, target genes were cloned into pGEM (Promega) under a T7 promoter, transcribed in vitro from *HincII*-digested plasmids using T7 RNApol (NEB), purified, and quantified by spectrophotometry. RNA transcripts were used as positive controls and nuclease-free water as negative control.

RESULTS

Sequence analysis

One hundred twenty full-length F sequences and a published F-targeted assay were aligned (Supplemental Figure 1)[19]. While the target sequences were generally conserved, there were several polymorphisms detected in numerous strains of HMPV. Thus, nucleotide changes were introduced into the forward and reverse primers as shown in Table 1 to increase degeneracy. The probe sequence was reversed and complemented to increase the number of cytosine residues, and extended slightly to increase the Tm.

Three hundred fifty-two partial or full N sequences were aligned with published real-time RT-PCR assays targeting the N gene[14-18,20,21]. Many of these were partial and non-overlapping, so that the number of viral sequences compared against any single primer/probe ranged from 45 to 85 (not shown). Some published assays had several mismatches with diverse viruses and were not pursued further. However, two published assays were generally well matched to N sequences (Supplemental Figures 2 and 3)[14,21]. One of these (UR for) contained a “CG” that was likely a typographic error in the published manuscript[14], since all N sequences encoded “GC” at this position. This and other nucleotide modifications were made to increase degeneracy or adjust Tm. Both the NL-N and UR probes were reversed and complemented to increase the number of cytosine residues (Table 1).

Assay performance against 4 HMPV prototypes

We prepared serial ten-fold dilutions of RNA extracted from each of four fully sequenced HMPV prototypes representing each of the four subgroups: TN96-12 (A1), TN94-49 (A2), TN98-242 (B1), and TN99-419 (B2)[27,28]. The infectious titer of the stocks ranged from 1.4×10^6 to 3.7×10^6 pfu/ml. We tested each of the primer/probe sets initially against dilutions of viral RNA from 10^{-2} to 10^{-5} (Figure 1A-D). All six assays reacted against all four subgroups, though the original NL-N assay performed poorly against subgroup B viruses and the VU assay was less sensitive for subgroup A viruses. The other assays tested exhibited linear performance and were tested at dilutions from 10^{-2} to 10^{-8} (Figure 1A-D). In this series of experiments, all four assays performed well. The NL-N 2 assay exhibited detection at slightly lower Ct for A1, A2, and B1 viruses, with the original CDC assay also performing well against B1 virus.

Performance against clinical specimens and clinical isolates

We tested the NL-N 2 assay against a panel of 222 clinical specimens collected from adults hospitalized with ARI[37-39] that had been tested previously with the original NL-N assay. The original assay detected three (1.4%) HMPV-positive specimens; the NL-N 2 assay detected 11 (5%) HMPV-positive specimens. We also tested the NL-N and NL-N 2 assay against a collection of 222 specimens obtained from infants with ARI[40,41]. The original NL-N assay detected two (1%) HMPV-positive specimens, while the NL-N 2 assay detected seven (3.2%) HMPV-positive specimens. All clinical specimens tested positive for RNAse P. Finally, we tested the NL-N 2 assay against a panel of 42 cultured HMPV isolates collected between 1982 to 2003, comprising all four subgroups, and the assay detected all

isolates. The NL-N 2 assay did not react with RNA extracted from cultured isolates or clinical specimens positive for influenza viruses A/B, human rhinovirus, respiratory syncytial virus, and parainfluenza viruses 1-3 (not shown). The NL-N 2 assay was tested against serial 10-fold dilutions of RNA runoff transcripts and the limit of detection was <50 copies.

Performance against External Quality Assessment (EQA) panels

Two previous QCMD panels were available for HMPV testing, the 2006 MPV.RSV06 and 2009 MPV.RSVRNA09 (www.qcmd.org)[40, 42]. The 2006 EQA panel comprised cultured A1 and B1 viruses at dilutions from 10^{-3} to 10^{-7}; all were detected by the NL-N 2 assay except one sample containing A1 at 2×10^{-6} dilution. This sample tested negative in all three QCMD in-house reference laboratories and was only detected by 27% of QCMD participating laboratories. The 2009 EQA panel comprised A1 and B2 viruses at dilutions from 10^{-4} to 10^{-6}; all were detected by the NL-N 2 assay, including low titer specimens that were infrequently detected by other QCMD participating laboratories. RSV-positive specimens and negative controls in both EQA panels tested negative for HMPV with the NL-N 2 assay.

DISCUSSION

Human metapneumovirus is a leading cause of ARI in children and adults worldwide. Reliable diagnosis for clinical or research use is dependent on genome detection methods, since the virus is difficult to culture and there are no commercially available rapid antigen tests. Here we report a modified HMPV real-time RT-PCR assay based on more recently available sequence data. HMPV, like other RNA viruses, exhibits a substantial rate of mutation due to the error-prone RNA polymerase[44] and thus significant genetic diversity[22, 23, 27, 28, 31]. The increase in available HMPV sequences facilitates the analysis of multiple strains to optimize oligonucleotide design.

A strength of this study is the use of sequences from disparate geographic sites and times, dating back to 1982. While HMPV, like other paramyxoviruses, undergoes mutations and genetic variation, these nucleotide changes do not result in progressive antigenic “drift” over time[24,31,45,46]. Thus, sequence-based assays for HMPV targeting conserved regions are likely to remain capable of detecting future circulating strains. Further, N is the most highly conserved HMPV gene[27].

There are limitations to our study. We did not test all published primer/probe sets, and some might have performed well despite nucleotide mismatches; *in silico* analysis does not always predict *in vitro* performance. However, we tested assays that have been used in multiple studies of HMPV epidemiology[14,19,21]. All assays performed well, though with sequence-based modifications the performance was enhanced. We tested these primer/probe sets with two chemistries with similar results, though some viral assays exhibit extreme variability in performance with different chemistries[47]. Validation with specific instruments and kits is necessary for all molecular assays.

In summary, we developed an improved HMPV real-time RT-PCR assay based on comparison of hundreds of HMPV gene sequences. The assay exhibited linear performance against all four viral subgroups, detected viruses from diverse lineages over time, enhanced detection of HMPV in clinical specimens, and performed well in EQA proficiency panels. This assay should be useful for ongoing epidemiology studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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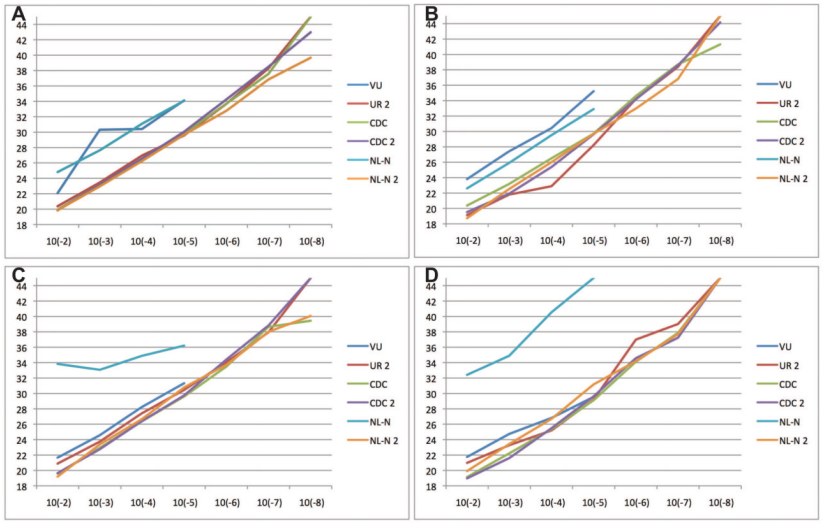

Figure 1. Performance of assays tested against prototype HMPV strains

Plots of cycle threshold (C_T) versus RNA serial 10-fold dilutions. **A**) TN96-12 A1. **B**) TN94-49 A2. **C**) TN98-242 B1. **D**) TN99-419 B2. Y axis = C_T, x-axis = dilution.

Table 1

Primer and probe sequences tested.

Assay	Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Probe (5′ to 3′)	Ref.
NL-N	N	CATATAAGCATGCTATATTAAAAGAGTCTC	CCTATTTCTGCAGCATATTTGTAATCAG	TGYAATGATGAGGGTGTCACTGCGGTTG	21
NL-N 2	N	CATAYAARCATGCTATATTAAAAGAGTCTC	CCTATYTCWGCAGCATATTTGTAATCAG	CAACHGCAGTRACACCYTCATCATTRCA	*
CDC	F	CAAGTGTGACATTGCTGAYCTRAA	ACTGCCGCACAACATTTAGRAA	TGGCYGTYAGCTTCAGTCAATTCAACAGA	19
CDC 2	F	CAARTGYGACATTGMTGAYCTRAA	AYTGCCGCACAACATTTAGRAA	CTTCTGTTGAATTGACTGAAGCTRACRGCCA	*
UR 2	N	CATGCTATATTAAAAGAGTCTCA	TCWGCAGCATATTTGTAATCAG	CAACHGCAGTRACACCYTCATCAATGCA	14 *
VU	N	ATGTCTCTTCAAGGGATTCACC	ATYTCTTGYTGCAATGATGARG	TCATAYAARCATGCTATATTAAAAGAGTCTCARTACACA	§

Primers were purchased from Invitrogen and were normal phase chromatography desalted. Probes were purchased from Operon and were reverse phase high performance liquid chromatography (HPLC) purified. Probes were 5′-labeled with 6-FAM and 3′-labeled with Black Hole Quencher-1 (BHQ-1).

* = modified from published sequences as described in text; UR = University of Rochester; NL = Netherlands; VU = Vanderbilt University

§ = this study

H = A, C, or T; R = A or G; W = A or T; Y = C or T